Phenotypic and Molecular Characterization of Non-typhoidal *Salmonella* Species in Humans and Animals in Central Ethiopia and Inhibition of Biofilm Formation Using Small Molecule Adenosine Mimetics

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A thesis submitted to

The Department of Pharmacology and Clinical Pharmacy

Presented in Fulfillment of the Requirements for the

Degree of Doctor of Philosophy (Pharmacology)

Addis Ababa University

Addis Ababa, Ethiopia,

June, 2016

Addis Ababa University

School of Graduate studies

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Abstract

Phenotypic and Molecular Characterization of Non-Typhoidal *Salmonella* Species in Humans and Animals in Central Ethiopia and Inhibition of Biofilm Formation Using Small Molecule Adenosine Mimetics

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This dissertation reports prevalence, serotype distribution and phenotypic and genotypic characteristics of non-typhoidal *Salmonella* (NTS) in humans and animals in central Ethiopia and effect of small molecule adenosine mimetic compounds on *Salmonella* biofilm formation. Farm level and animal level *Salmonella* prevalence was (7.6%, 2.3%) in dairy; (14.6%, 4.7%) in poultry; and (42.6%, 4.4%) in swine farms. The prevalence was 7.2% in diarrheic patients from primary health centers and 2.1% from hospitals. *S.*Typhimurium (27.6%) was the most frequently isolated serotype, followed by *S.* Saintpaul (21.7%), *S.* Virchow (18.4%) and *S.* Kentucky (6.6%). *Salmonella* isolation was significantly associated with detection of diarrhea in dairy cattle (p=0.012), and consumption of raw vegetables in humans (OR=1.91, 95% CI=1.29-2.83, χ^2=4.74, p=0.025). Drug resistance was more common in dairy farms in Addis Ababa than outside (p=0.009) and overall antimicrobial resistance was more common in animals than in humans. Clonally related genotypes of *S.*Virchow, *S.*Typhimurium, *S.*Kentucky, *S.*Braendurp and *S.* Miami were circulating among humans and animals as determined by pulsed-field gel electrophoresis (PFGE). MLST analysis showed 3 novel allele types and 5 novel sequence types among 21 strains examined. The dominant beta-lactamase enzyme was *bla*TEM type. *Bla*OXA10 and *bla*CTX-15 were detected only in a single MDR *S.* Concord strain. Double mutation in *gyr*A (Ser83-Phe and Asp87-Gly) as well as *par*C (Thr57-Ser + Ser80-Ile) subunits of quinolone resistance determining region (QRDR) was the primary mechanism for resistance to quinolones and was detected in all *S.* Kentucky isolates resistant to both nalidixic acid and ciprofloxacin from animals (n=8) and humans (n=2). Although decreased susceptibility to ciprofloxacin and/or nalidixic acid was observed in some isolates, no mutation in QRDR nor plasmid mediated quinolone resistance (PMQR) genes were detected. Majority of *Salmonella*

isolates exhibited robust biofilm formation (89%) and displayed red dry and rough (RDAR) morphotype. Detection of class 1 integron was correlated with expression of multicellular behavior and the extent of MDR. Screening of an ATP-mimetic library, gave a single compound (7955004) capable of significant inhibition of *Salmonella enterica* and *Acinetobacter baumannii* biofilm formation. The compound was not bactericidal or bacteriostatic toward *S.* Typhimurium nor cytotoxic to mammalian cells. GroEL and DeoD were found to be the potential protein-binding targets of the compound as identified by ATP-sepharose affinity matrix. Circulation of clonally related NTS serotypes in food animals and humans, abundance of MDR in isolates from food animals, co-dominance of MDR and multicellular behavior in *Salmonella* isolates in the study area, increased the risk of spreading resistant *Salmonella* strains and resistance genes to human population. Integrated surveillance of NTS in humans and animals and implementation of appropriate pathogen control strategy along critical points in food animal production from farm to bench is recommended. The identification of a lead compound with biofilm inhibitory capabilities toward *Salmonella* provides a potential new avenue of therapeutic intervention against *Salmonella* and other bacterial pathogens. Further activity guided evaluation of compound 7955004 and its derivatives with the goal of increasing its potency and broadening its spectrum of activity against additional biofilm forming pathogens should be conducted.

Key words: Antimicrobial resistance, Biofilm, Non-typhoidal *Salmonella*, Prevalence, Serotype

Acknowledgments

First and above all, I praise the almighty God for His all rounded blessings and guidance in my life and for giving me patience and strength throughout this study. I would like to express my sincere gratitude to my supervisors, Dr. Ephrem Engidawork (Addis Ababa University), Prof. John S. Gunn (The Ohio State University), Prof. Wondwossen A. Gebreyes (The Ohio State University) and Dr Daniel Asrat (Addis Ababa University), for continuous encouragement and guidance throughout the study period. I am grateful for time and energy you spent in guiding me to come to this point, starting from the inception of the study and for your unreserved contribution in editing the manuscripts and this dissertation. My many thanks also go to Dr Josephine Birungi and Dr Appolinaire Djikeng of Biosciences eastern and central Africa (BecA), for their close supervision during my stay at BecA, Nairobi. I would also like to thank Dr Johanna Marshall and Dr Geoffrey Gonzalez-Escobedo, for sharing their experience when I was working at the Ohio State University.

This dissertation would not have been successfully completed without the funding provided by The Ohio State University-NIH Fogarty International, through providing one and half year NIH Fogarty fellowship at the Ohio State University where I learned a lot about molecular techniques and did part of the research and for providing laboratory supplies and financial assistance for part of the work conducted in Ethiopia. Biosciences Eastern and Central Africa (BecA) is also highly appreciated for offering me African Biosciences Challenge Fund fellowship through which I was funded for research work and accommodation for 9 months at BecA-ILRI Hub, Nairobi, Kenya where I did most of the molecular characterization work. I would also like to thank US Food and Drug

Administration (FDA) for conducting whole genome sequencing of *Salmonella* isolates. I am also grateful for financial assistance obtained from WHO/AGISAR, and Addis Ababa University for the field work in Ethiopia. My many thanks also go to my home Institute, Aklilu Lemma Institute of Pathobiology for giving me study leave.

I am also grateful for the technical assistance of many individuals during field and laboratory work. Mr Haile Alemayehu, Mr Nega Nigusei, Mr Adane Worku, Mr Aboma Zewude from Aklilu Lemma Institute of Pathobiology and laboratory technicians in health centers and hospitals in Addis Ababa; Mrs Aditi Bhatiya, Mrs Haley Adcox from Gunn lab, Center for Microbial Interface Biology, The Ohio State University; Dr Bayleyegn Molla, Dr Julius Medardus, and Mr Ismael Nanna from Infectious Diseases Molecular Epidmiology Laboratory, Department of Preventive Veterinary Medicine, The Ohio State University, and Mr Moses Njahira, Mrs Martina Kyalo, Mrs Joyce Njuguna from BecA.

Finally, I would like to convey my heartfelt thanks to my family particularly my wife Dr Adey Feleke for shouldering my family responsibilities and continued encouragement throughout my long study period. I am also grateful to my children Deborah and Nathanael for their understanding and encouragement throughout this study period.

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List of Abbreviations

AMR- antimicrobial resistance

BDAR- Brown dry and rough

BLAST- Basic Local alignment search tool

BSAM- Brown smooth and mucoid

BPW- buffered peptone water

CFU- colony forming units

CLSI- Clinical and Laboratory Standards Institute

CRISPR -clustered regularly interspaced short palindromic repeat

2CSTS- two component signal transduction system

CVA-crystal violate assay

ddH_2O -double distilled water

DHPS- dihydropteroate synthase

ECM-Extracellular matrix

FDA- Food and Drug administration

FMHACA- Ethiopian Food, Medicine, Health care Administration and Control Authority

HI-FBS - Heat inactivated fetal bovine serum

LB- Luria-Bertani

LBNS- Luria-Bertani without salt medium

LDH -lactate dehydrogenase

MDR-Multidrug resistant

MLST- Multilocus sequence typing

NTS -Non-typhoidal *Salmonella*

OD- optical density

PBS-Phosphate buffered solution

PCR-Polymerase chain reaction

PFGE- Pulsed-field gel electrophoresis

PKIs- protein kinase inhibitors

PMQR-Plasmid mediated quinolone resistance

QRDR -quinolone resistance determining region

RDAR- Red dry and rough

RVB- Rappaport-Vassiliadis enrichment Broth

SAW- Smooth and white

SCV-*Salmonella* containing vacuole

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

SGI1-*Salmonella* genomic Island1

SPI – *Salmonella* Pathogenicity Island (SPI).

TASH-Tikur Anbessa Specialized Hospital

T3SS - Type three secretion system

TTB -Tetrathionate broth

WGS- whole genome sequencing

WHO- World Health Organization

List of Original Articles

This dissertation is primarily based on the following published articles and manuscripts under preparation, as well as works that are yet to be published. These articles will be referred in the text as **Paper 1, Paper 2, Paper 3, Paper 4, Paper 5** and **Paper 6**.

Paper 1 : Eguale T., Engidawork E., Gebreyes A.W., Asrat D., Alemayehu H., Medhin G., Johnson R.P., Gunn J.S., 2016. Fecal prevalence, serotype distribution and antimicrobial resistance of *Salmonellae* in dairy cattle in central Ethiopia. *BMC Microbiology 16, 1-11.*

Paper 2: **Eguale T**, Gebreyes WA, Asrat D, Alemayehu H, Gunn JS, Engidawork E. 2015. Non-typhoidal *Salmonella* serotypes, antimicrobial resistance and co-infection with parasites among patients with diarrhea and other gastrointestinal complaints in Addis Ababa, Ethiopia. *BMC Infectious Diseases 15 (1), 49.*

Paper 3: Eguale T, Birungi J, Asrat D, Nijahira M, Nana I, Gebreyes WA, Gunn JS, Djikeng A. Engidawork, E. Comparative phenotypic and genotypic characterization of temporally related non-typhoidal *Salmonella* isolated from humans and food animals in central Ethiopia *Manuscript under preparation.*

Paper 4: Eguale T, Birungi J, Asrat D, Nijahira M, Gebreyes WA, Gunn JS, Djikeng A. Engidawork, E. Molecular mechanisms of resistance to beta-lactam and quinolone antimicrobials in non-typhoidal *Salmonella* from humans and animals in central Ethiopia. *Manuscript under preparation.*

Paper 5: Eguale T, Marshall J, Molla B, Bhatiya A, Gebreyes WA, Engidawork E, Asrat D, Gunn JS. 2014. Association of multicellular behavior and drug resistance in *Salmonella*

enterica serovars isolated from animals and humans in Ethiopia. *Journal of Applied Microbiology* 117: 961-971.

Paper 6: Koopman JA, Bhatiya A, **Eguale T**, Kwiek JJ, Gunn, JS, 2015. Inhibition of *Salmonella enterica* biofilm formation using small-molecule adenosine mimetics. *Antimicrobial agents and chemotherapy 59 (1), 76-84*

1. Introduction

1.1 Salmonella overview

1.1.1 General microbiological characteristic of Genus *Salmonella*

Salmonella is a rod-shaped, Gram-negative, oxidase negative, non-spore forming, facultative anaerobic and predominantly motile bacteria. The genus *Salmonella* are approximately 0.7 to 1.5 µm wide and 2.0 to 5.0 µm in length. *Salmonella* can grow between 8 $^{\circ}$C and 45°C, the optimum temperature being 37°C but cannot survive temperature higher than 70°C. Optimum pH ranges from 4 to 9. *Salmonella* produces hydrogen sulfide when grown on agar media; black precipitate being used as the indicator of hydrogen sulfide production (Mastroeni and Maskell, 2006). *Salmonella* ferments glucose, manitol, arabinose, maltose, dulcitol and sorbitol producing acid and gas except *S.* Typhi and *S.* Gallinarum which produce only acid and no gas. They are indole negative, urease negative and citrate positive except *S.* Typhi and *S.* Paratyphi A which are citrate negative as they need tryptophan as growth factor. *Salmonella* decarboxylates the amino acid lysine and arginine, but not glutamic acid. Except *S.* Typhi, all have orithine decarboxylase and paratyphi A lacks lysine decarboxylase (Kumar, 2012).

Most of the members of this genus are motile by peritrichous flagella except *S.* Pullorum and *S.* Gallinarum. *Salmonellae* are frequently facultative intracellular parasites. *Salmonella* are non-capsulated except *S.* Typhi, *S.* Paratyphi C and some strains of *S.* Dublin. These bacteria can resist dehydration for a very long time (Brenner et al., 2000).

1.1.2 *Salmonella* classification and antigenic structure

The genus *Salmonella* obtained its name from the American veterinarian Daniel Elmer Salmon, who first isolated *Salmonella enterica* serotype Choleraesuis from pigs in 1885 (Rabsch *et al.*2003). There are 2 species of *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further classified into 6 subspecies (*enterica*, *salmae*, *arizonae*, *diarizonae*, *hautenae* and *indica*). Most of the *Salmonella* serotypes are part of subspecies *enterica*, and over 99% of human and animal infections are caused by serotypes under this subspecies (Uzzau et al., 2000). Serotypes belonging to *S. bongori* have been found predominantly associated with cold-blooded animals, but it can infect humans (Fookes et al., 2011) (Fig 1).

Salmonella have somatic antigen O, flagellar antigen H, and capsular antigen Vi (only in selected serotypes). Somatic O-antigen is the side-chain of repeating sugar units projecting outwards from the lipopolysaccharide layer on the surface of the bacterial cell wall. O-antigen is a mosaic of two or more antigenic factors. The type and number of these antigens are used to classify *Salmonella* into different serogroups. About 67 different O antigens have been recognized and they are designated by Arabic numerals. The O antigens are heat stable, and are not affected by heating for 2.5 h at 100^{o}C, and alcohol stable withstanding treatment in 96% ethanol at 37°C (Parija, 2009). The H- antigens represent a determinant group on flagellar protein. They are heat labile and alcohol sensitive. *Salmonella* is unique among the Enterobacteriaceae in that it commonly has two distinct phases of H antigens Phase 1 and phase 2.

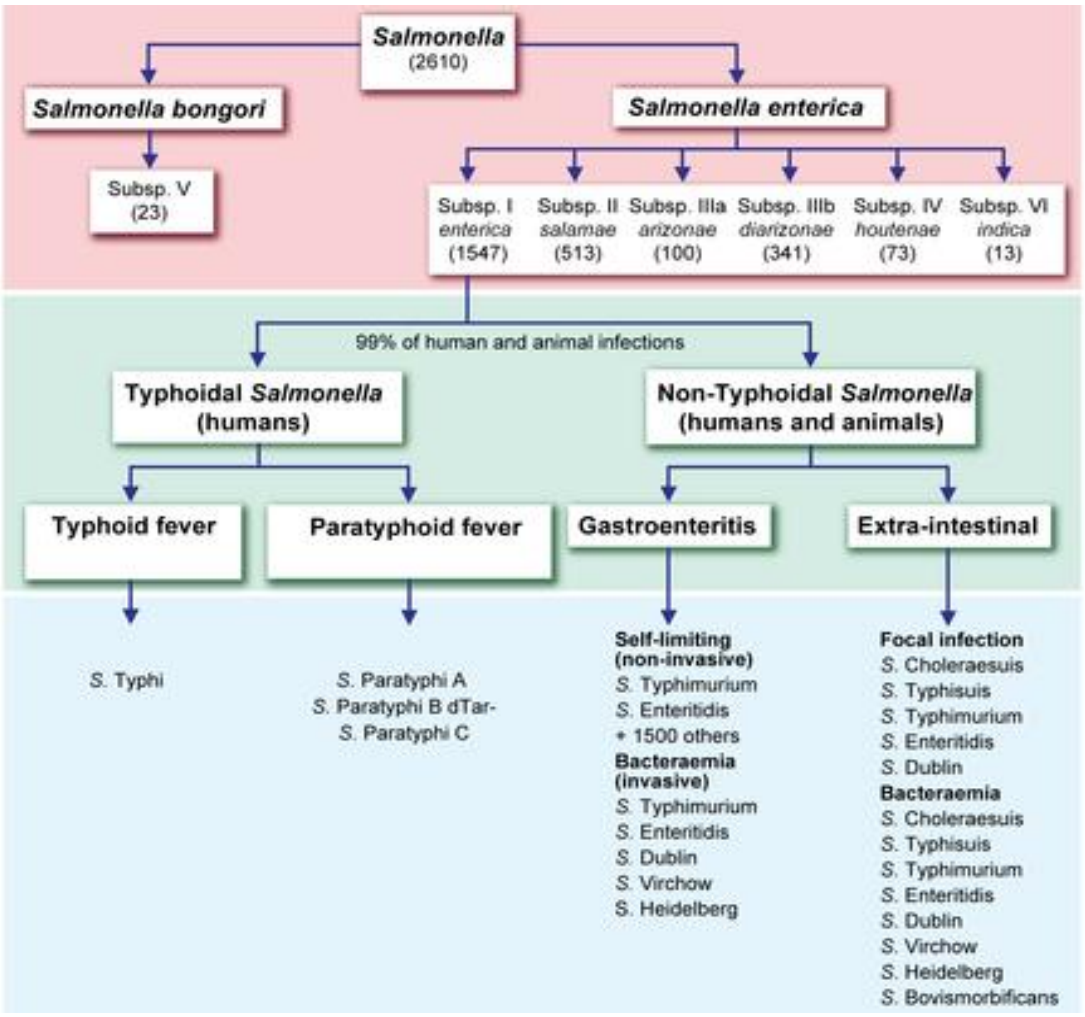

Figure 1 .Classification of *Salmonella*

Adopted from Achtman et al (2012)

The phase 1 protein encoded by *fli*C gene and phase 2 by *fljB* gene are coordinately regulated such that only one phase of flagellar antigen is expressed at a time in a single cell (Smith and Selander, 1991). Some H antigens are composed of multiple antigens, for example, H: e,n,x is the designation for a flagellar antigen that consists of three separate factors, e, n, and x, that occur together in one flagellum . The flagellar antigens of phase I are labeled with lower case letters (a to z and z1 z2 z3 etc.) and phase 2 with a mixture of

lower case letters and Arabic numerals. There are about 114 H variants of antigens (Wattiau et al., 2011).

The antigenic structure of *Salmonella* is described as an antigenic formula which has three parts, describing the O antigens, the phase I and II flagellar antigens. Nomenclature of *Salmonella* serotypes is therefore based on the antigenic formula summarizing the combination of antigens associated with a given serotype. According to Kauffmann-White scheme, each *Salmonella* serotype is recognized by its possession of a particular lipopolysaccharide (LPS) or O antigen and a flagellar or H antigen. For example the antigenic formula for serotype Typhimurium is **1,4,5,12:i:1,2** which means there are 4 types of O-antigens **1,4,5** and **12**; **i** - stands for phase 1 H-antigen; the last **1** and **2** stands for phase 2 H-antigens (Grimont and Weill, 2007). Diagrammatic representation of antigenic structure of *Salmonella* is shown in Fig.2.

The Vi antigen is a homopolymer of N-acetyl galactosaminouronic acid. Almost all strains of *S.* Typhi form Vi antigen as a covering layer outside the cell wall. Identical antigens have also been found in *S.* Paratyphi C and some strains of *S.* Dublin (Parija, 2009).

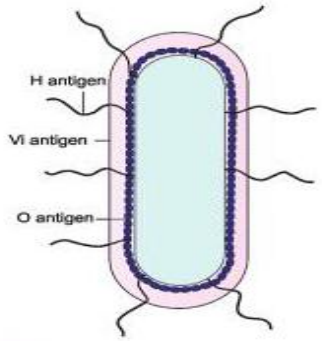

Figure 2. Antigenic structure of *Salmonella*
adopted from (Kumar, 2012)

1.1.3 Source and route of *Salmonella* infection

Salmonella can be acquired from contaminated food, water, contact with infected animals, human-to-human contact and from the environment. The major reservoir for *Salmonella* is the intestinal tract of a wide range of food animals and various food of animal origin (Bartholomew et al., 2014; Bouchrif et al., 2009a; Fey et al., 2000). Plant products contaminated with intestinal content of food animals are also potential sources of *Salmonella* infection (Bayer et al., 2014; Lienemann et al., 2011). The ease with which people can travel between distant countries and the global trade of food items has contributed significantly to the regional outbreak and global spread of *Salmonella* (Davis et al., 2002). Feco-oral route is the primary route of *Salmonella* infection in humans and other animal species. The minimum required infectious dose varies from 30 to more than 10^9 colony forming units (CFU) depending on the food type and serotype involved. *Salmonella* in food with high fat content such as cheese or ice cream needs low infectious dose (Foley and Lynne, 2008; Vought and Tatini, 1998).

1.1.4 Pathogenesis and virulence factors of non-typhoidal *Salmonella*

The outcome of NTS infection depends on bacterial infective dose, virulence of the strain and immune status of the host (van Asten and van Dijk, 2005). Once ingested with contaminated food or water, NTS strains that survived the effect of gastric acid gain access to the intestine inducing inflammatory changes in the intestinal epithelium, including the infiltration of neutrophils and fluid into the intestinal lumen, resulting in inflammatory diarrhea. This inflammatory reaction induces release of tetrathionate which serves as nutritional sources by NTS, providing the pathogen with a growth advantage over the

intestinal microbiota (Zhang et al., 2003). The bacterium induces its own uptake by epithelial cells and by traversing the epithelial barrier through microfold cells (M cells) that overlie intestinal lymphoid tissues known as Peyer's patches. Intracellular lifestyle of *Salmonella* within epithelial cells and macrophages (in sub-epithelial spaces) protects the bacteria from neutrophil-mediated killing which is essential for pathogenesis (Keestra-Gounder et al., 2015; van Asten and van Dijk, 2005).

The pathogenesis of *Salmonella* is primarily due to various virulence genes carried on *Salmonella* Pathogenicity Islands (SPIs). Because of difference in the GC content and presence of remnants of bacteriophage and transposon insertion sequences at the borders of the islands, SPIs are believed to be acquired from other species (Shames et al., 2009). Over 20 different SPIs have been described of which, SPI-1 and SPI-2 are the dominant ones (Blondel et al., 2009). SPI-1 is a 40 KB chromosomal DNA region encoding for a type – three secretion system (T3SS) which is used to inject bacterial effecter proteins such as SipA, SipC, SopB, SopE and SopE2, in to the cytosol of the target cell leading to uptake of the bacterium (Hayward and Koronakis, 2002). After internalization, *Salmonella* establishes an intracellular niche inside a modified phagosome known as the *Salmonella* containing vacuole (SCV). In the SCV, proteins such as SifA, SseF and SseG are involved in the intercellular survival of bacterial cells. These proteins are encoded by the second T3SS carried on SPI-2 (van Asten and van Dijk, 2005).

Several other virulence factors such as those encoded by plasmids; *Salmonella* virulence plasmids (*spv*), (Rotger and Casadesus, 1999; Rychlik et al., 2006b) fimbrial associated

genes (Velge et al., 2012) and flagella-related genes (McGhie et al., 2009) are also important for pathogenesis of *Salmonella.*

1.1.5 Host range and diseases caused by *Salmonella*

Salmonella commonly colonizes a range of animal hosts such as mammals, amphibians, reptiles, birds and insects (Hoelzer et al., 2011). Based on host range, *Salmonella* serotypes are divided into two groups; host adapted and ubiquitous (non-adapted). Host-adapted serotypes usually cause systemic diseases in a limited number of related species. These include *S.* Typhi, *S.* Gallinarum and *S.* Abortusovis. These serotypes almost exclusively cause systemic diseases in humans, birds and ovine respectively. These serotypes are referred to as host restricted serotypes

Some host adapted serotypes such as *S.* Dublin and *S.* Choleraesuis although they are known by causing severe systemic diseases in cattle and pigs respectively; they can also infrequently cause disease in other mammalian hosts including humans. Such serotypes which are prevalent in one particular host species but which can also cause disease in other host species are referred as host-adapted (HA) serotypes. Majority of serotypes such as *S.* Typhimurium and *S.* Enteritidis are capable of causing disease in a wide range of unrelated host species and are referred as host un-restricted serotypes (UR) (Uzzau et al., 2000).

Human infection by *Salmonella* spp. results in two primary clinical manifestations: typhoid fever and gastroenteritis. Typhoid fever is host -specific febrile illness caused by *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A. It affects only humans and transmission

from person to person is through consumption of contaminated food or water with feces of infected person.

Post ingestion of *S.* Typhi, the patient is usually asymptomatic for 7-14 days. Common symptoms of typhoid fever after development of bateremia include progressive fever, headache, abdominal pain, anorexia, coated tongue; tender abdomen, hepatomegaly, and splenomegaly. A few rose spot lesions on the abdomen and chest, approximately 2 to 4 mm in diameter, are also reported in 5 to 30% of cases. Typhoid fever can also lead to severe complications such as gastrointestinal bleeding, intestinal perforation, and typhoid encephalopathy (Parry et al., 2002).

Non-typhoidal *Salmonella* (NTS) gastroenteritis caused by a large number of *Salmonella* serotypes is usually self-limiting and normally do not cause systemic diseases. The most common manifestation of non-typhoidal salmonellosis is mild to moderate gastroenteritis, consisting of diarrhea, abdominal cramps, vomiting and fever. Typically, symptoms of gastroenteritis develop within 6 to 72 h after ingestion of the bacteria (Foley and Lynne, 2008). However, NTS may spread systemically in immunocompromised, young and elderly patients (Dutta et al., 2000). *Salmonella* serotypes responsible for non-typhoidal salmonellosis in human also cause disease in other species or use other hosts as reservoir or transitional hosts.

1.2 Epidemiology of non-typhoidal Salmonella species

1.2.1 Global epidemiology

Non-typhoidal *Salmonella* (NTS) species is ubiquitous in every corner of the world with varying level of prevalence and serotype distribution. Globally, NTS species are a leading bacterial cause of acute gastroenteritis causing estimated 93.8 million cases of gastroenteritis and 155,000 deaths annually (Majowicz et al., 2010). NTS causes most illnesses among the major bacterial enteric pathogens in young children and was shown to be the top among the seven leading foodborne pathogens in USA in causing disability adjusted life year (Scallan et al., 2015). A five-year surveillance in China involving 5 viral, 8 bacterial and 3 protozoal pathogenic etiologies of diarrhea in under five children involving 213 participating hospitals have shown that NTS accounted for 4.3% of the cases. NTS was the 5th pathogen preceded by rotavirus (29.7%), norovirus (11.8%), diarrhogenic *E.coli* (5%) and adenovirus (4.8) (Li et al., 2014; Yu et al., 2015). Another study in China showed that 17.2% of the children with acute gastroenteritis were positive for NTS followed by *Camplylobacter* (7.1%) and *Shigella* (5.7%) (Li et al., 2014; Yu et al., 2015).

In general, the epidemiology of NTS is characterized by the temporal dominance of certain successful clones followed by a decline and replacement with another clone (Lan et al., 2009). Different serotypes have been reported to be dominant in different countries at different times. For example, the dominant serotypes in Shangai, China were *S.* Entritidis followed by *S.* Typhimurium during 2010 and 2011 in children with acute gastroenteritis (Li et al., 2014). In USA *S.* Typhimurium followed by *S.* Entertidis were the dominant serotypes isolated from human during 2000-2005 (Callaway et al., 2008). However , in 2009, the dominant serotype was *S.* Enteritidis followed by *S.* Typhimurium (CDC, 2009), in 2012 *S.* Entritidis was the dominant serotype followed by *S.* Typhimurium (CDC, 2012).

Surveillance data involving 23 European countries between 2006 and 2007 in humans also showed *S.* Entritidis to be the first but with decreasing trend followed by *S.* Typhimurium with fairly consistent trend over time (Hendricksen, 2010).

1.2.2 Epidemiology of non-typhoidal *Salmonella* spp. in Africa and Ethiopia

In sub-Saharan Africa, *S.* Typhimurium is the most prevalent serotype and it is highly invasive (Brent et al., 2006; Graham et al., 2000; Okoro et al., 2012). Study in the Congo indicated that 79% of the serotypes causing invasive NTS recovered from blood of young children were Typhimurium (Lunguya et al., 2013). It was later on confirmed that 96% of these belonged to a specific ST313 (Ley et al., 2014). In Ethiopia, although there are a few studies on *Salmonella* in humans, animals and food of animal origin, there is no integrated surveillance and monitoring to establish the major serotypes responsible for non-typhoidal salmonellosis in humans. Most of the studies conducted in humans were based on diarrheic patients from hospitals and did not involve serotyping of isolates (Ashenafi and Gedebou, 1985; Asrat, 2008; Mache et al., 1997). Those that conducted serotyping indicated that *S.* Concord and. *S.* Typhimurium were the dominant NTS serotypes isolated from patients with diarrheal illness (Beyene et al., 2011; Gebre-Yohannes et al., 1987). Prevalence of *Salmonella* was 11.5% among diarrheic patients in Harrar hospital, east Ethiopia (Reda et al., 2011), 10.5% among diarrheic patients in Butajira health center, west Ethiopia (Mengistu et al., 2014). In study conducted in pediatric diarrheic outpatients in Addis Ababa and Jimma (west Ethiopia), prevalence of 5.3% was reported (Beyene et al., 2011) and 4.5% among diarrheic adult outpatients from various hospitals and clinics (Ashenafi and Gedebou, 1985).

The major problem in ascertaining the real situation of *Salmonella* in a country and especially in developing countries like Ethiopia is the lack of coordinated surveillance. Even where there is a laboratory-based surveillance system, it provides only trend information underestimating disease burden (Flint et al., 2005; Hall et al., 2005). The laboratory based surveillance system uses the results of patients who sought medical care, submitted a specimen, for whom the laboratory tested for the pathogen, reported positive findings and the result reported to the public health authorities and it does not take into account the patients who did not pass through all these steps. This shows that laboratory-based surveillance represents only fraction of the prevalence of the disease at the community level (Majowicz et al., 2010).

1.2.3 Risk factors for non-typhoidal salmonellosis

Different factors such as contaminated feed and drinking water (Paul and U., 2013), rodent infestation of the farm (Davies and Wray, 1995) exposure of animals to stress factors (Holt et al., 1995), open- sided housing conditions, large herd/flock size (Davison et al., 2006), geographical location and feeding brewers' products (Kabagambe et al., 2000) were reported to be associated with increased occurrence of farm level *Salmonella* infection in food animals. The major risk factors for human salmonellosis are consumption of contaminated food products, mainly those of animal origin such as poultry, egg, beef, pork and dairy products, as well as direct contact with infected animals (Bartholomew et al., 2014; Bouchrif et al., 2009b; Fey et al., 2000). Consumption of plant produces contaminated with *Salmonella* was also reported to be associated with *Salmonella* infection in some countries (Bayer et al., 2014; Jackson et al., 2013; Lienemann et al., 2011). Travel to Sub-Saharan

African counties and Middle East was also reported to be high risk factors to be hospitalized with NTS bacteremia in developed countries (Ekdahl et al., 2005; Koch et al., 2011). Drinking untreated water from private wells and recreation in surface waters, were also reported to be risk factors for sporadic salmonellosis in children (Denno et al., 2009).

1.3 Laboratory diagnosis and typing of Salmonella

1.3.1 Phenotypic methods

1.3.1.1Culture and Biochemical tests

Various enrichment and selective media are used to isolate *Salmonella* from different clinical specimens. NTS is commonly cultured from stool specimen. But when bacteremia is suspected, blood culture is indicated (Feasey et al., 2012). The best recovery of *Salmonella* species from fecal samples can be achieved by inoculating on enrichment broths such as Rappaport-Vassiliadis (RV) and Tetrathionate broth (TTB) and plating on one or more selective agar plates such as Xylose Lysine Tergitol 4 (XLT-4), Xylose Lysine Deoxycholate agar Agar (XLD), Deoxycholate agar (DCA) and Brilliant green agar. The common biochemical tests used to confirm the suspected *Salmonella* colonies are Lysine Iron agar, urea, citrate and triple sugar iron agar tests. Most of the NTS serotypes are urease negative and citrate positive. Isolation and identification of *Salmonella* take 4–7 days. Sensitivity of cultures can also be affected by antibiotic treatment, inadequate sampling, and a low number of viable organisms in feces (Miller and Mallinson, 2000).

1.3.1.2. *Serotyping and phage typing*

For the efficient epidemiological investigation of *Salmonella enterica,* detailed strain identification is essential. Serotyping has been used for a long time to type *Salmonella. Salmonella* serotyping is usually conducted by slide agglutination test using different *Salmonella* antisera produced against O-antigens followed by agglutination test with known antisera of phase1 and phase 2 flagellar (H) antigens. The disadvantages of serotyping include low throughput, high expense, and a requirement for considerable expertise and numerous antibodies (Achtman et al., 2012). Although serotyping cannot provide a sensitive fingerprint for tracing during an outbreak and to define phylogenetic relationships, it is still commonly used as an initial screening, followed by molecular subtyping to identify outbreak-related strains (Wattiau et al., 2011).

Phage typing is used to discriminate between *Salmonella* strains belonging to the same serotype. *Salmonella* phage types are assigned based on the ability of a given phage to lyse the investigated strain. *Salmonella* isolates that reacted with the phages but did not conform to any recognized phage type are designated atypical (AT), while isolates that do not react with any of the typing phages are designated untypable (UT) (Anderson et al., 1977). Phage typing is available only for a limited number of serotypes such as *S.* Typhimurium, *S.* Entertidis and few other serotypes.

1.3.1.3 Anti-biogram

Strains of *Salmonella* can also be typed based on their susceptibility profile to different antimicrobial drugs. It is common practice in clinical microbiology laboratory. Based on the level of previous exposure to different antimicrobial agents, strains of *Salmonella* might be susceptible, intermediate or resistant to different antimicrobial agents (CLSI, 2013). It has

poor discriminatory power, because epidemiologically related strains may have different antimicrobial susceptibility due to loss of plasmids carrying drug resistance genes or acquisition of new genetic materials over time. Isolates that are not genetically and epidemiologically related might also have similar anti-biogram phenotype (Singh et al., 2006).

1.3.2 Molecular typing

The modern typing methods are based on characterization of the genotype of the organism. The basis behind this typing system is that epidemiologically related isolates are derived from the clonal expansion of a single ancestor and share characteristics that differ from those of epidemiologically unrelated isolates. The usefulness of a particular genotypic characteristic for typing is related to its stability within a strain and its diversity within the species, reflecting the evolutionary genetic diversity arising from random, nonlethal mutations over time (Liebana et al., 2001)**.** Serotyping together with one or more molecular typing techniques, appear to provide the most reproducible and comparable discrimination of epidemiologically linked isolates (Harbottle et al., 2006). These methods are based on restriction endonuclease digestion, DNA amplification, DNA sequencing and gel electrophoresis techniques. The common molecular techniques used for *Salmonella* typing include pulsed-field gel electrophoresis (PFGE), Multilocus sequence typing (MLST), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), Insertion sequence typing and Plasmid profiling (Singh et al., 2006).

1.3.3.1Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is the technique by which the whole genome of bacteria is digested by rare-cutting restriction endonuclease enzymes and run through agarose gel subjecting them to electric fields that alternate in two directions at defined intervals. The commonly used restriction endonuclease in PFGE for *Salmonella* is XbaI. The PFGE yield different number of DNA fragments with strain specific patterns (Ribot et al., 2006). Several reports have shown that PFGE is highly discriminatory in successfully tracking the source of *Salmonella* infections for outbreak investigation as well as for epidemiological studies (Farias et al., 2014; Harbottle et al., 2006; Liebana et al., 2001). PFGE has been considered as the "gold standard" genotyping technique for molecular typing of *Salmonella* and is being used by CDC pulseNet program (Harbottle et al., 2006). The other advantage of PFGE is the development of bionumeric software which enables the analysis of gel images of the PFGE finger prints. However, PFGE is time-consuming and labor-intensive and does not display equal sensitivity with different serotypes (Kérouanton et al., 2007).

1.3.3.2 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is a molecular typing strategy that compares DNA sequences from portions of housekeeping genes which varies due to mutation or recombination events. MLST based on fragments from 7 housekeeping genes was first developed in 2002 to study the clonality of *S.* Typhi. The 7 housekeeping genes were *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*. The basis for selection of these genes was due to their scattered positions on the chromosome, the presence of genes with known functions in the surrounding DNA, and lack of any diversifying selection pressure.

Nucleotide differences in the individual genes leading to different alleles are combined and used to determine the differentiation of strains (Kidgell et al., 2002). MLST is useful for long-term epidemiological studies or phylogenetic analyses.

Later on, different schemes of MLST i) based on 4 polymorphic genes (16S RNA, *pduF*, *glnA*, and *manB*) with efficient serotype discriminating power (Kotetishvili et al., 2002); ii) based on variable genes (*manB*, *fimA*, and *mdh*) proved efficient for discriminating below serotype level (Alcaine et al., 2006; Sukhnanand et al., 2005); iii) based on prophage loci with more discriminatory power than PFGE or MLST based on housekeeping genes (Ross and Heuzenroeder, 2005); iv) MLST scheme based on virulence genes (*sseL* and *fimH*) and two clustered regularly interspaced short palindromic repeat (CRISPR) loci with capacity to differentiate outbreak strains/clones of the major serotypes of *Salmonella* were developed (Liu et al., 2011).

However, the first MLST scheme developed by (Kidgell et al., 2002) is more popular and is widely employed mainly because of the fact that it has central online public data base enabling global comparison of allele and sequence types hosted by the University of Warwick (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). The major limitations of MLST are the time and cost associated with laboratory work to amplify, determine, and proofread the nucleotide sequence of the target DNA fragments (Wattiau et al., 2011).

1.3.3.3 Plasmid profiling

Plasmid profile analysis was one of the earliest DNA-based subtyping schemes. The detection method is based on the isolation of plasmids followed by agarose gel

electrophoresis and gel imaging (Liebana et al., 2001). Its importance lies on the fact that most of the plasmids harbour virulence and antimicrobial resistance genes in *Salmonella*. The number and size of plasmid contained in an isolate is the basis for plasmid profiling. The different plasmid profiles within a serotype points the lateral transfer by gaining or losing the plasmids. The plasmids with 2–200 kb with different functions have been reported in *Salmonella* (Rychlik et al., 2006a). The major limitations of plasmid profiling is that plasmids can be lost and acquired during outbreak/storage, they can appear in different forms (linear, open circular, closed circular) which affects the migration of the DNA in agarose gel. Strains may also contain more than one plasmid species of the same size (Liebana et al., 2002).

1.4 Antimicrobial resistance

The extensive use of antimicrobials in human and veterinary medicine has created selection pressure and promoted the emergence and spread of antimicrobial resistant pathogens worldwide. Resistant organisms and genes responsible for development of resistance can easily circulate among humans, animals, food, water and the environment in a country and globally through trade, travel and migration at an unprecedented pace. To date, most of the pathogenic bacteria of animal and human origin have developed resistance to first line antimicrobial agents, including extended-spectrum cephalosporins, aminoglycosides, and fluoroquinolones. The recent WHO report indicated that this serious threat is no longer a prediction for the future and the damage caused by resistant bacteria is already happening in every region of the world (WHO, 2014b). This report emphasized that unless urgent coordinated action is taken, the world is heading to a post-antibiotic era, in which common

infections and minor injuries which have been treatable for decades can once again kill. In June 2014, the Ministerial Conference on Antibiotic Resistance that took place in the Netherlands, made a global call to take action on antimicrobial resistance, acknowledging it as a global threat to effective prevention and treatment of infections (WHO, 2014a).

1.4.1 Antimicrobial agents mechanism of action and resistance in *Salmonella*

Antimicrobials are classified according to their mechanism of action. The mechanisms of action for the major classes of antimicrobial agents include interference with cell wall synthesis (e.g., beta-lactams and glycopeptides), inhibition of protein synthesis (aminoglycosides, macrolides and tetracyclines), interference with nucleic acid synthesis (fluoroquinolones) and inhibition of a metabolic pathway (trimethoprim-sulfamethoxazole). Bacteria may be intrinsically resistant to one or more class of antimicrobials, or may acquire resistance by *de novo* mutation or via the acquisition of resistance genes from other organisms or from the environment. Acquired resistance genes may enable a bacterium to produce enzymes that destroy the antibacterial agent, to express efflux systems that prevent the drug from reaching sufficient concentration in its intracellular target, to modify the drug's target site, or to produce an alternative metabolic pathway that bypasses the action of the drug (Tenover, 2006). Below, the common antimicrobial agents and their mechanism of resistance are briefly discussed.

1.4.1.1 Resistance to beta-lactams

Beta-lactams are a large class of antimicrobials that have a beta-lactam ring in their structure and they are the most widely used antimicrobials in human and veterinary medicine. Beta-lactams include penicillin derivatives, cephalosporins, monobactams, and carbapenems.

Mode of action of beta-lactams is via penicillin binding proteins (PBPs) by inactivating the final stage of cell wall synthesis through acylation of the terminal amine group of one peptide chain onto the D-alanine residue of the next peptide chain. Resistance to beta-lactams in Gram-negative bacteria including *Salmonella* involves mainly the production of beta-lactamase enzymes capable of hydrolyzing beta-lactam ring (Bush et al., 1995b), followed by alterations of permeability, extrusion by efflux pumps, and to a lesser extent through alteration in penicillin binding protein (PBP)(Beceiro et al., 2013).

There are two ways of classifying beta-lactamases based on protein sequence similarity or based on functional characteristics. Based on protein sequence, beta-lactamases are classified in to four molecular classes A, B, C, and D. Classes A, C, and D also called (serine beta-lactamases) include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B beta-lactamases are metalloenzymes that utilize at least one active-site zinc ion to facilitate beta-lactam hydrolysis. Functional classification is based on substrate and inhibitor profiles in an attempt to group the enzymes in ways that can be correlated with their phenotype in clinical isolates. This involves group 1 (class C cephalosporinases) inhibitor resistant; Group 2 (classes A and D) broad-spectrum, inhibitor-resistant, and extended-spectrum serine carbapenemases; group 3 involves metallo beta-lactamases (Class B) (Bush and Jacoby, 2010; Bush et al., 1995a).

The enzymes discovered early such as TEM-1 and SHV-1 beta-lactamases were capable of inactivating few penicillins and first generation cephalosporins. But subsequent variants emerged with a range of amino acid substitutions in and around their active sites with

capacity to hydrolyze multiple beta-lactams including extended spectrum beta-lactams. To date, there are 222 different variants of TEM and 191 variants of SHV beta-lactamases (http://www.lahey.org/Studies/). Widespread use of third generation cephalosporins and aztreonam is believed to be the major cause of mutations in these enzymes that has led to the emergence of the extended spectrum beta-lactamases (ESBLs) (Paterson and Bonomo, 2005). In addition, there are several other variants such as OXA, CTX-M, CMY, and KPC with variable level of beta-lactam hydrolyzing capacity. Several different beta-lactamases have been reported from *Salmonella* in different countries including extended spectrum beta-lactamases capable of hydrolyzing third generation cephalosporins such as CTX-M, OXA, CMY, AmpC and others with different substrate specificity and geographic distribution (Carattoli, 2008; Li et al., 2007; Seiffert et al., 2014).

1.4.1.2 Resistance to aminoglycosides

The mechanism of action of aminoglycosides is related to binding to the 30S subunit of ribosomal RNA, which leads to loss of translational fidelity and thus to the accumulation of erroneous proteins and bacterial cell death. Resistance to amino glycosides is usually due to production of aminoglycoside-modifying enzymes (acetyl transferases, AACs; adenyl transferases, AAD (Barton Behravesh et al., 2011) and phosphoryltransferases (APHs) (Smith and Baker, 2002). Aminoglycoside phoshotransferases, Aph (6)-Ia (also called *strA*) and aph (6)-Id (*strB*) are reported to be widely distributed in *Salmonella* and other Gram-negative bacteria. These genes have been reported to be part of transposon Tn5393 (Pezzella et al., 2004). Genes encoding for aminoglycoside resistance modifying enzymes are often located on different mobile genetic elements such as plasmids and integrons and they are

usually co-selected with other antibiotic resistance genes, such as those encoding for beta-lactamases (Beceiro et al., 2013; Shakil et al., 2008).

1.4.1.3 Resistance to tetracycline

Tetracyclines are bacteriostatic antibiotics that bind reversibly to the 30S subunit of ribosome, thereby inhibiting the initiation of protein synthesis (Beceiro et al., 2013). The main resistance mechanisms for tetracycline are mono-component efflux systems specific for tetracyclines encoded by the *tet* genes. There are several types of *tet* genes. The most common types reported in *Salmonella* are *tetA, tetB, tetC, tetD* and *tetG*. The *tetG* gene has been identified in chromosome of *S.* Typhimurium DT104 located in *Salmonella* genomic island 1(SGI1) (Pezzella et al., 2004). Other mechanisms of resistance to tetracyclines are ribosomal protection and antibiotic modification (*tetX*). Efflux resistance genes and genes involved in ribosome protection are generally found on plasmids and self-transmissible chromosomal conjugative transposons, respectively (Speer et al., 1992).

1.4.1.4 Resistance to sulfonamides and trimethoprim

The target of sulfonamides and the basis for their selectivity is the enzyme dihydropteroate synthase (DHPS) in the folic acid pathway. Mammalian cells are not dependent on endogenous synthesis of folic acid and generally lack DHPS. Trimethoprim inhibits dihydrofolate reductase, the enzyme required to convert dihydrofolic acid to tetrahydrofolic acid, the precursor of folic acid. The mechanism of resistance to sulfonamides and trimethoprim is due to the horizontal spread of resistance genes; variants of the target enzymes dihydropteroate synthase and dihydrofolate reductase characterized by reduced

affinity to sulfonamides and trimethoprim, respectively. Two genes, *sul1* and *sul2*, encoding for drug insensitive dihydropteroate synthase, mediated by transposons and plasmids, induce high level resistance to sulfonamide in human clinical isolates while *sul3* was found to be prevalent in farm animals. *Sul1* is frequently associated with mobile genetic elements such as class 1 and 2 integrons and SGI1 (Perreten and Boerlin, 2003). For trimethoprim, several *dfr* genes expressing trimethoprim insensitive dihydrofolate reductases have been characterized (Sköld, 2001).

1.4.1.5 Resistance to Fluoroquinolones

Fluoroquinolones are potent, broad-spectrum antibiotics used to treat infections caused by wide range of Gram-positive and Gram-negative pathogenic bacteria. Fluoroquinolones differ from quinolones by the replacement of the eighth carbon atom of the backbone with a nitrogen atom and the addition of a fluorine atom at the sixth position, giving them more potent antibiotic action and a broader spectrum of activity. Fluoroquinolones target type II topoisomerase (DNA gyrase) mainly in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria. DNA gyrase contains two subunits *gyrA* and *gyrB* whose function is to relax the supercoiled DNA ahead of DNA replication fork by creating negative supercoiling. Topoisomerase IV contains *parC* and *parE* subunits with the function of decatinating (unlinking) replicated double stranded DNA (Drlica et al., 2008).

Fluroquinolones inhibit this activity by binding to the active sites of these enzymes leading to inhibition of DNA replication and cell death. The major mechanism of resistance to fluoroquinolones is through mutation in quinolone resistance determining region (QRDR) of

DNA gyrase and topisomerase IV subunits mainly *gyrA* and *parC* (Redgrave et al., 2014). The other mechanisms of quinolone resistance include efflux pumps (multidrug efflux pump and quinolone specific plasmid mediated efflux pump encoded by *qep* gene), *Qnr* (plasmid-mediated quinolone resistance), porins, and quinolone-modifying enzyme(aac(6')-Ib-cr) (Redgrave et al., 2014). Plasmid-mediated quinolone resistance determinants, specially *qnr* genes, has recently been reported from different parts of the world in different Gram-negative organisms including *Salmonella* (Jacoby et al., 2014). These genes encode for pentapeptide proteins which protects bacterial topoisomerase from the effect of quinolones. They do not induce high level resistance but their presence leads to mutation in the QRDR(Robicsek et al., 2006).

1.4.2 Mobile genetic elements and their role in dissemination of drug resistance

Acquired antimicrobial resistance genes are frequently contained within a mobile DNA segment that is capable of translocation from one part of a genome to another or between genomes. These mobile elements could be conjugative and mobilizable elements. Transduction, the process by which drug resistance determinant bacterial DNA is packaged in to bacteriophages and injected into the recipient bacterium also play a role in the spread of DNA between bacteria. Bacteria can also acquire resistance genes by a process called transformation where naked DNA is taken up by the recipient bacteria and either incorporated into the host genome by homologous recombination or transposition (van Hoek et al., 2011).

1.4.2.1Plasmids

Plasmids are extrachromosomal DNA elements that contain their own origin of replication. Plasmid mediated resistance is the dominant means of resistance acquisition in bacteria and is responsible to the rapid spread and accumulation of the prominent resistance mechanisms leading to a concomitant challenge to antibacterial therapy. Many plasmids carrying antimicrobial resistance genetic elements are capable of transferring to other organisms within the same or different species in addition to vertical transfer to the cell's progeny increasing the potential for the spread of resistance genes carried on plasmids (Smillie et al., 2010).

1.4.2.2 Integrons and Salmonella genomic island

Integrons are genetic units capable of capturing and incorporating diverse resistance genes into the bacterial genome via site-specific recombination mediated by the *intI*-encoded integrase (Hall and Stokes, 1993). Class 1 integrons play an important role in the global spread of antibiotic resistance by aiding the transfer of cassettes encoding multidrug resistance throughout the Gram-negative bacterial population while class 2 integrons are commonly detected in Gram-positive organisms although it can also be found in Gram-negative bacteria (Fluit and Schmitz, 1999). In *Salmonella* one or more class I integrons can be found in an isolate carrying one or more drug resistance genes. Class I integron is often associated with MDR (Leverstein-Van Hall et al., 2002).

Salmonella Genomic island 1 (SGI1) is a 43-KB mobilizable complex class 1 integron of 44 open reading frames located on chromosome in *Salmonella enterica* serotypes between

the *thdF* and *yidY* genes or between *thdF* and *int2* in the retron sequence of *S.* Typhimurium. The antibiotic resistance genes on this island have been localized to a 13-kb segment of the 3'end of SGI1 termed the MDR region. The first classical SGI1 reported from *S.* Typhimurium DT104 confers resistance to 5 drugs: ampicillin (*bla*PSE), chloramphenicol/florfenicol (*floR*), streptomycin/spectinomycin (*aadA2*), sulfamethoxazole (*sul1*), and tetracycline *tet*(G). This resistance cluster is made up of two class 1 integrons carrying *bla*PSE and *aadA2* with variable regions of 1.2 kb and 1.0 kb, respectively and *tet*(G) and *flo*(R) genes located in between the two integrons (Boyd et al., 2000). Letter on, several variants of SGI1 with different antimicrobial resistance gene clusters were detected worldwide in different *Salmonella* serotypes and in *Proteus mirabilis* (Ahmed et al., 2007; Doublet et al., 2003). A previous report indicated an association between strong biofilm formation and the presence of a complex class I integron found within SGI1 in *Salmonella enterica* (Malcova et al., 2008).

1.4.3 Use of antimicrobial agents in food animals and its human health implication

Globally, more than half of all antimicrobials are used in the production of food animals. Antimicrobial agents are used in animal production in three ways: therapy, prophylaxis and for growth promotion (Schwarz et al., 2001). The use of antimicrobial agents for management of sick animals is inevitable. Although prophylactic herd treatment is criticized for its role on the selection of resistance among pathogenic bacteria, antimicrobial prophylaxis at the key periods of disease incidence is highly beneficial. The most critical one is the use of antimicrobial agents for growth promotion. Several countries have already banned the use of antimicrobial agents as feed additives (Castanon, 2007; Phillips et al.,

2004). The mode of action of antimicrobial growth promoters is not fully established. However, it is believed to be a reduction of the growth of bacteria in the intestinal tract and thereby less microbial degradation of useful nutrients, and the prevention of infections with pathogenic bacteria (Dibner and Richards, 2005).

Although it is widely accepted that overuse of antimicrobial agents in veterinary medicine can be a major driver of resistance in microbes (McEwen and Fedorka-Cray, 2002), the evidence linking antibiotic use in farm animals with resistance in humans is still controversial (Gilbert, 2012; McEwen, 2012). A review of published data in the area by group of workers indicated that the actual danger of using antibiotics in animal agriculture is small. Their argument is that though resistance can be selected in food animals, and resistant bacteria can contaminate animal-derived food, adequate cooking can destroy them (Phillips et al., 2004). Risk assessment study also indicated withdrawing animal antibiotics can cause far more human illness-days than it would prevent (Cox and Popken, 2006).

1.4.4 *Salmonella* and antimicrobial resistance in Ethiopia

Studies conducted in Ethiopia show the prevalence of several serotypes of *Salmonella* in various food animals, food products (Addis et al., 2011; Alemayehu et al., 2003; Molla et al., 2004; Molla et al., 2006; Zewdu and Cornelius, 2009) and humans (Ashenafi and Gedebou, 1985; Asrat, 2008; Gebre-Yohannes et al., 1987; Mache et al., 1997) and high level of drug resistance has also been reported. For instance, 75%, 59.4%, 46.9%, 40.6%, and 40.6% of NTS isolates from food items and personnel in Addis Ababa were resistant to streptomycin, ampicillin, tetracycline, spectinomycin and sulfisoxazole, respectively (Zewdu

and Cornelius, 2009). On the other hand among NTS human isolates from Addis Ababa, 81.2%, 86.4%, 83.7%, 100%, 75.6%, 37.8%, 81.1%, 94.5%, were reported to be resistant to ampicillin, cephalothin, chloramphenicol, erythromycin, gentamycin, nalidixic acid, sulphonamide, and tetracycline respectively (Asrat, 2008).

Most recently, MDR and invasive strain of *S.*Concord was reported to be the major cause of salmonellosis in young children in Ethiopia (Beyene et al., 2011). This serotype had been repeatedly reported from children adopted from Ethiopia to different European countries and the USA (Hendriksen et al., 2009). This serotype is not common in other countries and most of the countries who reported this strain have direct or indirect contact with Ethiopians. Out of the 78 people from whom *S.*Concord was isolated during the year 2003-2007 in Europe and USA, 98% were children adopted from Ethiopia. Eighty one percent of the isolates were reported to be resistant to 3 or more drugs (Hendriksen et al., 2009). Originally *S.* Concord was suspected to be localized to specific orphanages where these children spent some of their time in Ethiopia. However, the report by Beyene et al (2011) indicated that the strain is the most prevalent, multidrug resistant *Salmonella* responsible for diarrhea in children in two geographically unrelated hospitals in the country. According to Beyene et al (2011) most of *S.* Concord isolates (70%) were resistant to several antibacterial agents (sulfamethoxazole+trimethoprim, ceftriaxone, chloramphenicol and gentamycin). Thirty percent of the isolates were reported to be invasive.

1.5 Salmonella biofilm formation and its implication

Bacterial biofilms are defined as a collection of aggregated microbial sessile communities that are bound to a surface and embedded in a self-produced polymeric matrix (Costerton et al., 1999; Vestby et al., 2009a). Extracellular structures of *Salmonella* that contribute to biofilm formation include curli fimbriae, cellulose, colanic acid, O-antigen capsule, extracellular DNA and lipopolysaccaride (LPS). *Salmonella* biofilms have been reported to occur in the intestine and on gallstones of the gallbladder in addition to its occurrence on environmental inanimate objects (Crawford et al., 2008; de Rezende et al., 2005; Solano et al., 2002). Formation of *Salmonella* biofilms on plants leading to persistence of the organism on plant surfaces and resistance to disinfectants are reported to be the major causes of plant product associated outbreaks (Yaron and Römling, 2014). It is estimated that 99.9% of the bacteria in nature are attached to a surface in the form of a biofilm (Murphy and Kirkham, 2002). Strains capable of biofilm formation frequently form colonies of the RDAR (red, dry and rough) morphotype which is caused by the co-expression of curli fimbriae and cellulose, BDAR (brown dry and rough) which produce curli but not cellulose, PDAR (pink dry and rough) which produce cellulose but not curli. Those strains which cannot produce biofilms are characterized by SAW (smooth and white) morphotype (Malcova et al., 2008; Römling, 2005).

Different serotypes of *Salmonella* have been reported to vary in biofilm forming ability. A study also indicated a correlation between biofilm forming ability and persistence in fish meals and feed factories suggesting biofilm forming ability to be an important factor for persistence of *Salmonella* in the factory environment (Vestby et al., 2009c). The planktonic and biofilm cells of MDR *Staphylococcus aureus* and *S.* Typhimurium were reported to be

more resistant to beta-lactams than antibiotic susceptible *S. aureus* and *S.* Typhimurium at pH 5.5 and pH 7.3 and they have shown differential gene expression (He, 2011). Other reports also indicated direct association of biofilm formation with the increase in antibiotic resistance of bacteria (Kim and Wei, 2007; Kwon et al., 2008).

1.5.1 Methods to inhibit bacterial biofilm formation

Bacterial biofilms are relatively resistant to antibacterial drugs, disinfectants and the host immune system compared to planktonic cells (Fux et al., 2003; Hoyle and Costerton, 1991; Janssens et al., 2008). Various efforts are being made to find chemicals that can inhibit biofilm formation or eliminate the formed bacterial biofilm to enhance the susceptibility of bacteria to antibacterial drugs. Some of these are enzymes that can degrade exopolysaccaride or extracellular matrix such as DNAse (which degrade the extracellular DNA), and other novel compounds from chemical libraries (Jiang et al., 2011; Tetz et al., 2009).

1.5.2 Kinase inhibitors as potential antibiofilm agents

Kinases are components of signal transduction pathways that are critical in the biology of all cells. Because of this role, kinases are established drug targets, particularly for cancer. Recent works in the area have led to approval of several kinase inhibitors by FDA for cancer treatment (Zhang et al., 2009). Kinases are thus anticipated to serve as new targets for therapeutics aimed at drug resistant pathogens. Protein phosphorylation in bacteria is performed mainly by two component signal transduction systems (2CSTSs) and eukaryotic-like serine/threonine kinases or bacterial tyrosine kinases. 2CSTSs occur in most bacterial species, and composed of an environmental sensing protein (histidine kinase) and a DNA-

binding protein (the response regulator). Histidine kinases are composed of two domains: an N-terminal signal input domain that often possesses sub-domains with recognized signaling functions and a C-terminal autokinase domain that includes phosphotransferase and an ATP-binding sub-domain. The response regulators are composed of a conserved N-terminal regulatory domain and a C-terminal effector domain (Stock et al., 2000). In the prototypical two-component system, a sensor histidine kinase catalyzes its autophosphorylation and then subsequently transfers the phosphoryl group to a response regulator, which can then effect changes in cellular physiology, often by regulating gene expression (Wolanin et al., 2002).

Studies have shown signal transduction phosphorelay networks as key components of the regulatory machinery that coordinates gene expression during biofilm development in response to environmental signals (Zakikhany et al., 2010). *Salmonella* spp. contains numerous well-characterized two-component regulatory systems such as SirA/BarA, EnvZ/OmpR, SsrA/SsrB, PhoP/PhoQ PreA/PreB, and PmrA/PmrB which are involved directly or indirectly in regulating genes involved in invasion, antimicrobial peptide resistance and biofilm formation (Prouty and Gunn, 2000). Bacteria with an incomplete lipopolysaccharide (LPS), those with defect in motility, and those deficient in quorum sensing are unable to form biofilms (Prouty and Gunn, 2003).

1.6 Rationale for the study

Consumption of raw milk, inadequately pasteurized milk, improperly cooked beef from culled dairy cattle, contaminated water and direct animal contact are the major routes of acquiring dairy associated salmonellosis in human (Fey et al., 2000). In Ethiopia, there are

large numbers of small-scale animal farming mainly peri-urban dairy farms situated close to areas of public residence. Most of these farms are located very close to Addis Ababa, capital city of the country, or reside within the city in a very close proximity with human populations. Previous *Salmonella* prevalence study conducted in Addis Ababa showed farm level and animal level prevalence to be 47.8% and of 7.7% respectively (Addis et al., 2011). However, this study involved small sample size and the isolates were not serotyped. Information on the prevalence, serotype distribution and antimicrobial susceptibility of *Salmonella* in dairy farms and other food animals is vital to implement appropriate strategies to prevent introduction and spread of the pathogen in the farm as well as to reduce the risk of human salmonellosis.

Human infections with *Salmonella* spp. result in significant morbidity, mortality and economic burden worldwide. Salmonellosis constitutes a major public health burden and represents a significant cost in many countries. Millions of human cases are reported worldwide every year. The primary sources of human *Salmonella* infection are food-producing animals such as cattle, poultry and swine (Kagambèga et al., 2013). The sources and transmission routes of *Salmonella* in Ethiopia are poorly understood due to lack of coordinated national epidemiological surveillance system. *Salmonella* serotypes commonly affecting humans and the relative contribution of various risk factors as a source of *Salmonella* infection to humans is not clearly understood. A recent report also indicated the increasing prevalence of invasive drug resistant *S.* Concord in two regions of the country (Beyene et al., 2011). Despite wide occurrence of this MDR serotype in human patients, so far, no information is available on its occurrence in domestic animals. Most of prevalence

studies conducted in humans involved diarrheic children from hospitals (Ashenafi and Gedebou, 1985; Asrat, 2008; Beyene et al., 2011) and little is known on the prevalence, serotype distribution and antimicrobial susceptibility of *Salmonella* isolates from primary health centers.

Over use and misuse of antimicrobial agents in humans and animals is reported to be the major contributing factors for increasing the rate of selection for resistant strains. In Ethiopia, antimicrobial agents are indiscriminately used in both humans and animals and high level of resistant *Salmonella* has been reported in both sectors (Alemayehu et al., 2003; Beyene et al., 2011; Mache et al., 1997; Molla et al., 2004; Molla et al., 2006). The fact that animals and humans live in close proximity; absence of strict adherence to drug withdrawal in veterinary medicine, indiscriminate use of antimicrobial agents in both humans and animals as well as consumption of raw and under cooked meat and other animal products suggests possibility of transfer of resistant strains/genes from animal to humans or vice versa.

Despite reports of occurrence of drug resistant *Salmonella* from animal products and humans, the phenotypic and genotypic relatedness of *Salmonella* isolated from animals and humans is not established. Research into such relationships will provide information on transfer of resistant pathogens between animals and humans and the genes responsible for specific antimicrobial resistance. Characterization of spatially and temporally related *Salmonella* serotypes isolated from humans and animals using phenotypic and molecular techniques supported by evidence on antimicrobial use in humans and animals could provide

reliable information on contribution of different livestock sectors to human salmonellosis and transfer of drug resistant *Salmonella* from animals to humans or vice versa.

The major mechanism of resistance to beta-lactam antimicrobial agent in Gram-negative bacteria is through production of beta-lactamase enzymes capable of hydrolyzing and inactivating beta-lactam ring of drug. There are different verities of these enzymes with substantial difference in their ability to hydrolyze different beta-lactams (Beceiro et al., 2013). The major mechanism of resistance to fluoroquinolones is through mutation in quinolone resistance determining region (QRDR). Plasmid-mediated quinolone resistance determinants, specially *qnr* genes, has recently been reported from different parts of the world in different Gram-negative organisms including *Salmonella* (Jacoby et al., 2014). However, little is known on molecular mechanism of resistance to beta-lactam and fluoroquinolones in *Salmonella* isolates from Ethiopia. Characterization of beta-lactam resistant and quinolone resistant *Salmonella* isolates from various hosts using molecular techniques for the type of beta-lactamase enzymes and quinolone resistant genetic determinants commonly involved in conferring resistance to isolates will be useful to implement appropriate control measures.

Biofilms play a significant role in several infectious diseases including *Salmonella* and treatments of these infections cost several billion dollars annually. Strong biofilm forming ability of *Salmonella* contributes to persistence in the food, food processing surfaces, environment and host. They are also resistant to antimicrobial agents, disinfectants and host immune response (Janssens et al., 2008; Vestby et al., 2009b). *Salmonella* serotypes and strains vary in biofilm forming ability. Despite several reports on the occurrence of MDR

in *Salmonella* serotypes from humans and animals in Ethiopia, little is known about biofilm forming ability and their relationship with antimicrobial susceptibility. Study on biofilm forming ability and drug resistance phenotype and genotype of isolates from various sources could provide insight into the association of multidrug resistance and biofilm formation (multicellular behavior) of *Salmonella* from different sources.

As there is limited or no protective vaccine against *Salmonella* infection, development of effective antimicrobial agents that circumvent the resistance problem is highly essential. The identification of novel molecular targets important for biofilm formation will facilitate the discovery of new drug candidates displaying antibiofilm activity against drug-resistant *Salmonella* and other organisms.

Bacterial kinases participate in the regulation of cellular processes known to be important for biofilm formation, such as quorum sensing (Aubert et al., 2013; McLoon et al., 2011; Ray and Visick, 2012; Rubinstein et al., 2012). In recent years, kinases have been assigned a high priority as candidate drugable targets due to their endogenous small-molecule binding motif and importance in cellular functions (Hopkins and Groom, 2002). Due to the reliance of several biofilm-associated processes on bacterial kinases and other ATP-requiring proteins, screening of chemical libraries of small-molecule ATP-mimetic compounds could come up with promising lead compounds with potential use to inhibit biofilm formation and for disruption of established biofilms of *Salmonella* and other bacterial pathogens in the host and the environment.

2. Objectives of the Study

The general objective of this study was to contribute to the reduction of the public health burden caused by drug resistant *Salmonella* infections through generating information on phenotypic and genotypic characteristics of *Salmonella* serotypes in humans and animals and through finding potential compound with antibiofilm activity.

The specific objectives were:

- To determine fecal prevalence, serotype distribution and antimicrobial resistance of *Salmonella* in dairy cattle, slaughtered cattle ,poultry and swine in central Ethiopia
- To determine prevalence and antimicrobial resistance of non-typhoidal *Salmonella* serotypes and co-infection with parasites, among patients with diarrhea and other gastrointestinal complaints in Addis Ababa, Ethiopia
- To investigate the phenotypic and genotypic diversity of *Salmonella enterica* from humans and food animals in central Ethiopia
- To determine the major types of beta-lactamase enzymes common in isolates resistant to beta-lactam antimicrobial agents and to investigate mechanism of quinolone resistance in isolates with reduced sensitivity to nalidixic acid and ciprofloxacin
- To investigate association of multicellular behavior and drug resistance in *Salmonella enterica* serotypes isolated from animals and humans in Ethiopia
- To Evaluate small molecule kinase inhibitors for antibiofilm activity on *Salmonella enterica*

3. Materials and methods

3.1 Study Design and Study area

A cross-sectional *Salmonella* prevalence and antimicrobial resistance study was conducted in dairy, poultry and swine farms in Addis Ababa and five towns of Oromia region at the outskirt of Addis Ababa: Sebeta, Barake, Welmera, Sululta and Adaa from May 2013 to January 2014. All these five towns are located at the outskirt of Addis Ababa and in these areas; the interaction between the animal and human populations is very high due to high density of the human population and the large number of peri-urban dairy farming facilities. In Addis Ababa, ten representative government-owned health centers out of 36 were randomly selected; one from each sub-city and Tikur Anbessa Specialized Hospital (TASH) was also included. Fecal samples were also collected from slaughtered cattle at Addis Ababa Abattoir. *Salmonella* isolation and antimicrobial susceptibility study were conducted at Aklilu Lemma Institute of Pathobiology (ALIPB). Molecular characterization work was conducted at Center for Microbial Interface Biology, The Ohio State University, USA and Biosciences Eastern and Central Africa, (BecA) Nairobi, Kenya. Characterization of multicelluar behavior and drug resistance as well as screening of kinase inhibitors for antibiofilm assay was conducted at The Ohio State University, USA.

3.2 Sample size for Salmonella prevalence study

For study on prevalence of *Salmonella* in dairy cattle, 1203 fecal samples were collected. Sampling was conducted from healthy as well as diarrheic cattle irrespective of age and sex

in 132 dairy herds (Addis Ababa n=38, Adaa n=12, Sebeta=21, Sululta=24, Welmera=18). Inclusion of farms in the sampling population was based on representation of the area under study, willingness of the owners, as well as accessibility. Each farm represented a single herd. The minimum herd size of a farm to be included in the study for dairy cattle was 5 cattle. In such small sized farms, all or most of the animals were sampled. The largest herd size contained 398 heads of cattle. Farms were categorized in to small, medium and large based on the herd size of (5-20), (21-50), and greater than 50 heads of cattle, respectively. In larger farms, a minimum of 10% of the animals in the farm were sampled. For poultry, 549 pooled fecal samples (from 3 chicken each) were collected from a total of 48 farms (Adaa district n= 33, Addis Ababa n=6, Sebeta n=6, Barake n=6). In the case of swine, 180 fecal samples were collected from 7 farms, 4 from Adaa district and 3 from Addis Ababa. In addition, 282 fecal samples from cattle ready to be slaughtered at the Addis Ababa slaughter house were also randomly collected.

For human study sample size (n=576) was calculated based on the previous study in Addis Ababa, with a prevalence of 6.4% (Mache et al., 1997) using the formula $(Z0/2)/\Delta)\ 2p(1-p)$ at 95% CI, margin of error of 2%. However, to increase the number of isolates, a total of 765 patients from health centers were included. From each health center, a minimum of 71 and a maximum of 82 diarrheic patients of any age referred to laboratory for stool examination who did not take antibiotics during the last 2 weeks were recruited (n=765). In addition, 192 patients who presented with various gastrointestinal complaints (diarrhea, abdominal pain and gastritis) and submitted stool samples to the parasitology and

microbiology laboratory were included in the study. Only 98 (51%) of the stool samples from TASH were diarrheic.

3.3 Sample and data collection

Fecal samples from dairy cattle, slaughtered cattle and swine were collected directly from rectum using sterile disposable gloves into sterile zippered plastic bag. Pooled fresh fecal samples (from 3 chickens) were collected from poultry farms. Stool samples from each human patient were collected in clean screw capped plastic containers. Samples were transported to ALIPB using ice box within 3-4 h of collection. Information such as herd size, age, sex and use of antimicrobial agents and presence of diarrhea in a farm was recorded in each farm during sample collection. Information on patients' history such as consumption of raw meat, milk and vegetables during the last 2 weeks was also collected by interviewing the patients during sample collection from health centers. All demographic, clinical and laboratory data obtained from study participants at health facilities such as stool consistency and laboratory examination results of stool specimens for other pathogens were also recorded. The stool consistency was determined in the health center and hospital laboratories immediately after samples were received according to the Bristol stool consistency scale (type 5, 6 and 7) defined as loose, mucoid and watery, respectively (Lewis and Heaton, 1997). As it was difficult to collect patient associated information from hospital patients, analysis for association of *Salmonella* infection status and various risk factors was conducted only for patients from health centers.

3.5 Laboratory Investigation

3.5.1 Microscopic examination of stool specimens

Direct microscopic stool examination of samples from human patients was performed for detection of ova and parasites by laboratory technicians in health centers and hospital laboratories where the samples were collected immediately prior to transportation to ALIPB and the laboratory result was recorded.

3.5.2 Culture and identification of *Salmonella* species

Isolation and identification of *Salmonella* species was conducted according to WHO Global Foodborne Infections Network laboratory protocol (WHO, 2010). Briefly, 10 g of feces was suspended in 90 ml of buffered peptone water (BPW) and incubated for 24 h at 37°C. In case of human samples 5 g of feces was suspended in 45 ml of BPW. One hundred µl of this suspension was transferred to 10 ml of Rappaport-Vassiliadis enrichment Broth (RVB), (Oxoid, USA) and incubated for 24 h at 37°C. One ml of suspension was also transferred to 10 ml of Tetrathionate broth (TTB) (Oxoid, USA) and incubated for 24 h at 42°C. The sample from these two broths was streaked on to Xylose Lysine tergitol 4 (XLT-4) selective media and the plates were incubated at 37°C for 24 h. Presumptive *Salmonella* colonies were then further investigated biochemically using Triple Sugar Iron agar, Urea, Citrate and Lysine Iron Agar slants. Those colonies with typical *Salmonella* biochemical properties were then further confirmed by genus specific PCR (Cohen et al., 1993). *Salmonella* recovered from both RVB and TTB of a single sample were first considered as different strains until the isolates were tested for antimicrobial susceptibility. When differences in

antimicrobial susceptibility were observed, both isolates were considered as different strains. On the other hand, if the isolates showed similar susceptibility pattern, only one isolate was considered for further analysis. *Salmonella* isolates were grown overnight in trypton soya broth(TSB) which was then frozen in 20% glycerol stock at -80°C till further analysis.

3.5.3 Salmonella serotyping and phage typing

Salmonella isolates were serotyped and phage-typed at the Public Health Agency of Canada, World Organization for Animal Health (OIÉ) Reference Laboratory for Salmonellosis, Guelph, Ontario, Canada. Briefly, the somatic (O) antigens were determined by slide agglutination tests (Ewing, 1986) and flagellar antigens were determined using a microplate agglutination technique (Shipp and Rowe, 1980). The antigenic formulae of Grimont and Weill (2007) were used to identify and assign the serotypes of the isolates. Phage typing of *S.*Typhimurium isolates was performed by the methods developed by Callow (1959) and extended by Anderson et al (1977) with reference phages obtained from the Public Health England, Gastrointestinal Bacteria Reference Unit, Colindale, England and the Public Health Agency of Canada, National Laboratory for Enteric Pathogens, Winnipeg, Canada. *Salmonella* isolates that reacted with the phages but did not conform to any recognized phage type were designated atypical (AT).

3.5.4 Antimicrobial susceptibility testing

Susceptibility of the isolates to 18 antimicrobials was determined using the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). Briefly, frozen isolates were subcultured on tryptic soy agar (Becton, Dickinson and

Company, USA) from which 3 to 4 pure colonies were inoculated to a tube containing 5 ml of tryptic soy broth (TSB) (Becton, Dickinson and Company, USA) and mixed gently using sterile inoculating loop. It was then incubated at 37°C for 4-5 h. The turbidity of the suspension was then adjusted to the optical density of a McFarland unit of 0.5 using sterile saline to standardize the inoculum size. Sterile cotton swab was dipped and rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum. It was then inoculated to Mueller Hinton Agar plate (Oxoid, Ltd) by streaking the swab over the entire surface of the plate. The inoculated plates were left at room temperature to dry for 5-10 minutes and antimicrobial discs were dispensed by pressing on the plate with sterile forceps and the plates were inverted and incubated at 37°C overnight. Diameters of the zone of inhibition were measured to the nearest millimeter using a plastic ruler.

The following antimicrobials (Sensi-Discs, Becton, Dickinson and Company, Loveton, USA) and disc potencies were used: amikacin (An) (30 µg), amoxicillin + clavulanic acid (Amc) (20/10 µg), ampicillin (Amp) (10 µg), cefoxitin (Fox) (30 µg), ceftriaxone (Cro) (30 µg), cephalothin (Cf) (30 µg), chloramphenicol (C) (30 µg), ciprofloxacin (Cip) (5 µg), gentamicin(Gm) (10 µg), kanamycin(K) (30 µg), nalidixic acid (Na) (30 µg), neomycin (N) (30 µg), nitrofurantoin (Nitro) (100 µg), streptomycin(S) (10 µg), sulfisoxazole (G) (1000 µg), sulfamethoxazole + trimethoprim (Sxt) (23.75/1.25 µg), trimethoprim (Trm)(5 µg) and tetracycline (Te) (30 µg). The interpretation of the categories of susceptible, intermediate or resistant was based on the CLSI guidelines (CLSI, 2013). In this study, isolates were regarded as multi-drug resistant (MDR) when they are resistant to at least one or more drugs

among 3 antimicrobial categories (Magiorakos et al., 2012). For the purpose of analysis, all readings classified as intermediate were considered as resistant unless indicated. *E. coli* ATCC 25922 was used as a quality control organism.

3.5.5 Conjugation assay

To determine whether integrons of the *Salmonella* isolates were on conjugative DNA elements, class 1 integron positive isolates were conjugated with Rifampicin-resistant (Rif^R) and sulfamethoxazole-susceptible (Sul^S) *E. coli* (MG1655). Both donor (*int1*-positive sulfamethoxazole-resistant *Salmonella* isolates) and recipient bacteria were cultured in Luria-Bertani (LB) broth (OD_{600}= 0.6). The mating of donor (sulfamethoxazole-resistant isolates) and recipient was conducted by mixing in a 1:9 ratio (v/v) in 2 ml of LB broth followed by incubation overnight in a water bath at 37°C. The transconjugants were selected by plating 50 µl of the mating culture onto MacConkey agar plates containing both rifampicin (50 µg/ml) and sulfamethoxazole (512 µg/ml). Colonies were then selected based on their resistance to both antimicrobials and purified by subculture on LB agar with and without antibiotic. The transconjugants were further tested for their antimicrobial susceptibility patterns and the presence of class 1 integrons by PCR (Vo et al., 2010).

3.6 Molecular characterization of Salmonella isolates

3.6.1 Bacterial DNA extraction

Isolates were grown on LB agar (37°C, overnight). A single colony was inoculated to LB broth and grown in shaking incubator at 37°C for 16-18 h. Genomic DNA was then extracted using QIAGEN genomic DNA extraction kit according to the manufacturer's

recommendation for most of the experiments. For some of the experiments, bacterial genomic DNA template was obtained by resuspending and boiling a single fresh colony of each isolate in 50 µl of 2mM NaOH at 95°C for 5 min.

3.6.2 Detection and characterization of Class 1 Integron

All isolates were screened using PCR with primers specific to *Inti1* gene as described previously (Leverstein-Van Hall et al., 2002). For all *Inti1* positive isolates and all isolates resistant to sulfisoxazole, the presence of integron carrying integrated drug resistance gene cassettes was determined by PCR amplification of the two conserved segments (5'-CS and 3'-CS primer sets) (Lévesque et al., 1995). PCR products were separated by agarose gel electrophoresis and visualized under UV-light. In the cases where there were more than one variable segments in an isolate, each segment was cut from the gel and purified with a QIAGEN gel extraction kit, (QIAGEN, USA). Single band PCR products were then purified using PCR purification kit (QIAGEN, USA) and submitted for sequencing to Plant Microbe Genomics Facility of The Ohio State University. DNA sequences were then analyzed by accessing the Genbank database via the BLAST network service (USA).

3.6.3 Detection and characterization of *Salmonella* genomic Island and its variants

All *inti1* and class 1 integron positive *Salmonella* isolates were analyzed for the presence of *Salmonella* genomic Island 1(SGI1). First the presence of the left and right junction of SGI1 was examined by PCR using primers described by Doublet (2003). Primer sets used for amplification of the left junction incorporate segments of the *thdf* and *inti1* genes. The right junction of SGI1 was amplified using primers targeting the direct repeat portion of the SGI1

and the second Integron in *S.*Typhimurium. In other serotypes, since they do not have the second integron, the reverse primer for the detection of the right junction of SGI1 was part of the *yidY* gene. The order of the antimicrobial resistance gene cluster was tested as previously described (Doublet et al., 2008; Leverstein-Van Hall et al., 2002; Lévesque et al., 1995). All PCR reaction conditions were performed following the original references for each reaction. PCR products were then purified, sequenced and sequences were analyzed using BLAST and compared with previous reports on GenBank (accession number AF261825 and AY463797). List of primers used to screen for integrons and SGI1 are indicated in Table 1.

Table 1 Primers used to characterize class 1 integrons and SGI1.

Primer	Gene	Amplification	Size (bp)	Nucleotide sequence (5'-3')	Reference
Inti FP	*Inti1*	*Inti*	242	TCTCGGGTAACATCAAGG	(Leverstein-Van Hall et a
Inti RP	*Inti1*			CTTCAGGAGATCGGAAGACCTC	2002)
5'-CS	*variable*	Integron	variable	GGCATCCAAGCAGCAGCAAG	(Lévesque et al., 1995)
3'CS				AAGCAGACTTGACCTGA	
LJ-FP	*Thdf*	Left Junction	500	ACACCTTGAGCAGGGCAAG	(Doublet et al., 2003)
LJ-RP	*Int*			AGTTCTAAAGGTTCGTAGTCG	
RJ-FP	*S044*	Right Junction		TGACGAGCTGAAGCGAATTG	(Doublet et al., 2003)
RJ-RP	*int2*		515	AGCAAGTGTGCGTAATTTGG	
RJ-RP2	*yidY*		500	ACCAGGGCAAAACTACACAG	
Int1	*Inti1*	*Inti1-aadA2*	1135	GCTCTCGGGTAACATCAAGG	(Doublet et al., 2003)
Aad	*aadA2*			GACCTACCAAGGCAACGCTA	
Sul	*Sul* delta	*Sul* delta-*floR*	942	GACCTACCAAGGCAACGCTA	(Doublet et al., 2003)
Flo	*FloR*			AAAGGAGCCATCAGCAGCAG	
Flo	*FloR*	*floR-tetR*	598	TTCCTCACCTTCATCCTACC	(Doublet et al., 2003)
tetR	*tetR*			TTGGAACAGACGGCATGG	
orf2 delta	*orf2* delta	*orf2* delta-*merA*	2735	ACTGACGAAGACGGCGAATG	(Doublet et al., 2008)
merA	*merA*			GTGCCGTCCAAGATCATG	
	merR	*merR-tetA*	2280	TGCTGTCTGAACTCGTGTGCG	(Doublet et al., 2008)
Tet A	*tetA*			CGGCAGGCAGAGCAAGTAGA	

3.6.4 Detection and characterization of beta-lactamase genes

PCR and DNA sequencing were performed for the detection and characterization of beta-lactamase (*bla*) genes in isolates resistant to one or more of the beta-lactam antimicrobial agents (Ampicillin, Amoxycillin and clavulanic acid, Cephalothin, Cefoxitin and ceftriaxone) examined with oligonucleotide primers previously described for *bla*TEM, *bla*SHV, *bla*PER, blaPSE , *bla*OXA1, *bla*OXA4 , *bla*OXA10, *blaCMY,* and *bla*CTX-M genes (Table 2). Group specific primers were used to characterize *bla*CTX-M enzymes (Johan et al., 2004). All PCR reactions were conducted using ready to use AccuPower® PCR PreMix (Korea) containing all reagents.The PCR condition for all reactions involved initial denaturation for 3 minutes at 95°C followed by 30 cycles of (95°C for 30 s, specific annealing temperature for 1 m, and 72°C for 30 s) followed by final extension at 72°C for 5 m. Specific annealing temperature for each PCR reaction is shown in Table 2. All amplicon sequences were translated to amino acid sequences using CLC Main Work Bench and compared with protein sequences in the Genbank database and classification of *bla*TEM enzymes was based on beta-lactamase classification database (http://www.lahey.org/Studies/temtable.asp Lahey).

3.6.5 Investigation of mechanism of quinolone resistance

All isolates with reduced susceptibility to nalidixic acid and/or ciprofloxacin were examined for presence of known quinolone resistance determinants. Quinolone resistance determining region (QRDR): *gyrA, gyrB, parC and parE* genes were amplified using PCR. PCR was also used to examine for various plasmid mediated quinolone resistance genes: *qnrA, qnrB, qnrD, qnrS, qepA,* and *aac(6')-Ib* (Park et al., 2006). PCR amplicons were purified using QIAGEN gel extraction kit and PCR purification kit (QIAGEN, USA) and sequenced.

Presence of mutation in the QRDR was examined by translating nucleotide sequences into proteins and aligning against reference sequence of *S.*Typhimurium strain LT2 on NCBI database (Accession Number AE006468). The primers used for this investigation are shown in Table 3.

Table 2 List of primers used for detection of beta-lactamases and to characterize the groups of CTX-M beta-lactamases

Gene/target	**Primer**	**Sequence 5'-3'**	**Amplicon size**	**AT °C**	**Ref**	**Remark**
*bla*Tem Gene	TEM-F1	ATGAGTATTCAACATTTCCG	862-bp	55	(Naiemi et al., 2005)	
	TEM-R1	GACAGTTACCAATGCTTAATCA				
	blaTEM-F2	TAA CCA TGAGTGATAACACT			(Naiemi et al., 2005)	For
	blaTEM-R2	CCGATCGTT GTCAGAAGTAA				sequencing
bla SHV gene	Bla SHV-F1	CTTTACTCGCCTTTATCG	827-bp	56	(Naiemi et al., 2005)	
	Bla SHV-R1	TCCCGC AGATAAATCACCA				
	blaSHV-F2	ACTGCCTTTTTG CGCCAGAT			(Naiemi et al., 2005)	For
	blaSHV-R2	CAGTTCCGTTTCCCAGCGGT				sequencing
bla OXA-1	OXA-1-F	ATGAAAAACACAATACATATCAAC	755-bp	48	(Edelstein et al., 2004)	
	OXA-1-R	TTTCCTGTAAGTGCGGACAC				
bla OXA -4	OXA-4-F	TCAACAGATATCTCTACTGGT	216bp	54	(Edelstein et al., 2004)	
	OXA-4-R	TTTATCCCATTTGAATATG				
bla OXA-10	OXa 10-F	TCAACAAATCGCCAGAGAAG	277bp	57	(Edelstein et al., 2004)	
	Oxa-10-R	TCCCACACCAGAAAAACCA				
bla PER	Per1-F	AATTTGGGCTTAGGGCAGAA	925 bp	55	(Weldhagen et al., 2003)	
	Per1-R	ATGAATGTCATTATAAAAGC				
*bla*PSE	blaPSE-F	TGCTTCGCAACTATGACTAC			(Riano et al., 2006)	
	blaPSE-R	AGCCTGTGTTTGAGCTAGAT				
*bla*CYM	blaCMY2-F	TGGCCGTTGCCGTTATCTAC	868	57	(Chen et al., 2004)	
	blaCMY2-R	CCCGTTTATGCACCCATGA				
CTX-M group I	CTXM1-F3	GACGATGTCACTGGCTGAGC	499	55	(Pitout et al. 2004	
	CTXM1-R2	AGCCGCCGACGCTAATACA				
CTX-M group II	TOHO1-2F	GCGACCTGGTTAACTACAATCC	351	55	(Pitout et al. 2004)	
	TOHO1-1R	CGGTAGTATTGCCCTTAAGCC				
CTX-M group III	CTXM825F	CGCTTT GCCATGTGCAGCACC	307	55	(Pitout et al. 2004)	
	CTXM825R	GCT CAGTACGATCGAGCC				

CTX-M group IV	CTXM914F	GCTGGAGAAAAGCAGCGGAG	474	62	(Pitout et al. 2004)
	CTXM914R	GTAAGCTGACGCAACGTCTG			

Table 3. List of primers used for detection of quinolone resistance mechanism

Gene	**Primer name**	**Primer sequence (5′ to 3′)**	**Product size**	**AT in ºC**	**References**
gyrA	GyrAFP	AAATCTGCCCGTGTCGTTGGT	344bp	58	(Fabrega et al., 2009)
	GyrARP	GCCATACCTACTGCGATACC			
gyrB	GyrB FP	GAATACCTGCTGGAAAACCCAT	446bp	57	(Fabrega et al., 2009)
	GyrB RP	CGGATGTGCGAGCCGTCGACGTCCGC			
parC	ParC FP	AAGCCGGTACAGCGCCGCATC	395bp	57	(Fabrega et al., 2009)
	ParC RP	GTGGTGCCGTTCAGCAGG			
ParE	ParE FP	TCTCTTCCGATGAAGTGCTG	240bp	55	(Eaves et al., 2004)
	ParE RP	ATACGGTATAGCGGCGGTAG			
qnrA	qnrA FP	ATTTCTCACGCCAGGATTTG	516bp	53	(Robicsek et al., 2006)
	qnrA RP	GATCGGCAAAGGTTAGGTCA			
qnrB	qnrB FP	GATCGTGAAAGCCAGAAAGG	469bp	53	(Robicsek et al., 2006)
	qnrB RP	ACGATGCCTGGTAGTTGTCC			
aac(6′)-Ib	aac(6′)-Ib FP	TTGCGATGCTCTATGAGTGGCTA	482-bp	55	(Park et al., 2006)
	aac(6')-Ib-RP	CTCGAATGCCTGGCGTGTTT			
	aac(6')-Ib-cr-seq	CGTCACTCCATACATTGCAA (for sequencing of aac(6′)-Ib-cr			
qepA	QepA FP	CGTGTTGCTGGAGTTCTTC	403bp	59	(Cattoir et al., 2008)
	QepA RP	CTGCAGGTACTGCGTCATG			
QnrD	QnrD FP	CGAGATCAATTTACGGGGAATA	565bp	53	(Cavaco et al., 2009)
	QnrD RP	AACAAGCTGAAGCGCCTG			
QnrS	QnrS FP	ACGACATTCGTCAACTGCAA	417bp	53	(Robicsek et al., 2006)
	QnrS RP	TAAATTGGCACCCTGTAGGC			

3.7 Salmonella genotyping

3.7.1 Pulsed-field gel electrophoresis

Representative isolates (n=47) were systematically selected to represent source and serotypes and genotyped using pulsed-field gel electrophoresis (PFGE) to investigate genetic relatedness of *Salmonella* serotypes obtained from food animals in Addis Ababa and surrounding districts and those obtained from diarrheic patients from Addis Ababa. Two out-group *Salmonella enterica* strains from Kenya and USA both isolated from swine were also included in this analysis. PFGE was performed according to Center for Disease Control and Prevention (CDC) PulseNet), as previously described (Ribot et al., 2006) using a contour clamped homogeneous electric field (CHEF)-Mapper (Bio-Rad Laboratories, Hercules, CA). Briefly, Bacterial culture was grown overnight in TSB. The culture was suspended and mixed with meted 1% SeaKem Gold: 1%SDS agarose at 56°C. Agarose plugs were made in disposable plug molds. Cells were lysed in 50 ml tubes using lysis buffer (50mM Tris, 50mMEDTA, Ph8 +1%Sarcosyl) plus protinase K. DNA digestion was performed using XbaI restriction enzyme in agarose plugs. Pieces of small plugs about 2mm was cut and loaded on to the comb. The gel was poured, comb removed and PFGE was run for 18-20 hrs. After staining with ethidium bromide, DNA fragments were visualized under UV trans-illumination (Gel Doc 2000, Bio-Rad Laboratories, Hercules, CA, USA). Gel images were photo documented using the Quantity one 1D analysis software (Bio-Rad Laboratories). PFGE gels were then analyzed using BIONUMERICS software V. 4.61 (Applied Maths NV, Keistraat, Belgium) using dice coefficient similarity index and unweighted pair group average (UPGMA) cluster analysis. Image analysis was conducted based on 2.2% tolerance and 1.5% optimization. The plausible genetic threshold for clustering was 88%.

3.7.2 Multilocus sequence typing

Sixteen representative isolates originating from human patients and different food animals were analyzed using multilocus sequence typing (MLST). Seven housekeeping genes *aroC* (chorismate synthase), hemD (uroporphyrinogen III cosynthase), *dnaN* (DNA polymerase III beta subunit), *hisD* (histidinol dehydrogenase), *sucA* (alphaketoglutarate dehydrogenase), *purE* (phosphoribosyl aminoimidazole carboxylase), *thrA* (aspartokinase + homogenize dehydrogenase) were amplified by PCR according to the recommendation of the MLST database for *Salmonella enterica* (http://web.mpiib-berlin.mpg.de/mlst). The list of primers and amplification protocols including PCR conditions indicated in the database were used for each PCR reaction. Amplification products were purified using Qiagen PCR purification kit and sequenced using forward and backward primers.

The sequences were assembled and analyzed using CLC Main Work Bench. The sequences were then trimmed to fit to the size of the MLST database requirement and submitted to the MLST database website (http://web.mpiib-berlin.mpg.de/mlst) and assigned existing or novel allele numbers and sequence types (STs). For five *Salmonella* isolates, whole genome sequencing (WGS) was conducted by Food and Drug administration (FDA), USA. DNA samples were prepared using Nextera XT Kit and sequencing using Illumina Miseq technology according to manufacturer's recommendation. Fastq files of raw reads of the sequence were uploaded to Centre for Genomic Epidemiology web server which analyzes the data and assign specific allele and sequence types (Larsen et al., 2012).

3.8 Biofilm assay and investigation of antibiofim activity of kinase inhibitors

3.8.1 Screening *Salmonella* isolates for multicellular behavior.

Biofilm forming ability of the isolates was assessed using the crystal violet staining method (Djordjevic et al., 2002; Malcova et al., 2008) at 20°C and 37 °C in Tryptic Soy Broth 20 times diluted (TSB 1:20). Overnight broth cultures of bacteria were diluted 100 times in TSB 1/20 medium. Two hundred µl of this was inoculated into a flat bottomed 96-well polystyrene microtitre plate. Some wells were inoculated with 200 µl of TSB 1:20 medium only as a negative control. The plates were incubated statically for 24 h. The planktonic bacteria were then removed by vacuum aspiration and the plates washed with 200 µl of 1X phosphate buffered saline (PBS). The bound biofilm cells were fixed by heating at 60^0C for 1 h. Each well was then stained with 200 µl of 1% crystal violet for 5 min and the excess stain was removed by washing with 200 µl of 1X PBS. The attached biofilm bacteria were solublized by 200 µl of 33% acetic acid. The amount of biofilm formation in each isolate was then determined by reading the optical density (OD) value of each well using spectrophotometer at wave length of 570 nm and subtracting the mean OD reading of the medium alone. The result for each isolate was obtained from mean reading of 8 wells per experiment and the experiment was conducted twice.

Bacterial colony morphology was investigated by growing the isolates on LB agar plates without salt (LBNS) supplemented with Congo red (40 µg/ml) and Coomassie brilliant blue dyes (20 µg/ml) for 6 days at room temperature. The plates were then inspected visually for the morphotype and recorded (White et al., 2006).

Cellulose production was assessed on LBNS agar containing 20 µg/ml of calcofluor (Fluorescent Brightener 28, Sigma, USA). After 6 days of growth at 20°C, the plates were visualized under UV light at 366 nm (White et al., 2006). In order to quantify the level of cellulose production by different strains, a modified broth calcofluor binding assay was used. Briefly, overnight broth cultures were normalized by optical density and diluted 1:100 in LBNS liquid medium containing calcofluor (20µg/ml) in black 96-well microtiter plates (Corning Costar®, USA) and incubated at 20°C for 6 days. The optical density (600 nm) and fluorescence (excitation =366 nm, emission= 565 nm) of bacteria in each well was then measured using a Spectra Max M2 Plate reader. Wild type *S.* Typhimurium (JSG210=ATCC 14028s and JSG1748 *ΔbcsA*::kan cellulose mutant (Prouty and Gunn, 2003) strains were used as positive and negative controls, respectively. Readings less than that of JSG1748 were considered negative for the production of cellulose. Ability to form pellicle at liquid air interface was assessed by inoculating 0.5ml of over-night culture of the bacteria in LB medium into glass test tubes containing 4.5 ml of the LBNS medium. It was then kept at room temperature for 6 days and pellicle formation was then visually inspected at the end of the experiment (Solano et al., 2002).

3.8.2. Screening of kinase inhibitors for antibiofilm activity

3.8.3.1 Bacterial strain, growth conditions and chemical inhibitors.

The *Salmonella* strains used in this study were wild-type *S.* Typhimurium (standard reference laboratory strains) 14028s (JSG210) and *S.*Typhi strain TY2 (JSG624). *Acinetobacter baumannii* 19606 (WLS2401), *Staphylococcus aureus* Strain USA300), and *Pseudomonas aeruginosa* (strain PAO1) were also used for biofilm assay. Bacteria were

stored at -80°C in LB broth containing 20% glycerol. Overnight cultures were grown in LB or TSB medium at 37°C with aeration. It was then normalized to OD_{600nm} 0.8 and the culture was diluted 1:100 in TSB diluted 1:20 or undiluted super optimal broth (SOB) for *S*.Typhi and grown at 30°C in static conditions.

Out of 450,000 chemical libraries of adenosine mimetics, 3000 compounds were selected and purchased from ChemBridge (San Diego, CA). Selection of library of putative kinase inhibitors was based on the predicted probability of binding to the ATP binding region of proteins and allosteric sites of kinase targets. The compounds in the library were scored based on statistical significance of chemical similarity to ATP by tanimoto score (Baldi and Nasr, 2010). Low-energy conformations of 5'-O-methyl adenosine were used in the compounds to mimic the adenosine portions of ATP, without providing energy for cellular processes. Compounds were aliquoted at a 5mM concentration in Dimethyl sufoxide (DMSO) and stored in the dark at 4°C.

3.8.3.2 High-throughput screening of compounds for antibiofilm assay

The original high-throughput screen was conducted using 5µM drug concentration utilizing an automated BioMek 2000 robot platform with dilutions of the drug made in 1X PBS in 96 well polystyrene plates, Corning Inc. (Corning, NY) according to the method described previously (O'Toole and Kolter, 1998). Controls were treated with 0.1% DMSO (solvent) final concentration and PBS alone. Each plate contained 80 drugs, leaving 16 wells for controls. Cultures and drugs were added simultaneously and the BioMek 2000 carried out assay in a similar manner to the rapid attachment biofilm assay described below.

3.8.3.3 Rapid attachment biofilm assay, IC_{50} determination, and biofilm dispersion assay.

To determine biofilm inhibition by the compounds, an overnight culture of the desired reference laboratory strains of bacteriawere grown in TSB medium at 37°C on a roller drum or shaker. Cultures were normalized to OD_{600nm} of 0.8 and a 1:100 dilution of the normalized culture in TSB 1:20 and incubated at 30°C in static conditions to promote biofilm formation for 24 h. The OD_{600nm} was then read to observe growth of planktonic bacteria. Plates were washed in double distilled water (ddH_2O) and heat fixed at 60°C for 1 h. Biofilms were stained for visualization with 33% gentian violet solution (10 ml gentian violet, 18 ml 1X PBS, 1 ml methanol, 1 ml isopropanol) for 5 min, followed by a release of stain by 33% acetic acid (33 ml glacial acetic acid, 67 ml 1X PBS). The released dye was measured at OD_{570nm}. The half maximal inhibitory concentration of the compound was determined by finding the concentration at which 50% of the total observed biofilm inhibition occurred using drug concentrations between 0.125µM to 50µM. Biofilm dispersion assays were carried out in a similar manner, except 30µM compound 7955004 was added after a 24 h biofilm was established and allowed to incubate at 30°C in static conditions for another 24 h. Biofilms were quantified by gentian violet staining and dye release. Out of 3000 chemical libraries examined, only one compound (Compound 7955004), [3-(2-furylmethyl)-2-{[(5-hydroxy-1H-pyrazol-3-yl)methyl]thio}-3,5,6,7-tetrahydro-4H-cyclopenta[4,5]thieno[2,3-d] pyrimidin-4-on) showed over 50% biofilm inhibition and further analysis was conducted only for this compound.

3.8.3.4 Determination of bacterial viability following exposure to compound 7955004.

In order to establish potential bactericidal or bacteriostatic effects of compound 7955004, cultures were grown in the presence or absence of the drug and plated for viability. Overnight cultures were grown as described above, back diluted 1:100 and incubated for 24h (TSB, 37°C with aeration) in the presence or absence of 30µM compound 7955004. Following 0 h, 2 h, 4 h, 8 h, 16 h and 24 h, 10µl aliquots were removed, serially diluted and plated for viability.

3.8.3.5 Eukaryotic cellular cytotoxicity assay

Effects on eukaryotic cells were quantitatively assessed by 12 and 24 h LDH-release experiments using lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Clontech, Mountain View, CA). The Hep G2 hepatocyte cell line was grown in modified essential medium (MEM) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) and 2mM L-glutamine and incubated in a humidified 5% CO_2 incubator at 37°C. Cells were seeded at 1 x 10^4 cells ml^{-1} into a polystyrene 96-well tissue culture plate and allowed to adhere overnight. Adherent Hep G2 cells were subjected to 12 and 24 h of drug (50µM and 30µM) in triplicate. Negative and positive controls consisted of equal volumes of 0.1% DMSO in final concentration and 2% Triton-X, respectively. LDH release was measured by a colorimetric assay at A_{490nm}.

3.8.3.6 ATP-binding proteins captured by ATP-Sepharose affinity matrix

In order to determine potential ATP-binding protein-targets of compound 7955004, bacterial lysates were prepared from wild-type *S.* Typhimurium cultures grown in biofilm-inducing conditions (TSB 1:20, 30°C, 3 ml). Cells were centrifuged and resuspended in 1 ml of lysis buffer (50mM HEPES, pH 7.4, 120mM NaCl, 20mM $MgCl_2$, 1mM DTT, 0.1% Triton-X, and 1X complete protease inhibitor) and prepared by FastPrep system and bacterial lysis column B containing 0.1mm silica spheres for extraction of DNA, RNA, and proteins. The lysate was run through ATP-sepharose resin (Graves et al., 2002), and allowed to bind for 1 h. Low salt (120mM, 2X) and high salt (300mM, 2X) and low salt (120mM, 2x) rinses were used to wash off non-specifically binding proteins. Washes were optimized such that no proteins were present in eluent when visualized in silver stained gels. The column was subsequently washed with a solution of 500µM of compound 7955004 to compete protein targets off the column, followed by a 10µM ATP wash to clear the column of remaining ATP-bound proteins. Proteins were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver nitrate (Shevchenko et al., 1996) to determine the optimal number of wash steps and to identify protein bands of interest. Column elution fractions following the wash with compound 7955004 were subsequently electrophoresed (10% SDS-PAGE) and stained with SYPRO Red dye. These protein bands were excised and submitted to The Ohio State University Mass Spectrometry and Proteomics Facility. Identified peptides were analyzed using Mascot Deamon V2.2.1 (Boston, MA) and compared to the NIH/NCBI database.

3.9 Ethical consideration

The study protocol involving human and animal subjects was ethically approved by the Institutional Review Board of College of Health Sciences, Addis Ababa University and National Research Ethics Review Committee (Permit#3-10/474/05 dated 29-03-2015). For study involving human subjects, informed consent and children's assent were obtained from adults and parents or guardians for children, respectively before inclusion into the study. Permission to export *Salmonella* isolates to USA, Canada and Kenya were obtained from Plant and Animal Health Regulatory Department of Federal Ministry of Agriculture. Permission on the use of 87 *Salmonella* strains originally collected from food animals in Ethiopia was obtained from strains archive, Infectious Diseases Molecular Epidemiology Laboratory, College of Veterinary medicine, The Ohio State University. The isolates were stored in glycerol stock at -80°C.

3.10 Data analysis

For study involving prevalence and risk factors of *Salmonella* in dairy cattle, probability of selecting a given animal from a given herd was considered as a weighting variable. Data analysis method that fits to survey data as it is implemented in STATA version 12 was used to estimate prevalence of *Salmonella* and to investigate its association with pre-specified background characteristics. Animal level prevalence of *Salmonella* was calculated as the weighted percentage of *Salmonella* culture-positive fecal samples among the total number of animals examined. Herd level prevalence of *Salmonella* was calculated as the percentage of herds with one or more *Salmonella* culture-positive fecal samples among the total number of

herds sampled. Association of weighted animal level prevalence and selected background characteristics was assessed using person chi-square within survey command of STATA software. Association of herd level *Salmonella* positivity and pre-specified characteristics was assessed using person chi-square.

Prevalence of non-typhoidal *Salmonella* in humans was calculated as a percentage of *Salmonella* culture-positive stool samples among the total number of samples examined. Associations of putative risk factors as well as occurrence of protozoan pathogens with *Salmonella* infection in patients from health centers were assessed using both univariable and multi-variable logistic regression, accounting for clustering of patients at the health center level by using cluster-robust standard errors. Comparison among different means was conducted by one way analysis of variance (ANOVA) or student t-test. Results were reported statistically significant whenever the p-value was less than 0.05

Association of drug resistance determinants and biofilm formation/multicellular behavior was analyzed using chi-square test. For antibiofilm experiments all experiments were conducted in triplicate, and data represent a minimum of 3 biological replicates. The 50% effective concentration (EC50) values, were calculated using GraphPad Prism 6 software by plotting the data as a function of the log10 compound concentration, and standard sigmoidal dose-response curves were fit to the data; 95% confidence intervals (CIs) are reported to indicate the error of each EC50 determination.

4. Results

The main findings of this study are published in 4 articles (Paper 1, Paper 2, Paper 5 and Paper 6) and 2 manuscripts prepared for publication (Paper 3 and 4) which are referred to in the text. Some of the results that are not depicted in the articles are presented in Tables and figures and corresponding texts in the dissertation. In addition, some tables and figures that were presented in the papers were also presented here in the dissertation to make a better documentation and discussion of the findings. The term serovar and serotype were used interchangeably in the papers whereas only serotype was used in the dissertation.

4.1 Salmonella spp. prevalence in animals and humans

4.1.1 Prevalence in animals

Farm level and individual animal level prevalence of *Salmonella* in dairy cattle is presented in **Paper 1.** Weighted animal level and farm level prevalence of *Salmonella* was (30/1203; 2.3% and (10/132; 7.6%), respectively. Significant difference in the prevalence of *Salmonella* was observed across different age groups (p= 0.023) and the largest was observed in cattle less than 6 months. Prevalence of *Salmonella* in poultry farms, swine farms and slaughtered cattle is shown in Table 4. *Salmonella* was recovered from 7 (14.6%) of 48 poultry farms investigated whereas 3 (42.9%) of the 7 swine farms were positive for *Salmonella*. Out of 549 pooled fecal samples of poultry, 26 (4.7%), were positive for *Salmonella*. Individual animal level prevalence of *Salmonella* in swine was 4.4%. Out of 282 fecal samples collected from slaughtered cattle at Addis Ababa Abattoir, 20 (7.1%) were positive for *Salmonella*.

Table 4. Farm level and individual sample level fecal prevalence of *Salmonella* in poultry farms, swine farms in Addis Ababa and surrounding districts and slaughtered cattle in Addis Ababa Abattoir

Commodity type	District	No. of farms	No. of samples[a]	No. (%) positive samples	No. (%) positive farms
Poultry	Adaa	33	464	25(5.4)	6(18.2)
	Addis Ababa	6	45	1(2.2)	1(16.7)
	Barake	3	18	0	0
	Sebeta	6	22	0	0
	Total	**48**	**549**	**26(4.7)**	**14.6**
Swine	Adaa	3	100	2(2)	1(33.3)
	Addis Ababa	4	80	6(7.5)	2(50)
	Total	**7**	**180**	**8(4.4)**	**42.9**
Slaughtered cattle	Addis Ababa	NA	282	20(7.1)	NA

[a] Samples for poultry were pool of fecal droppings from 3 chicken, NA-not applicable

4.1.2 Prevalence in humans

Prevalence of *Salmonella* among diarrheic human patients from health centers in Addis Ababa was (55/765; 7.2%) while in those from TASH was (4/192; 2.1%). The overall prevalence of *Salmonella* among human patients was 59(6.2%) (**Paper 2**).

4.2 Intestinal parasites and concomitant infection with Salmonella Spp.

Microscopic examination of stool samples revealed infection with *Entamoeba histolytica* (19 %), *Giardia lamblia* (8.1 %), egg of *Hymenolepis nanna* (0.9 %) and egg/larvae of *Strongyloides stercoralis* (0.7 %) in patients from health centers, whereas only *E. histolytica* (0.5 %), *G. lambia* (4.3 %), and egg of *Tanea* species (1.1 %) were detected in patients from TASH. Although *Salmonella* was commonly detected in patients positive for *E. histolytica*

(9.7 %) compared to negative ones (6.6 %) *Salmonella* infection was not significantly associated with any of the parasites (**Paper 2**).

4. 3 Major risk factors associated with Salmonella spp. colonization

4. 3.1 Risk factors for *Salmonella* carriage in dairy cattle

In dairy cattle, prevalence of *Salmonella* was significantly higher in animals less than 6 months of age compared to other age groups (p= 0.023). Herd level prevalence of *Salmonella* in dairy farms was significantly higher in large sized herd (p=0.047). All *Salmonella* isolates were obtained from herds which kept their dairy cattle completely indoors, while none was recovered from farms which allowed their animals to graze outside occasionally or those where cattle were totally outdoors. Detection of diarrhea in an animal was also significantly associated with animal level *Salmonella* carriage and herd level prevalence of *Salmonella* in dairy farms. Out of 255 fecal samples collected from 24 diarrheic herds, 22 (8.63%) were positive for *Salmonella*, whereas, only 8 (0.84%) of the 948 fecal samples collected from 108 non-diarrheic herds were positive for *Salmonella*. Six of 24 (25%) of diarrheic herds were positive for *Salmonella* while only 4 of 108 (3.7%) of non-diarrheic herds were positive for *Salmonella* (p<0.001) (**Paper 1**).

4. 3.2 Risk factors for *Salmonella* in humans

Human *Salmonella* infection status among diarrheic patients from health centers was significantly associated with consumption of raw vegetables. Multivariable logistic regression analysis after adjusting for all pre-specified variables revealed statistically significant association of *Salmonella* infection status with consumption of raw vegetables

(OR=1.91, 95% CI=1.29-2.83, χ^2 =4.74, p=0.025). Symptom of watery stool consistency was also significantly associated with recovery of *Salmonella* compared to those patients with loose and mucoid stool consistency (OR=3.05, 95% CI=1.22-7.6; p=0.005) (**Paper 2**).

4. 4 Salmonella serotype and Phage type distribution

All together, 20 different serotypes were recovered from animals and humans. *S.*Typhimurium and *S.* Saintpaul were detected in all types of food animals and humans. The dominant serotypes isolated from humans and animals were *S.*Typhimurium 42 (27.6*%), S.* Saintpaul 33 (21.7%), *S.* Virchow 28 (18.4%), and *S.* Kentucky 10 (6.6%). *Salmonella* Saintpaul, although was the most dominant serotype isolated from poultry and swine, 20 (76.9%), 2 (25%) respectively, and second dominant serotype 10 (20%) isolated from cattle, only one *S.* Saintpaul was detected from humans. The frequency of *Salmonella* serotypes according to host species is shown in Table 5. *S.* Typhimurium was the dominant serotype recovered from all host species representing 27 (39.7%) of the human infection and 32 (17.9%) of the total animal infection followed by *S.* Saintpaul representing 1(1.5%) of human infection and 32 (38.1%) of animal infection. *S.* Virchow was the second dominant serotype isolated from humans and among animals it was recovered only from dairy cattle and slaughtered cattle. *S.* Kentucky though was of low proportion, it was detected from dairy cattle, slaughtered cattle, poultry as well as humans (Table 5).

Among *S.* Typhimurium strains, 10 different known phage types (PTs) and 2 atypical PTs were identified. The dominant PT was PT126 representing 19.1%, followed by PT1 (14.3%). Interestingly, all *S.*Typhimurium PT126 were isolated from human patients

whereas other phage types were fairly distributed among host species. In addition, the two *S.* Enteritidis isolated from humans were atypical and one *S.* Heidelberg isolated from swine was PT2 (**Paper 3**).

Table 5 *Salmonella enterica* serotypes isolated from dairy cattle, slaughtered cattle, poultry, swine and human in central Ethiopia

Serotype	Dairy	Slaughtered cattle	Poultry	Swine	Animal Total	Human	Total (%)
Typhimurium	7	4	3	1	15	27	42(27.6)
Saintpaul	6	4	20	2	32	1	33(21.7)
Virchow	5	2	-	-	7	21	28(18.4)
Kentucky	5	1	2	-	8	2	10(6.6)
Kottbus	-	1	-	-	1	7	8(5.26)
Miami	-	-	-	2	2	3	5(3.29)
Haifa	-	3	1	-	4	-	4(2.63)
Braendruep	-	2	-	-	2	1	3(1.97)
Dublin	3	-	-	-	3	-	3(1.97
Newport	-	-	-	-	-	2	2(1.32)
Milkawasima	1	2	-	-	2	-	2(1.32)
Livingstone var.14+	1	-	-	1	2	-	2(1.32)
Aberdeen	1	-	-	-	1	-	1(.66)
Concord	-	-	-	-	-	1	1(.66)
Agona	-	1	-	-	1	-	1(.66)
Entertidis	-	-	-	-	-	2	2(1.32)
Heildelberg	-	-	-	1	1	-	1(.66)
I:6;7,14,I,w	1	-	-	-	1	-	1(.66)
V:ROUGH-O;-:-	-	-	-	-	-	1	1(.66)
I,Rough-O:I:1,2	-	-	-	1	1	-	1(.66)
Total	30	20	26	8	84	68	152

4.5 Antimicrobial susceptibility of Salmonella Spp. from animals and humans

4.5.1 Resistance phenotype

Detailed resistance profile of *Salmonella* isolates obtained from food animals and diarrheic human patients in central Ethiopia during 2013 to 2014 are presented in **paper 1, Paper 2** and **Paper 3.** As a whole, 140 (92.1%) of the 152 *Salmonella* isolates were resistant to one or more antimicrobials tested. These involve 50 (100%) of isolates from cattle, 24 (92.3%) of isolates from poultry, 6 (75%) of isolates from swine and 59 (86.8%) of isolates from humans (Table 6). Among all antimicrobials tested, frequency of resistance was more common to streptomycin (81.6%), nitrofurantoin (44.7%), kanamycin (44.7%), tetracycline (36.2%) and sulfisoxazole 53 (34.9%). The rate of occurrence of resistance to some antimicrobials was variable among the isolates collected from different sources. Frequency of resistance to tetracycline, ampicillin and cephalothin in *Salmonella* isolates from humans was relatively low compared to that seen in animals. For instance, resistance to tetracycline ranged from 34.6% - 70% in isolates obtained from food animals while only 13.4% of human isolates were resistant to tetracycline. Resistance to chloramphenicol, was detected in 42.3% of isolates obtained from poultry, most of these isolates belonged to *S.* Saintpaul. Despite long history of use of chloramphenicol in human medicine in the country, only 2 (3%) of isolates from human patients were resistant to chloramphenicol, one of which was *S.* Concord isolated from diarrheic child in TASH. Decreased susceptibility to amikacin was detected only in few isolates 6 (3.9%) all obtained from slaughtered cattle. Overall, rate of occurrence of resistance to antimicrobials in human was less common compared to isolates obtained from food animals (Table 6). Resistance pattern of each isolate is presented in **Paper 3.**

Table 6 Antimicrobial resistance profile of *Salmonella* isolates from different food animals and diarrheic human patients

Drug*	Source of *Salmonella* isolates					
	Dairy cattle, (n=30)	Slaughtered cattle (n=20)	Poultry (n=26)	Swine, (n=8)	Human, (n=68)	Total (152)
	No.(% R)	No.(%R)	No.(%R)	No.(%R)	No.(%R)	No.(%R)
An	-	6(30)	-	-	-	6(3.9)
Amp	10(33.3)	5(25)	11(42.3)	1(12.5)	11(16.4)	38(25)
Amc	8(26. 7)	5(25)	12(46.2)	1(12.5)	8(11.9)	34(22.4)
Cf	14(46. 7)	8(40)	12(46.2)	1(12.5)	12(17.9)	47(30.9)
C	-	-	11(42.3)	-	2(3)	13(8.6)
Cro	-	-	1(3.9)	-	2(3)	3(2)
Fox	-	-	-	1(12.5)	2(3)	3(2)
Cip	9(30)	2(10)	5(19.2)	2(25)	3(4.5)	21(13.8)
Gm	7(23.3)	2(10)	2(7.7)	-	5(7.5)	16(10.5)
K	14(46. 7)	16(80)	12(46.2)	3(37.5)	23(34.3)	68(44.7)
Tmp	1(3.3)	2(10)	1(3.9)	-	3(4.5)	7(4.6)
Sxt	-	2(10)	1(3.9)	-	3(4.5)	6(3.9)
Te	16(53.3)	14(70)	9(34.6)	5(62.5)	9(13.4)	55(36.2)
Su	18(60)	17(85)	24(92.3)	-	26(38.9)	53(34.9)
S	26(86.7)	18(90)	24(92.3)	6(75)	50(74.6)	124(81.6)
Nitro	18(60)	15(75)	5(19.2)	3(37.5)	27(40.3)	68(44.7)
Na	9(30)	1(5)	5(19.2)	2(25)	16(23.9)	33(21.7)
N	5(16.7)	4(20)	3(11.5)	-	9(13.4)	21(13.8)

An,amikacin;Amp,ampicillin; Amc, amoxicillin and clavulanic acid; Cf, cephalothin; C, chloramphenicol,Cro, ceftriaxone; Cip, ciprofloxacin; Gm, gentamicin; K, kanamycin; Tmp, trimethoprim; Sxt, sulfamethoxazole + trimethoprim Te, tetracycline, Su-sulfisoxazole; S, streptomycin; Nitro, nitrofurantoin; Na- nalidixic acid, N, neomycin

*All intermediately resistant isolates were considered resistant for this analysis

There was a statistically significant difference in the rate of occurrence of MDR between isolates obtained from dairy farms in Addis Ababa and outside of the city limits of Addis Ababa. The mean ± standard error of mean (SEM) number of antimicrobials to which *Salmonella* isolates obtained from dairy cattle in Addis Ababa were resistant was 7.23±1.32, while the value for isolates obtained outside of Addis Ababa was 4±0.62 (p= 0.01) (Fig 3) (**Paper 1**).

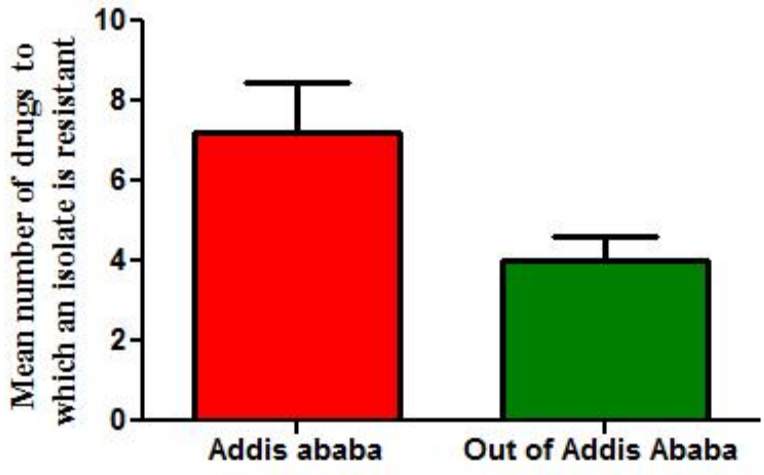

Figure 3. Level of occurrence of multidrug resistance in *Salmonella* isolated from dairy cattle in Addis Ababa and outside of Addis Ababa.

Mean±SEM number of drugs to which *Salmonella isolates* obtained from slaughtered cattle, dairy cattle, poultry, swine and human patients were resistant was 5.1±0.42, 5.33±0.7, 5.69±0.64, 2.88±0.74 and 3.1±0.35, respectively. The level of MDR was significantly higher in *Salmonella* isolates obtained from slaughtered cattle, dairy cattle and poultry compared to those obtained from humans ($p < 0.05$), while no significant difference was observed among isolates obtained from food animals. Fig 4 shows the mean number of drugs to which *Salmonella* isolates obtained from different sources were resistant.

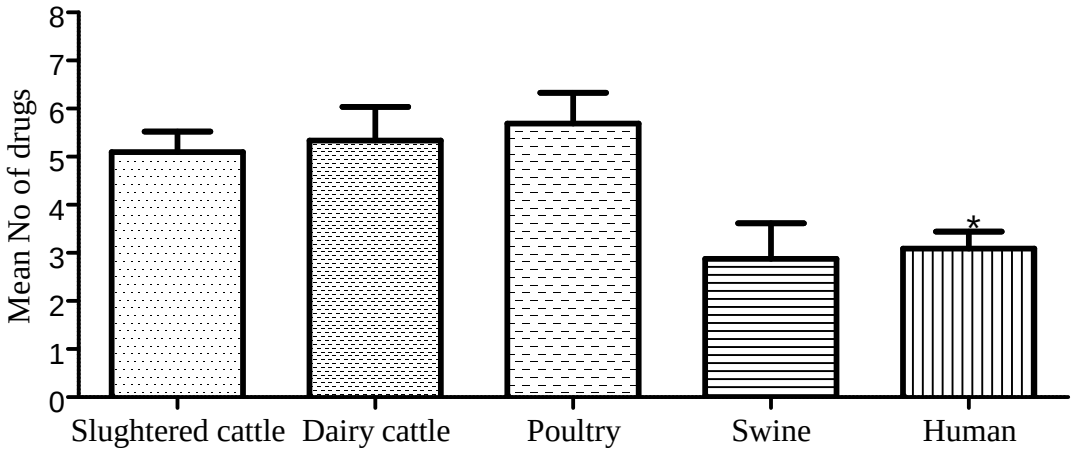

Figure 4. Level of MDR among *Salmonella* isolates from different food animals in central Ethiopia and diarrheic human patients in Addis Ababa

(Each bar represents mean ±SEM number of drugs to which isolates were resistant).

Level of multiplicity of antimicrobial resistance appeared to be associated with serotypes involved. For instance, *S.* Kentucky isolated from dairy cattle, slaughtered cattle, poultry as well as human patients were all resistant to several drugs and they were all completely resistant to nalidixic acid and ciprofloxacin. Resistance to chloramphenicol was common in *S.* Saintpaul isolated from poultry farms than other serotypes. *S.* Saintpaul and *S.* Virchow were resistant to more number of antimicrobials than other serotypes irrespective of the source of isolation (**Paper 3**).

Investigation of antimicrobial resistance for isolates collected from various food of animal origin and a few from human during 2000-2008 was also conducted. Majority of the isolates (n=74, 85.1%) exhibited MDR to the activity of between 3 and 11 of the 18 antimicrobials tested. Out of the 87 *Salmonella* isolates, resistance was detected to streptomycin (77%),

tetracycline (65.5%), sulfisoxazole (52.9 %), neomycin (41.4%), nalidixic acid (41.4%), nitrofurantoin (39.1%), cephalothin (33.3%), ampicillin (31%), sulfamethoxazole and trimethoprim (24.1%), amoxicillin + clavulanic acid (20.7%). Only one isolate (1.2%) was resistant to ceftriaxone while all isolates tested were susceptible to amikacin and cefoxitin (**Paper 5**).

Rate of resistance to some antimicrobials such as tetracycline, sulfisoxazole, nalidixic acid and neomycin was higher in isolates collected during 2000-2008 than those collected during 2013-14. On the other hand, resistance to kanamycin and streptomycin was higher during 2013-14. Overall mean±SEM number of drugs to which isolates were resistant was 5.9± 0.29 for isolates collected during 2000-2008 while for those collected in 2013-2014 were 4.3±.26 ($p<0.0001$) (Table 7).

Table 7 Comparative frequency of antimicrobial resistance to selected antimicrobial agents in isolates collected from carcasses of various food animals and in-contact humans during 2000-2008 and those collected from feces of various food animals and diarrheic patients during 2013-2014

Antimicrobial agent	No. resistant (%)*	
	Year of Collection	
	2000-2008	2013-2014
Amikacin	-	6(3.9)
Ampicillin	27(31)	38(25)
Amoxycillin+clavulanic acid	18(20.7)	34(22.4)
Cephalothin	29(33.3)	47(30.9)
Chloramphenicol	5(5.8)	13(8.6)
Ceftriaxone	1(1.2)	3(2)
Cefoxitin	-	3(2)
Ciprofloxacin	13(14.9)	21(13.8)
Gentamycin	13(14.9)	16(10.5)
Kanamycin	8(9.2)	68(44.7)
Trimethoprim	11(12.6)	7(4.6)
Sulfamethoxazone +trimethoprim	21(24.1)	6(3.9)
Tetracycline	57(65.5)	55(36.2)
Sulfisoxazole	46(52.9)	53(34.9)
Streptomycin	67(77)	124(81.6)
Nitrofuratoin	34(39.1)	68(44.7)
Nalidixic acid	36(41.4)	33(21.7)
Neomycin	36(41.4)	21(13.8)
Total	**87**	**152**

*All intermediately resistant isolates were also considered resistant in the analysis

4.5.2 Molecular mechanisms of resistance to beta-lactam and quinolone antimicrobials

Characterization of resistance mechanisms for beta-lactam and quinolone antimicrobials is presented in **Paper 4**. Among 152 *Salmonella* isolates from humans and animals 43 (28.3 %) were resistant to one or more of beta-lactam antimicrobials (ampicillin, cephalothin, cefoxitin, ceftriaxone and amoxicillin+clavulanic acid) and they were tested for various beta-lactamase enzymes and *bla* genes were detected in 35/43 (81.4%) of the isolates. The dominant *bla* gene responsible for resistance to beta-lactam antimicrobials in majority of *Salmonella* isolates (34, 94.4%) was found to be variants of *bla*TEM gene. Both phenotypic resistance to beta-lactam antimicrobials and detection of *bla* genes was more common in isolates collected from poultry compared to isolates from other sources. In one of the human isolates *S.* Concord, 2 *bla* genes (*blaOXA-10* and *blaCTX-M15)* were detected. Both these genes encode for the enzymes that are endowed with extended spectrum beta-lactamase activity (Table 8) (**Paper 4**).

Table 8. Occurrence of beta-lactamase genes in *Salmonella* isolates from different sources with reduced susceptibility to beta-lactam antimicrobials

Source	Total no. of isolates	[+]Resistant to ≥one of Beta-lactams (%)	*bla* genes tested**			Not detected	No.(%)positive for *bla* genes
			*bla*TEM	*bla*OXA10	*bla*CTX-M		
Dairy Cattle	30	11(36.7)	7	-	-	4	7(63.6)
Slaughtered cattle	20	5(25)	5			0	5(100)
Poultry	26	13(50)	12	-		1	12(92.3)
Swine	8	2(25)	1	-	-	1	1(50)
Human	68	12(17.7)	9	1	1	2	*10(83.3)
Total	**152**	**43(28.3)**	**33**	**1**	**1**	**8**	**34(81.4)**

[+]Ampicillin, Cephalothin, Cefoxitin, Ceftriaxone, amoxicillin and clavulanic acid

**Though all isolates were screened for *bla*TEM, *bla*SHV, *bla*OXA1, *bla*OXA4, *bla*PER, *bla*PSE and *bla*CMY2, none of them were positive for these genes

**blaOXA10* and *blaCTX-M15* were detected in a single isolate

Majority of the *bla*TEM enzymes were TEM-1 (18, 56.25%) followed by TEM-57 (10,31.25%) and all 10 TEM-57 were recovered from *S.* Saintpaul isolated from poultry, while those from *S.* Saintpaul strains obtained from cattle and human were all TEM-1. Two different TEM types with novel amino acid sequences (Gln6Ser, n=3) in two *S.* Kentucky and one *S.* Typhimurium and (Phe13Leu, n=1) in *S.* Typhimurium were detected. Despite change in amino-acid sequences, no difference in antimicrobial susceptibility to beta-lactam antimicrobial agents was seen among isolates carrying different variants of TEM enzymes **(Paper 4)**.

Out of 29 *Salmonella* isolates with reduced sensitivity to quinolones, high level resistance to both nalidixic acid and ciprofloxacin was observed only in 10 *S.* Kentucky isolates (34.5%). Over half (58.6%) of the quinolone resistant isolates were also resistant to at least one of the beta-lactam antimicrobials. All *S.* Kentucky isolates with high level nalidixic acid and ciprofloxacin resistance were MDR to several antimicrobials. All of these S. Kentucky isolates had double mutations in *gyrA* (Ser83Phe + Asp87Gly) and *ParC* (Thr57Ser + Ser80Ile). Single mutation in *gyr*A (Ser83Phe) was observed in 4 isolates (*S.* Livingstone var.14+(2), *S.* Virchow (1), I:6;7,14:-:I,w (1). All these isolates were resistant to nalidixic acid and intermediately resistant to ciprofloxacin. Single mutation in *gyr*A (Ser83Tyr) was detected in one *S.* Haifa from poultry with R-phenotype of resistance to nalidixic acid and intermediate resistance to ciprofloxacin.

Double mutation in *parC* (Thr57Ser + additional novel mutation Tyr83Phe) was detected in one *S.* Miami isolate obtained from swine. However, this additional mutation did not seem to contribute to a decrease in sensitivity to nalidixic acid, rather this strain was susceptible with zone of inhibition of 22 mm but intermediately resistant to ciprofloxacin. A strain of *S.* Agona with only single mutation at Thr57Ser was intermediately resistant to nalidixic acid but sensitive to ciprofloxacin. Double mutation in *gyrB* gene (Val423Gly+Asp459His) was detected in two isolates *S.* Mikawasima and *S.* Braenderup. *S.* Braenderup had additional substitution in *parC* gene (Thr57Ser) associated with intermediate susceptibility with both nalidixic acid and ciprofloxacin whereas *S.* Mikawasima was intermediately susceptible only to nalidixic acid. No mutation was detected in *parE* gene in all isolates examined (**Paper 4**).

None of the tested plasmid mediated quinolone resistance genes were detected in any of the isolates examined. Seven isolates belonging to *S.* Saintpaul, *S.* Typhimurium, *S.* Aberdeen, *S.* Virchow and *S.* Haifa with no mutation in QRDR were susceptible to nalidixic acid but had shown reduced susceptibility to ciprofloxacin with zone of inhibition ranging from 25-27mm according to CLSI (2013) cut-off points. There appeared to be other resistance mechanisms responsible for the observed decreased sensitivity (**Paper 4**).

4.5.3 Class 1 integron and *Salmonella* genomic Island.

Eighty -seven *Salmonella* isolates collected during 2000-2008 from food of animal origin and humans in Ethiopia, were screened for class I integron and *Salmonella* genomic Island (SGI1). A class 1 integron was detected in 29 (33.3%) of the 87 isolates examined, being found in serotypes Braenderup (6/6), Kentucky (14/15), Typhimurium (7/11), I: 6,7:e,h:- (1/1), and Newport (1/11). Two distinct integrons were detected only in *S.* Typhimurium. Representative gel image of class 1 integron of selected isolates is shown in Fig. 5 **(Paper 5)**

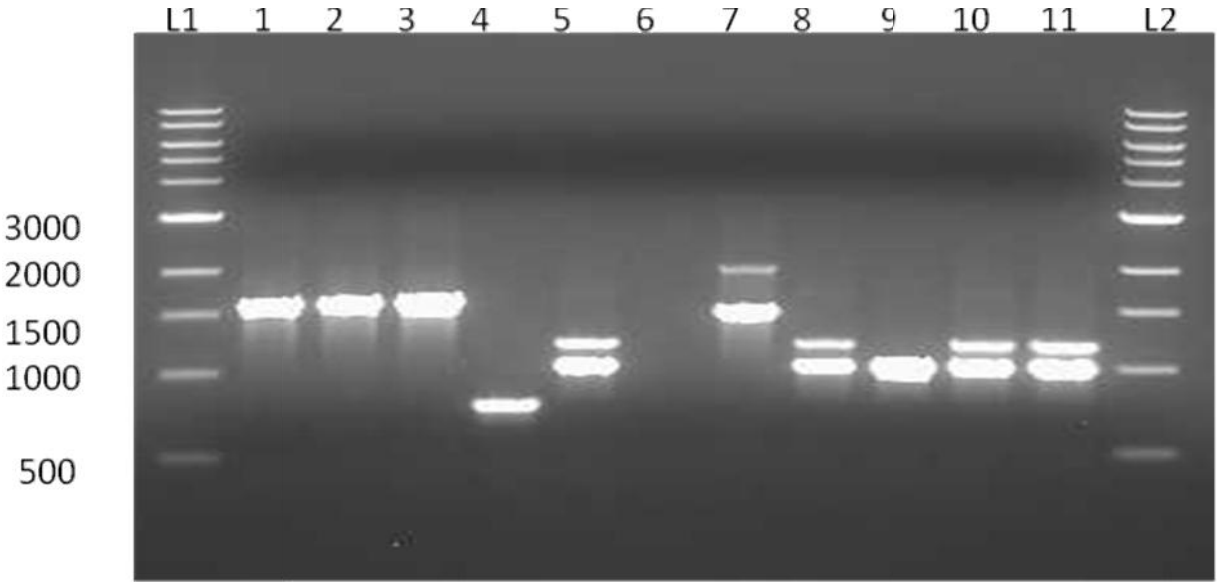

Figure 5. Representative image of polymerase chain reaction amplification of class 1 integron

L1(1kb ladder, lane 1-3 aac(3)-Id) and aadA7(1600); lane 4, B1dhfrVII gene (750); Lane 5, aadA2, blaPSE-1 (1000 &1200); Lane 6 Inti1 positive but integron negative; Lane 7, aadB+catB3 , blaOXA-1+ aadA1 (1600 & 2000**),** Lane 8 , aadA2, blaPSE-1 (1000&1200), Lane 9, aadA2 (1000), Lane 10 and 11, aadA2, blapse-1, (1000&1200)

There was a positive correlation between the presence of a class 1 integron and number of antimicrobial agents to which isolates were resistant (X^2=44.2, p<0.0001). Eight out of the 29 (27.6%) class 1 integron positive isolates were demonstrated to be carried on conjugative plasmids and have the capacity for rapid horizontal transfer of antimicrobial resistance among enteric pathogens. However, all SGI1 positive isolates and two of the *S.* Typhimurium isolates bearing a non-SGI1 integron were unable to transfer their integron to *E. coli.* This demonstrates that for most of the isolates, the class 1 integron was located on the chromosome; either incorporated in the SGI1, or found separately, and was not mobilizable **(Paper 5).**

Nineteen (65.5%) were positive for SGI1 or its variants. Classical SGI1 conferring resistance to ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfisoxazole and tetracycline) was detected in four serotype Typhimurium isolates: Typhimurium DT 104 (n=2), Typhimurium var. Copenhagen (n=1) and Typhimurium (n=1). Two variants of SGI were also detected, SGI1-K and SGI1-C. All serotype Kentucky isolates (n=14) carried SGI1-K and one Typhimurium isolate SGI1-C conferring resistance only to streptomycin and sulfisoxazole **(Paper 5).**

4.6 Genetic diversity of representative Salmonella isolates

In order to assess the genetic relatedness of *Salmonella* serotypes, 49 representative isolates were analyzed using PFGE. Large genotypic diversity with 11 genotypic clusters and 7 sporadic clones were obtained. Majority of the *Salmonella* isolates within a serotype are clonally related. This was clearly observed in *S.* Virchow, *S.* Kentucky, *S.* Kottbus, *S.* Braenderup and *S.* Typhimurium. In addition to genotypic clonality, some of these isolates especially those belonging to serotype Kentucky had similar drug resistance profile. A single *S.* Saintpaul isolated from human showed different PFGE finger print from other *S.* Saintpual strains obtained from animals (**Paper 3**).

Twenty-one representative isolates were genotyped using MLST. Three novel alleles (*aroC457*, *sucA432* and *thrA480)* and 5 new sequence types were found and deposited on the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). Both *S.* Kentucky from poultry and cattle were ST198 which is similar to the *S.* Kentucky strain commonly circulating in Africa and Asia. Most of *S.*Typhimurium from human, poultry and cattle belonged to the ST19, one *S.* Typhimurium vr. Copenhagen was ST313 while another isolate from human gave a novel sequence type ST1936. But all *S.* Typhimurium isolates belonged to the same clonal complex. Among the 6 *S.* Saintpaul, all isolates from poultry and cattle had the same novel ST1934, while one isolate from human was ST49. *S.* Braenderup, *S.* Haifa, and *S.* Kottbus examined were all with novel Sequence types (ST1932, ST1935 and ST1937), *S.* Virchow isolated from human gave ST303 **(Paper 3**).

4.7 Association of multicellular behavior and drug resistance in Salmonella enterica serotypes

The results presented under this section is based on the experimental study conducted to characterize and understand the association of biofilm formation and antimicrobial resistance phenotypes and genotypes in 87 *Salmonella* isolates collected from food animals and humans during 2000 -2008 from different parts of Ethiopia **(Paper 5).**

4.7.1 Morphotypes, biofilm formation, cellulose production and pellicle formation

When grown on Congo red agar for 6 days at 20°C, most of the isolates (n=63, 72.4%), exhibited red dry and rough morphotype (RDAR). The rest displayed brown smooth and mucoid (BSAM) (n=19, 21.8%), smooth and white (SAW) (n=3, 3.5%) and brown dry and rough (BDAR) (n=2, 2.3%). In spite of their differing host origins, most of the isolates belonging to a given serotype produced a similar morphotype. Isolates that expressed the RDAR morphotype at 20°C produced more biofilm as compared to those that expressed other morphotypes (p=0.0002). Moreover, biofilm formation of most of the isolates was higher at 20°C than at 37°C**.** Majority of isolates (n=84, 96.6%) produced a pellicle at 20°C except for two isolates of serotype Dublin and one Hadar. All of the isolates that produced RDAR morphotypes produced thick and stable pellicles that were not dispersed by agitation, while pellicles produced by isolates with BSAM and BDAR morphotypes were fragile and easily dispersed when agitated. All isolates that produced the RDAR morphotype showed calcofluor binding in an agar based calcofluor binding assay at 20°C, while all isolates that produced other morphotypes did not demonstrate appreciable levels of calcofluor binding. A

broth culture calcofluor binding assay was developed to better quantify presumed cellulose production and a strong correlation between qualitative and quantitative assessment methods of cellulose production at 20°C was demonstrated.

All isolates that failed to produce a pellicle (n=3, 3.5%) demonstrated SAW or BSAM morphotypes and showed no cellulose production at 20°C. Pellicle formation of representative isolates is shown in Fig 6. Although cellulose production appeared to be important for biofilm formation in isolates that expressed the RDAR morphotype, the level of biofilm formation was not directly related to that of cellulose production. For example, some serotypes such as Hadar and 1:6.8;-produced no detectable cellulose but formed robust biofilms. Alternatively, some serotypes that were the top cellulose producers (such as *S.* Enteritidis) were not the best biofilm producers **(Paper 5)**.

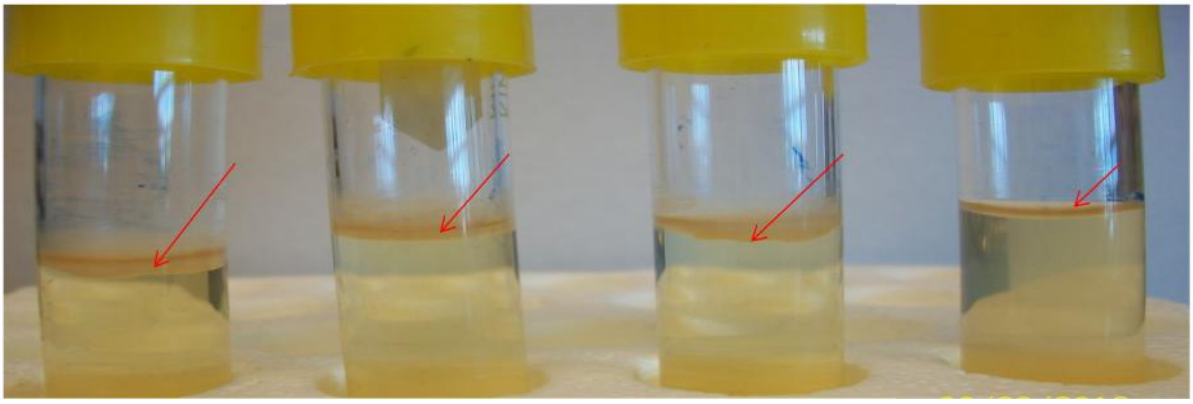

Figure 6. Pellicle formation of representative isolates incubated in LBNS medium at room temperature for 6 days

(A, B, C=pellicle at liquid air interface; D= no pellicle formation)

4.7.2 Antimicrobial resistance and multicellular behavior.

Majority of the isolates (n=74, 85.1%) exhibited MDR to the activity of between 3 and 11 of the 18 antimicrobials tested and 6 (7%) of the isolates were pansusceptible to all drugs tested. Interestingly, 3 (50%) of these isolates failed to exhibit multicellular behavior, demonstrated a SAW morphotype, showed no or little pellicle formation and exhibited minimal biofilm production. Analysis of the occurrence of resistance to selected antimicrobials by strains expressing different morphotypes showed that resistance to ampicillin, cephalothin, and sulfisoxazole, was more common in strains with the RDAR morphotype than the BDAR/BSAM morphotypes. On the other hand, resistance to gentamicin, streptomycin, kanamycin and nitrofurantoin was more common in isolates expressing BDAR/BSAM morphotypes **(Paper 5)**.

Occurrence of class 1 integron and SGI1 was more common in isolates producing RDAR morphotype when grown on Congo red agar. None of the isolates characterized by a BSAM, SAW or BDAR morphotype were positive for a class 1 integron and SGI1. At environmental temperature (20°C)**,** isolates carrying a class 1 integron and SGI1 (+/+) and those with a non-SGI1 integron (+/-) formed significantly more biofilm than isolates with no integron and SGI1 (-/-). There was no difference in biofilm forming capacity between (+/+) and (+/-) isolates. Collectively, the results indicated that isolates carrying a class I integron (SGI1 or otherwise) formed approximately 50% more biofilm than those lacking an integron **(Paper 5).**

4.8 Inhibition of Salmonella enterica biofilm formation using small molecule adenosine mimetics

4.8.1. Initial screening and identification of a candidate compound

The initial library comprised of 3,000 putative ATP-mimetic compounds were screened with a high-throughput screening assay and 34 compounds were found to inhibit biofilm formation by ≥ 30%, 5 compounds by ≥ 40% and 3 compounds by ≥ 50%. Manual testing of the 3 compounds using the rapid attachment biofilm assay in 3 separate experiments eliminated all but one compound, 7955004 (3-(2-furylmethyl)-2-{[(5-hydroxy-1H-pyrazol-3-yl)methyl]thio}-3,5,6,7-tetrahydro-4H cyclopenta [4,5]thieno[2,3-d]pyrimidin-4-on), which consistently demonstrated ~55% reduction of *S.* Typhimurium biofilm formation relative to untreated controls at a concentration of 30µM **(Paper 6).**

4.8.2 Dose dependent inhibition of biofilm formation by Compound 7955004

Biofilm assay using crystal violet assay technique in the presence of varying concentrations of compound 7955004 (0.625µM to 50µM) revealed a dose-dependent inhibition of biofilm formation in *S.Typhimurium* as well as *Acinetobacter baumannii* and minor inhibition in *Staphylococcus aureus.* This inhibition was not associated with bactericidal and bacteriostatic activity which was further verified through bacterial CFU enumeration in the presence or absence of 30µM drug **(Paper 6).**

4.8.3 Cytotoxicity of compound 7955004

To determine the potential cytotoxic effects of compound 7955004, lactate dehydrogenase (LDH) assay was performed on HepG2 cells that were treated with varying concentrations of the compound for 12h and 24h. No significant difference between LDH release in treated versus control wells was observed indicating no acute cellular cytotoxicity at 30µM and 50µM concentrations. This result was further confirmed by trypan blue exclusion assay. Thus, compound 7955004 showed no evidence of acute toxicity on human hepatocytes at a concentration of 50µM, which was over 9 fold greater than the established (IC_{50}) (Paper 6).

4.8.4 Identification of putative protein targets for compound7955004

To determine potential interacting proteins with compound 7955004 in the bacterial proteome, wild-type *S.* Typhimurium was grown overnight and bacterial lysates were prepared and run through an ATP-Sepharose affinity chromatography. Two protein bands (molecular weights 60 kDa and 25 kDa) were consistently eluted by compound 7955004. These protein bands were identified using mass spectrometry as *S.* Typhimurium GroEL (Hsp60) and purine nucleoside phosphorylase (DeoD), respectively **(Paper 6)**.

5. Discussion

Food animals are the primary source for transmitting non-typhoidal *Salmonella* to humans (Branham et al., 2005). This study reports occurrence of *Salmonella* serotypes commonly implicated for human salmonellosis in dairy cattle, slaughtered cattle, poultry and swine in highly populated town of Addis Ababa and surrounding districts and demonstrated co-occurrence of related serotypes in different livestock and human patients in Addis Ababa **(Paper 1, 2, 3).**

When individual animal level prevalence of *Salmonella* was compared in dairy cattle, slaughtered cattle, swine, and poultry, it was low in dairy and high in slaughtered cattle. On the other hand the highest farm level prevalence of *Salmonella* was detected in swine farms. However, diverse serotypes such as *S.* Typhimurium, *S.* Kentucky and *S.* Virchow detected from human patients in Addis Ababa during similar study period were more often isolated from dairy cattle than other food animals implying higher chance of human *Salmonella* infection from dairy cattle in Addis Ababa (**Paper 3**). This is probably because of the fact that most of the dairy farms were located in Addis Ababa where human samples were collected while most of the poultry and swine farms were located in Adaa district.

Salmonella was more frequently detected in human patients consuming raw vegetables in Addis Ababa. This might be due to contamination of vegetables from small scale livestock farms mainly, dairy in Addis Ababa residing in close proximity with small scale vegetable farms. Most of the vegetable farms are located at the periphery of the town, where small scale dairy farms are also located. Water used to grow and clean these vegetables might be

contaminated with *Salmonella* in the feces of animals from these farms in addition to cross-contamination in the kitchen (Hanning et al., 2009). The fact that *Salmonella* can form strong biofilms on plant surfaces, leading to prolonged persistence and resistance to disinfectants might have contributed to frequent recovery of *Salmonella* from these patients (Yaron and Römling, 2014) **(Paper 2)**.

Although *Salmonella* prevalence in conventional poultry is usually very high in different countries (Alali et al., 2010; Barua et al., 2012; Kagambèga et al., 2013) and commonly implicated as the major sources of human *Salmonella* outbreaks (Basler et al., 2014; Gaffga et al., 2012; Taylor et al., 2012), only 4 serotypes, dominated by *S.* Saintpaul (in 7 out of 48 farms) were detected in the current study ableit only one *S.* Saintpaul was detected in human. Even this strain was genotypically unrelated to all *S.* Saintpaul isolates from animal origins in the current study, suggesting other possible source of infection. The possible explanation for poultry not being a major source of *Salmonella* infection to human in the current studycould be due to the fact that most of the poultry farms in the current study were from Adaa district which is a little bit far from human population in Addis Ababa. The other possible reason could be strain of the dominant serotype detected from poultry, *S.* Saintpaul might be less virulent to human as it was shown to be genotypically different from the one isolated from human (**Paper 3**). Similarly individual animal level prevalence of *Salmonella* in swine in the current study was low (4.4%) compared to studies in other countries (Molla et al., 2010; Wales et al., 2013). Among 8 isolates recovered from pigs in the current study, there were 6 serotypes of which *S.* Typhimurium and *S.* Miami were also recovered from human during similar study period. *Salmonella* was recovered in13.9% of chicken and

11.3% of pork in Ethiopia previously (Zewdu and Cornelius, 2009). The high prevalence in chicken and pork than in poultry and swine feces could be due to contamination during slaughter, transportation and storage of the chicken and pork (**Paper 3**).

Significantly higher occurrence of MDR in *Salmonella* isolates obtained from food animals compared to those obtained from humans might be due to frequent use of these drugs in animals in the study area (**Paper 1**). This poses high risk of transmission of drug resistance and resistance genetic markers to humans. Most of *Salmonella* isolates from humans in the current study originated from patients from primary health centers where antimicrobial selection pressure is minimal. Previous studies (Beyene et al., 2011; Gebre-Yohannes, 1985) on human *Salmonella* isolates were based on hospital patients and showed high level of MDR compared to the current findings. Interestingly, 2 (50%) of the 4 *Salmonella* isolates from TASH in the current study were MDR to over 10 antimicrobials. One of the isolate, *S.* Concord resistant to ampicillin, cephalothin, cefoxitin and ceftriaxone was shown to produce *bla*CTX-M-15 and *bla*OXA-10 (**Paper 4**). The other MDR *S.*Typhimurium DT 193 isolated from diarrheic child in TASH was resistant to 11 drugs (AmpAmcCfKSxtTmpTeSuNitroSN) and whole genome sequence analysis of this isolate revealed the presence of acquired resistance genes: *aac(6')-Iy, aph(3')-Ia, strA, strB, blaTEM-1B, sul2, tet (A) and dfrA5* (data not shown). This suggests that high level MDR *Salmonella* infection in patients from hospitals in Ethiopia might be due to nosocomial infection. Previous study in TASH showed high load of MDR nosocomial pathogens including *Salmonella* carried by cockroaches in a neonatal intensive care unit (Tilahun et al., 2012). *Salmonella* nosocomial infection has been reported previously in different countries

(Lee and Greig, 2013; Smith et al., 2014). Therefore, strict pathogen control strategy and prudent use of effective antimicrobials in the hospital environment is essential to reduce the burden of resistant *Salmonella* species and other pathogens in hospitals.

On the other hand, the level of MDR in isolates collected from carcasses of food animals and few in contact animals during 2000-2008 was significantly higher than those collected during 2013-14 from feces of livestock and clinical human patients. Generally, local and global studies showed that, occurrence of resistance to antimicrobials is increasing in *Salmonella* spp. (Tadesse, 2014; Weinberger and Keller, 2005) and our finding is contrary to this usual trend. The probable reason could be due to the fact that most *Salmonella* isolates collected earlier were from carcasses of food animals at abattoir with high probability of exposure to drugs and resistant pathogens at fattening farms. Moreover, there might be contamination of meat products by persistent and resistant *Salmonella* strains at slaughter houses and butcheries. Most of the isolates collected during 2013-14 were however isolated from feces of animals at farm level and clinical patients at primary health centers. As the source of *Salmonella* isolates during these two periods were different, such comparison may not entail the true trend of antimicrobial resistance in *Salmonella* isolates in the country.

Although, *S.* Concord was the dominant serotype in human patients in previous reports from Ethiopia (Gebre-Yohannes, 1985; Beyene et al., 2011); *S.* Typhimurium was the dominant serotype in the current study, which is in line with studies conducted in other sub-Saharan African countries (Kariuki et al., 2006). In the present study, only one *S.* Concord was isolated from a one year old hospitalized child **(Paper 2)**. None of the *Salmonella* isolates from animals were also *S.* Concord. Moreover, no literature was available on occurrence of

Salmonella from animal and food of animal origin in Ethiopia despite widespread report of *S.* Concord in humans from Ethiopia and children adopted from Ethiopia (Beyene et al., 2011; Gebre-Yohannes, 1985; Hendriksen et al., 2010; Hendriksen et al., 2009) and a few human reports from other countries (Cattoir et al., 2007; Erdem et al., 2005; Hasman et al., 2005) most of which are linked to Ethiopian origin. Pubmed literature search for *S.* Concord from non-human origin has shown recovery of *S.* Concord only from chicken, sheep and sewage in Saudi Arabia in 1982 (Barbour and Nabbut, 1982; Nabbut et al., 1982). The fact that, most of the recent *S.* Concord isolates identified globally are linked to children adopted from Ethiopia, its high prevalence in young children in hospitals, absence in diarrheic patients from primary health centers, as well as food animals and food of animal origin so far tested in Ethiopia, suggests that *S.* Concord is probably circulating only among patients in hospital settings and orphanages in Ethiopia. The single isolate from the current study and most of the isolates from previous studies were MDR to several drugs, which probably was acquired in the hospitals and contributed to its persistence in the environment. This serotype was shown to cause invasive disease (Beyene et al., 2011) posing serious threat nationally and globally. Further investigation on the possible reservoir of this serotype should be conducted.

Salmonella serotypes frequently isolated from clinical human patients such as *S.* Typhimurium, *S.* Virchow, *S.* Kottbus and *S.* Kentucky were also isolated from spatially and temporally related food animals. Moreover, genotyping of selected isolates showed clonally related isolates of *S.* Kentucky from dairy cattle, slaughtered cattle, poultry and humans; *S.* Virchow from dairy cattle, slaughtered cattle and humans; *S.* Miami from swine and human;

S. Braedrup from slaughtered cattle and human. Though difficult to clearly indicate the direction of flow of the isolates as the study was cross-sectional and included different sources at around the same time, the current finding clearly showed clonal circulation of strains of these serotypes among humans and animals in the study area. Interestingly, all *S.* Kentucky strains in the current study were resistant to 9 antimicrobials in common (AmpAmcCfCipGmTeSuSNa) **(Paper 3)**. Similarly most isolates of *S.* Virchow from humans and animals in the current study had common antimicrobial resistance profile and representative isolates showed identical PFGE finger print, despite difference in geographic location, suggesting common source of infection. Infection of humans with MDR strains of *Salmonella* has been reported to lead to prolonged hospitalization, increased risk of invasive illness and poor health outcomes compared to those infected with susceptible strains (Helms et al., 2004; Martin et al., 2004; Varma et al., 2005). There is a need for appropriate control strategy to reduce spread of these MDR strains among animal and human population of the study area.

Molecular characterization of resistance to beta-lactam antimicrobials in the current study showed that the major resistance mechanism was due to *bla*TEM and most of which were *bla*TEM-1(**Paper 4)**. However, all *bla*TEMs from *S.* Saintpaul of poultry origin were *bla*TEM-57 with substitution of Gly to Asp at position 92 of amino acid sequences which was first reported from *Proteus mirabilis* (Bonnet et al., 1999) and latter on from *E. coli* in China (Yuan et al., 2009). Fortunately, all TEM types, including TEM-57 and the two novel TEM enzymes in the current study with mutation at position 6 (Gly to Ser) and 13 (Phe to Leu) were not associated with extended spectrum activity against second and third

generation cephalosporins. However, as most of the isolates in the current study were from livestock and diarrheic patients from primary health centers, this finding cannot represent the real status of all *bla* enzymes in *Salmonella* and other Gram-negative organisms in the country. Therefore, further study involving different Gram-negative organisms such as *E. coli, Pseudomonas and Klebsiella* species should be conducted in hospital and community settings.

Detection of similar amino acid substitutions in *gyrA* gene at codon 83 and 87 (Ser83Phe + Asp87Gly) and *ParC* gene at codon 57 and 80 (Thr57Ser + Ser80Ile) in all *S.* Kentucky isolates obtained from human and animal sources as well as carriage of *bla*TEM gene in all of them also strengthen our previous hypothesis of clonal spread of *S.* Kentucky strain in the human and animal population in the study area. *S.* Kentucky isolates in the current study also belonged to ST198 suggesting the clonal relatedness of our isolates to the internationally spreading clone of *S.* Kentucky (Weill et al., 2006). Contrary to previous experimental study which showed that mutation based fluoroquinolone resistance in *S.* Typhimurium ends up with loss or reduction of its invasive capacity important for its clonal success (Fabrega et al., 2009), this nationally and globally spreading clone of MDR fluoroquinolone resistant *S.* Kentucky should have some unique mechanisms which enabled its clonal success to spread globally. This might be associated with its strong biofilm forming ability or unknown genetic mechanism encoded by plasmid. Further study is recommended to figure out the mechanism associated with the clonal success of this MDR strain of *S.* Kentucky **(Paper 4).**

In study on multicellular behavior of non-typhoidal *Salmonella* isolates from animals and humans **(Paper 5),** majority of the isolates had shown RDAR morphotype (co-expression of curli and cellulose) and strong biofilm production on crystal violet assay which is in agreement with previous reports (García et al., 2004; Zogaj et al., 2001). However several isolates with the BSAM morphology exhibiting minimal cellulose production and over production of capsular polysaccharide (de Rezende et al., 2005; Malcova et al., 2008) were similarly able to form biofilms. Thus, while extracellular matrix (ECM) typically curli and cellulose are important for biofilm development, the exact ECM constituents that allow efficient biofilms to occur are not specific. Contrary to *Salmonella* isolates with RDAR morphotype which produced thick and stable pellicles, those characterized by a BDAR or BSAM morphotype on Congo red agar formed fragile and easily dispersed pellicles. *S.* Typhimurium lacking either cellulose (JSG1748, Δ*bcsA*::kan), or curli (JSG3132, Δ*csgG)* formed BDAR and PDAR morphotypes on Congo red agar, respectively. The cellulose-deficient strain (JSG1748) formed a fragile pellicle at the liquid air interface whereas the curli mutant (JSG3132) produced no pellicle at all. This suggests that, the primary requirement for pellicle formation is curli but formation of tight and relatively strong aggregation of the bacteria in the pellicle and expression of the RDAR morphotype is associated with co-expression of both curli and cellulose.

This study also developed a new calcofluor binding assay in liquid medium which was demonstrated to have a strong correlation between results obtained with the established qualitative calcofluor binding technique on agar plates. Thus, the liquid assay described here

provides a new, objective method to quantify the level of cellulose production by bacteria grown in broth culture **(Paper 5).**

Correlation of the occurrence of resistance to selected antimicrobials by strains expressing specific multicellular morphotypes could be due to co-existence of genes required for expression of specific morphotype and resistance phenotype. Correlation of MDR in strains with strong biofilm production compared to non-producers or reduced biofilm producers has also been reported previously for *Salmonella* (Kim and Wei, 2007), *Staphylococcus* (Kwon et al., 2008), mixed microbial isolates from the intraocular lens (Ravish K, 2012), and *Acinetobacter baumannii* (Rao et al., 2008). It has also been shown that loss or inhibition of one or more MDR efflux pumps in *S.* Typhimurium resulted in impaired biofilm formation demonstrating an association of efflux pump-mediated drug resistance and biofilm formation (Baugh et al., 2012).

Class I integron was detected in over 30% of 87 Salmonella isolates tested of which 27% demonstrated the potential for rapid horizontal transfer to *E. coli*. Class 1 integrons and SGI1were exclusively detected in Salmonella isolates with strong biofilm forming ability, with RDAR morphotypes, and also MDR phenotypes in most of these isolates. On the other hand, integrons and MDR phenotype were not detected in isolates with poor biofilm forming ability, those with SAW morphotype and poor pellicle formation. Although formal studies on antimicrobial usage are not available in the country, most of the dairy farms in the current study reported extensive use of oxytetracycline (94.6%), penicillin + streptomycin (81.8%) for therapeutic purose (Paper 1). Imprudent use of antimicrobials in Ethiopia may be selecting for both biofilm formation and antimicrobial resistant strains together. This could

be aided by easy DNA transfer through close association of bacteria in biofilm. Acquisition of drug resistance genetic markers such as class 1 integrons and/or the SGI1 may provide encoded factors that affect biofilm formation in a manner unrelated to antibiotic resistance (Golding et al., 2007).

In general, *Salmonella enterica* serotypes from Ethiopia are dominated by multicellular behavior and multidrug resistance. Both traits are important for bacteria to withstand environmental insults and may advance the dissemination of these isolates. It appears that indiscriminate antimicrobial use is selecting not only for more resistant strains but more persistent ones as well. This demands special emphasis on prudent use of antimicrobials in both veterinary and human health sectors in this country and suggests the need for integrated and sustainable surveillance on multicellular behavior and drug resistance in *Salmonella enterica* of animal and human origin in the region **(Paper 5).**

Biofilm formation has been demonstrated to result in increased tolerance to antimicrobials, which is not exhibited by genetically identical cells in the planktonic phase of growth (Foley and Gilbert, 1996; Lewis, 2001; Stewart, 2002). MDR and strong biofilm forming ability in *Salmonella* in the host and the environment are now common, and drug discovery is a high-priority research area in an effort to find novel ways to hamper dissemination of pathogens and fight infections (Hopkins and Groom, 2002). Here, 3000 chemical libraries of adenosine mimetic compounds were screened for antibiofilm activity and a single compound (Compound 7955004) exhibited dose dependent antibiofilm activity in *S.* Typhimurium, *S.* Typhi and *A. baumannii* without killing the bacteria. However, the compound did not disperse pre-formed biofilms suggesting its activity only on early stage of

biofilm formation. Further study on compound 7955004 or related compounds might come up with potential use of these chemicals in food processing environment and use in antimicrobial therapy to combat persistent infection **(Paper 6).**

Two proteins GroEL (Hsp60) and purine nucleoside phosphorylase (DeoD) were elucidated to be the major targets of compound 7955004 using the ATP-sepharose affinity chromatography technique. GroEL, a chaperone protein capable of interacting with and assisting in the proper folding of many proteins in eukaryotes, bacteria, and archea (Ryabova et al., 2013; Zhang et al., 2013) requires ATP-binding and hydrolysis for its function in the cell. GroEL was reported to be essential for biofilm formation in *Mycobacterium smegmatis* and nontypable *Haemophilus influenza* (Gallaher et al., 2006; Ojha et al., 2005). The fact that compound 7955004 had no effect on the planktonic growth of bacteria, suggests that it causes reduced functionality of GroEL but not complete loss of its function because previous studies has shown that complete deletion of GroEL is lethal in *E. coli*. The second protein target, DeoD is responsible for scavenging and breaking down nucleotides producing free purine bases and free sugar that can be utilized as a carbon source. DeoD was reported previously to be associated with biofilm in *E. coli* (Zhang et al., 2007), in *Vibrio* (Beyhan et al., 2007) and early attachment and biofilm formation in *Steptococcus* (Yadav et al., 2012).

Subsequent studies should focus on employing heterologous deletions in these putative target proteins to establish whether they are important in *Salmonella* biofilm formation as well and to better understand how compound 7955004 is working in the early stages of biofilm formation. Further activity guided evaluation of compound 7955004 and its

derivatives with the goal of increasing its potency and broadening its efficacy to additional biofilm-forming pathogens should be conducted.

6. Limitations of the study

Detailed information of stored *Salmonella* isolates collected during 2000-2008 was not available. The number of *Salmonella* isolates collected from patients from TASH was also not comparable with those obtained from primary health centers. The number of *Salmonella* isolates with poor biofilm forming ability, were small compared to those with strong biofilm producers. Because of such differences among groups, it was difficult to make comparison among groups and inference on the study findings. Since the study was conducted in central Ethiopia, in and around Addis Ababa, where there are large number of small scale livestock production, high human-livestock interaction and easy availability of pharmaceuticals, the findings cannot be generalized to the status of *Salmonella* in the whole country.

7. Conclusions and Recommendations

Similar serotypes, resistance patterns and clonally related serotypes of *Salmonella* were found in food animals and humans in central Ethiopia. Particularly, clonally related serotypes of *S.* Virchow, *S.* Typhimurium, *S.* Kentucky, *S.* Braendurp and *S.* Miami were found to circulate among food animals and humans in the study area. Human *Salmonella* infection was also significantly associated with consumption of raw vegetables suggesting the possible source of infection to humans. Presence of large number of small scale dairy farms and vegetable farms in close proximity in highly populated city of Addis Ababa might be facilitating easy transfer of *Salmonella* and other pathogens between animals, the environment and human population. The level of MDR was high in *Salmonella* isolates from food animals compared to those from diarrheic human patients from primary health centers, increasing risk of spread of resistant *Salmonella* strains and resistance genes to human population. Some of the clonally related *Salmonella* isolates from humans and animals share common MDR phenotype indicating transfer of drug resistance genes. Of particular concern is occurrence of MDR *S.* Kentucky in dairy cattle, slaughtered cattle, poultry; MDR *S.* Virchow in dairy cattle, slaughtered cattle and subsequent detection of clonally related isolates from diarrheic human patients in Addis Ababa. *S.* Kentucky isolated from food animals and humans were MDR to over 9 drugs and all were completely resistant to ciprofloxacin, which is of great concern as this antimicrobial is among the last options for treatment of complicated non-typhoidal salmonellosis in humans.

Level of MDR in *Salmonella* isolates from diarrheic human patients from primary health centers in the current study was low compared to the 2 human isolates (*S.* Typhimurium PT

193 and *S.* Concord) from TASH which showed a wider spectrum of resistance. These MDR isolates are probably acquired from the hospital environment. Co-occurrence of beta-lactamases with ciprofloxacin resistant determinants in MDR *S.* Kentucky with high level fluoroquinolone resistance mediated by double mutations in *gyrA* and *parC* genes in cattle, poultry, and humans suggests clonal spread of this strain in the study area.

Integrated laboratory based surveillance involving isolates from humans, food animals and food is the corner-stone for proper monitoring of Salmonella serovars and their antimicrobial resistance status. As the majority of NTS serotypes have broad host range and capacity to survive in different environmental conditions, there is a need for clear understanding of reservoirs of these pathogens and various risk factors to prevent infections. Prevention of transmission of NTS therefore is a reliable model of the One Health approach, since reducing human infections cannot be achieved without reducing *Salmonella* in animals and restricting transmission from the environment. Routine culturing for *Salmonella* is not a common practice among primary health centers and frequency of culturing for *Salmonella* is low in hospitals. Currently, there is no national surveillance network for NTS among hospital and regional laboratories and there is no centrally coordinated effort to serotype *Salmonella* isolates.

This study also demonstrated that *Salmonella* serotypes from Ethiopia are dominated by multicellular behavior and there was strong association of multicellular behavior and presence of MDR phenotype as well as MDR genetic determinants. Both MDR and multicellular behavior enable the bacteria to persist in environmental insults and may

advance the rapid dissemination of these isolates. Screening of adenosine mimetic compounds came up with a compound 7955004, with dose dependent inhibition of early stage biofilm formation of *S. enterica* and *A. baumannii* without causing effect on planktonic growth of bacteria. The identification of a lead compound with biofilm inhibitory capabilities toward *Salmonella* provides a potential new avenue of therapeutic intervention against *Salmonella*, with applicability to biofilms of other bacterial pathogens.

Recommendations

Detection of clonally related *Salmonella* isolates from food animals and humans as well as association of *Salmonella* recovery rate with consumption of raw vegetables warrants implementation of appropriate pathogen control strategy along critical points in food animal and vegetable production from farm to bench. There should be a strong national integrated monitoring and surveillance of NTS serotypes circulating in humans and livestock and their drug resistance status. In country with such a large human and livestock population, it is advisable to have at least one reference laboratory to serotype and conduct drug resistance characterization of *Salmonella* isolates from humans, animals and food so as to efficiently track the source of *Salmonella* outbreak in humans and to identify major risk factors on time. In addition, establishment of national surveillance network using One Health approach involving humans, food animals, food and the environment is recommended. Special emphasis should be paid to prudent use of antimicrobials in both veterinary and human health sectors in the country. To this effect, creation of awareness on the potential threat of emergence and spread of MDR in pathogens of veterinary and public health importance to

health professionals as well as the community is vital in prolonging the life span of available antimicrobials.

Improvement in hygiene and sanitation is the basic requirement for prevention of further spread of pathogens. Therefore, to control further spread of MDR resistant strains of *Salmonella* in the hospital environment, primary health centers, farms and slaughter houses; strict pathogen control strategy such as regular disinfection, proper cleaning and monitoring of susceptibility status of isolates should be conducted. Creating awareness with regard to the importance of hygiene for the patients and the health professionals is vital in reducing the risk of infection with MDR *Salmonella* and other health facility acquired infections. Further study on characterization of molecular resistance determinants including extended spectrum beta-lactamases and fluoroquinolone resistance mechanisms in *Salmonella* and other Gram-negative pathogens in hospital and community setting is recommended since the majority of the current isolates were from healthy animals at farm level and human patients from primary health care centers. Further detailed studies on association of biofilm formation and MDR, development of chemicals capable of inhibiting biofilm formation are a worthy area of future study.

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Paper 1

Eguale et al. BMC Microbiology (2016) 16:20
DOI 10.1186/s12866-016-0638-2

BMC Microbiology

RESEARCH ARTICLE Open Access

Fecal prevalence, serotype distribution and antimicrobial resistance of *Salmonellae* in dairy cattle in central Ethiopia

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Abstract

Background: *Salmonellae* are major worldwide zoonotic pathogens infecting a wide range of vertebrate species including humans. Consumption of contaminated dairy products and contact with dairy cattle represent a common source of non-typhoidal *Salmonella* infection in humans. Despite a large number of small-scale dairy farms in Addis Ababa and its surrounding districts, little is known about the status of *Salmonella* in these farms.

Results: *Salmonella* was recovered from the feces of at least one animal in 7.6 % (10/132) of the dairy farms. Out of 1203 fecal samples examined, 30 were positive for *Salmonella* resulting in a weighted animal level prevalence of 2.3 %. Detection of diarrhea in an animal and in a farm was significantly associated with animal level ($p = 0.012$) and herd level ($p < 0.001$) prevalence of *Salmonella*. Animal level prevalence of *Salmonella* was significantly associated with age ($p = 0.023$) and study location; it was highest among those under 6 months of age and in farms from Adaa district and Addis Ababa ($p < 0.001$). Nine different serotypes were identified using standard serological agglutination tests. The most frequently recovered serotypes were *Salmonella* Typhimurium (23.3 %), *S.* Saintpaul (20 %), *S.* Kentucky (16.7 %) and *S.* Virchow (16.7 %). All isolates were resistant or intermediately resistant to at least one of the 18 drugs tested. Twenty-six (86.7 %), 19 (63.3 %), 18 (60 %), 16 (53.3 %) of the isolates were resistant to streptomycin, nitrofurantoin, sulfisoxazole and tetracycline , respectively. Resistance to 2 drugs was detected in 27 (90 %) of the isolates. Resistance to 3 or more drugs was detected in 21 (70 %) of the isolates, while resistance to 7 or more drugs was detected in 11 (36.7 %) of the isolates. The rate of occurrence of multi-drug resistance (MDR) in *Salmonella* strains isolated from dairy farms in Addis Ababa was significantly higher than those isolated from farms outside of Addis Ababa ($p = 0.009$). MDR was more common in *S.* Kentucky, *S.* Virchow and *S.* Saintpaul.

Conclusion: Isolation of *Salmonella* serotypes commonly known for causing human salmonellosis that are associated with an MDR phenotype in dairy farms in close proximity with human population is a major public health concern. These findings imply the need for a strict pathogen reduction strategy.

Keywords: Dairy farm, Feces, *Salmonella enterica*, Multi-drug resistance

Background

Salmonella is a diverse bacterial species comprising over 2600 serotypes [1]. *Salmonella* commonly colonizes a range of animal hosts such as mammals, amphibians, reptiles, birds and insects [2]. There are 2 species of *Salmonella*: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further classified into 6 subspecies (*Salmonella enterica* subspecies *enterica*, *S. enterica* Subspecies *salmae*, *S. enterica* Subspecies *arizonae*, *S. enterica* Subspecies *diarizonae*, *S. enterica* Subspecies *hautenae* and *S. enterica* Subspecies *indica*). Most of the *Salmonella* serotypes are part of *S. enterica* subspecies *enterica*, and over 99 % of human and animal infections are caused by serotypes under this subspecies [3].

Diseases caused by *Salmonella* represent an important public health problem among the common bacterial

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foodborne pathogens worldwide. It is estimated that globally 93.8 million cases and 155,000 deaths are associated with gastroenteritis due to *Salmonella* species annually. Of these cases, 85.6 % were estimated to be foodborne [4]. Human salmonellosis has been associated with contaminated food products, mainly those of animal origin such as poultry, beef, pork and dairy products, as well as direct contact with infected animals [5–7].

Various serotypes of *Salmonella* have been isolated from the feces of apparently healthy dairy cattle. *Salmonella* in dairy animals may exist as a normal microbiota of the gastrointestinal population, or as a transient member of the gastrointestinal microbial population [8].

All sick, recovered and asymptomatic cattle can shed *Salmonella* through feces and the organism can survive for a long time in favorable environments outside the host [9]. Fecal shedding of *Salmonella* can increase intra-herd transmission, accidental spread to other herds, environmental contamination and risk of human infection [10]. Consumption of raw milk, inadequately pasteurized milk, improperly cooked beef from culled dairy cattle, contaminated water and direct animal contact are the major routes of acquiring dairy associated salmonellosis in humans [6].

In Ethiopia, there are large numbers of small-scale peri-urban dairy farms mainly situated close to areas of public residence. Most of these farms are located very close to Addis Ababa, capital city of the country, or reside within the city in a very close proximity with human populations. The consumption of raw milk and its derivatives is common in Ethiopia, posing high risk of infection with dairy-associated foodborne pathogens. Such pathogens include *Salmonella spp.*, *Klebsiella* spp., *Enterobacter* spp., and *Escherichia coli*, which have been identified in milk products in Ethiopia [11]. Gram positive pathogens such as *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes* and *Enterococcus* spp. have also been frequently isolated from milk [12, 13].

Occurrence of non-typhoidal *Salmonella* serotypes commonly infecting humans in dairy cattle, particularly, those stains resistant to antimicrobial agents commonly used in human medicine, are a serious threat to human health. Some multi-drug resistant (MDR) *Salmonella* outbreaks in humans have been linked to exposure to dairy farms or contaminated dairy products [6, 14]. Information on the prevalence, serotype distribution and antimicrobial susceptibility of *Salmonella* in dairy farms is vital to implementation of appropriate strategies to prevent the introduction and spread of the pathogen in the farm as well as to reduce the risk of human salmonellosis. Knowledge on the serotypes circulating in dairy farms would inform scientists/clinicians on the role of dairy cattle as a source of human *Salmonella* infections. A previous study conducted in Addis Ababa has shown farm level prevalence of 47.8 % and animal level prevalence of 7.7 % [15]. However, this study involved small sample size and the isolates were not serotyped. Given the relative lack of information concerning the prevalence and serotype distribution of *Salmonella* spp. in dairy farms in Ethiopia, the present study was designed to investigate animal level and herd level fecal prevalence of *Salmonella*, serotype distribution and antimicrobial resistance profiles of *Salmonella* in dairy farms in and around Addis Ababa, Ethiopia. It also attempted to investigate the association of farm size, occurrence of diarrhea in the farm and age of animals with prevalence of *Salmonella* in these dairy farms.

Methods

Study design, study area and sampling of study animals

A cross-sectional study was conducted in Addis Ababa and in five districts of the Oromia region located at the outskirt of Addis Ababa, namely: Sebeta, Barake, Welmera, Sululta and Adaa (Fig. 1). In these areas, the interaction between animal and human population is very high due to high density of the human populations and the large number of peri-urban dairy farming facilities. These areas are the major sources of dairy milk supply to Addis Ababa. Sampling of study herds and animals was conducted from June to December 2013. Study animals were selected from 132 dairy herds (Addis Ababa; $n = 38$; Adaa, $n = 12$; Sebeta, $n = 21$; Sululta, $n = 24$; Welmera; $n = 18$). Inclusion of herd in the sampling was based on representation of the area under study, willingness of the owners, geographical accessibility, and the herd having a minimum of 5 cattle. The largest herd size contained 398 head of cattle. Farms were categorized into small (5–20 animals in a herd), medium (21–50 animals in a herd) and large (more than 50 animals). Mean herd size of small, medium and large farms was 12.6, 31.7 and 100.4, respectively. In total 1203 fecal samples were collected from healthy as well as diarrheic cattle during the study period. The study design was cross-sectional implying a one point fecal sample collection from a given herd and hence there was no repeated fecal sample collection.

Sample collection, *Salmonella* isolation and identification

Fecal samples were collected directly from the rectum using disposable gloves into sterile zippered plastic bags and transported to the Microbiology Laboratory, Aklilu Lemma Institute of Pathobiology, in an ice box within 3–4 h of collection. Isolation and identification of *Salmonella* was conducted using conventional methods [16, 17]. Briefly, 10 g of feces was pre-enriched in 90 ml of buffered peptone water (BPW) (Becton Dickinson, Sparks, MD) and incubated overnight at 37 °C. A 100 μl pre-enriched suspension was added into 9.9 ml of Rappaport-Vassiliadis enrichment Broth (RVB)

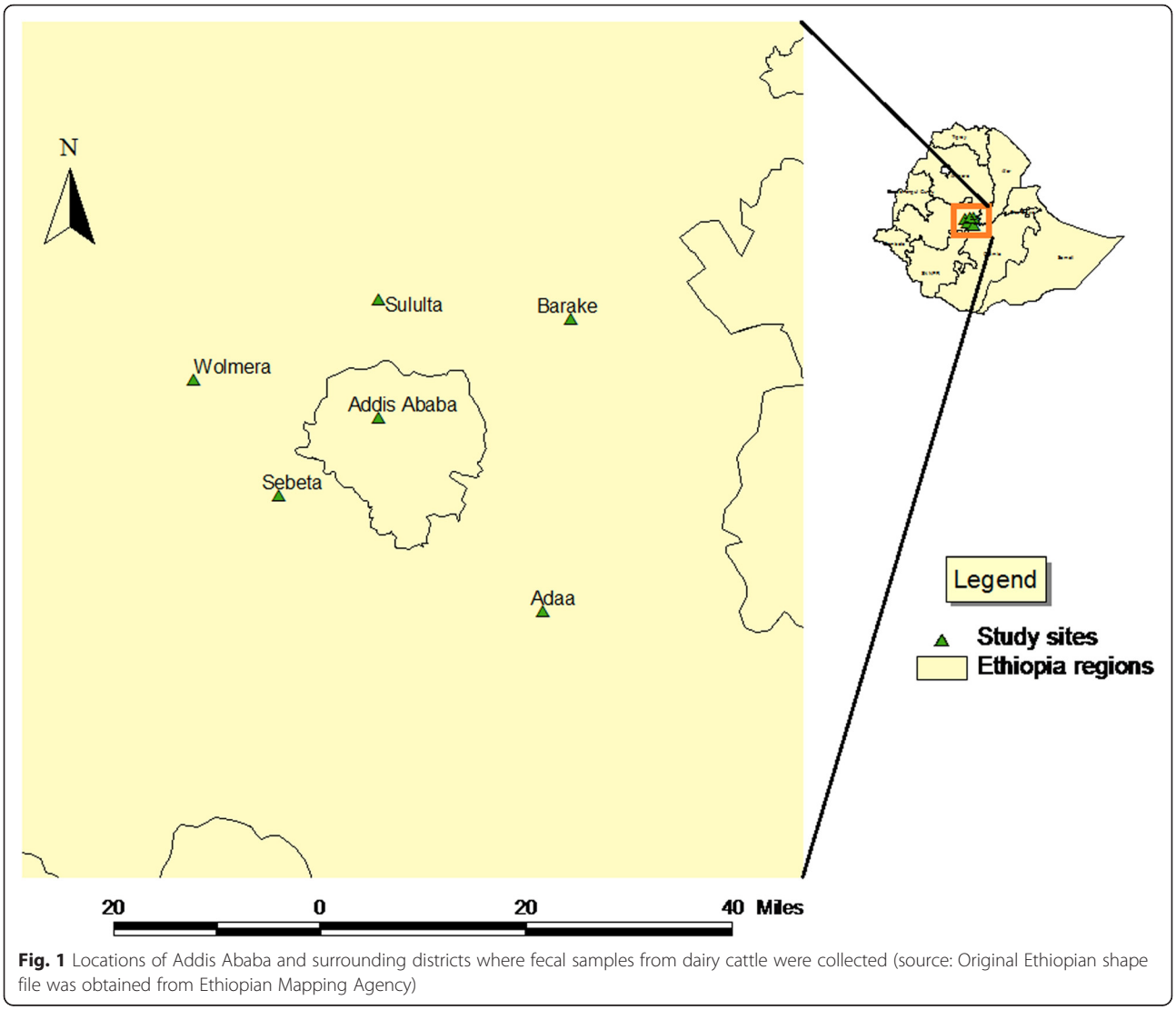

Fig. 1 Locations of Addis Ababa and surrounding districts where fecal samples from dairy cattle were collected (source: Original Ethiopian shape file was obtained from Ethiopian Mapping Agency)

(Oxoid, USA) and incubated at 42 °C for 24 h. At the same time, 1 ml of suspension was also transferred to 10 ml of Tetrathionate broth (TTB) (Oxoid, USA) and incubated for 24 h at 37 °C. It was then streaked from both RVB and TTB to Xylose Lysine Tergitol 4 (XLT-4) (Oxoid, USA) selective media and the plates were incubated at 37 °C for 24 to 48 h. Presumptive *Salmonella* colonies were further investigated biochemically using Triple Sugar Iron agar, Urea, Citrate and Lysine Iron Agar slants. Those colonies with typical *Salmonella* biochemical properties were then further confirmed by genus specific PCR [18]. A reference strain of *S.* Typhimurium (ATCC 14028) was used as a positive control during biochemical analysis and PCR. One confirmed *Salmonella* isolate from each positive sample was stored at −80 °C in 20 % glycerol until further testing.

Data collection

Information such as herd size, housing condition, types of antimicrobials commonly used in the farm, age, sex of animals and presence of diarrhea in a farm was recorded using a purposively designed questionnaire. A farm was categorized as a diarrheic farm if one or more animals in the herd were diarrheic at the time of sample collection. Collection of data was performed at the time of fecal sample collection from each farm.

Salmonella serotyping and phage typing

Salmonella isolates were serotyped and phage typed at the World Organization for Animal Health (OIÉ) Reference Laboratory for Salmonellosis of the Public Health Agency of Canada's National Microbiology at Guelph. Serovars were determined by serum agglutination

according to the White-Kauffmann-Le Minor scheme [19, 20], with identification of somatic (O) antigens by slide agglutination tests [21] and flagellar (H) antigens by a microplate agglutination technique [22]. *S.* Typhimurium isolates were phage typed by the methods developed initially by Callow [23] and extended by Anderson et al. [24] and Rabsch [25] with 30 reference phages obtained directly from the WHO Reference Laboratory for phage typing of *Salmonella* species at Public Health England or from the same source via Canada's National Microbiology Laboratory at Winnipeg. These typing phages were number 1–35 with discontinued use of phages 9, 30, 31, 33 and 34. Internal reference strains of phage type 1 (fully susceptible) and phage type 124 (susceptible to only one phage) were included as controls. Isolates that reacted with the phages but did not conform to any recognized phage type were designated atypical (AT), while those that did not react with any of the typing phages were designated untypeable (UT).

Antimicrobial susceptibility testing

Susceptibility of the isolates to 18 antimicrobials was determined using the Kirby-Bauer disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute [26]. The following antimicrobials (Sensi-Discs, Becton, Dickinson and Company, Loveton, USA) and disc potencies (μg) were used: amikacin (30), amoxicillin + clavulanic acid (20/10), ampicillin (10), cefoxitin (30), ceftriaxone (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), nitrofurantoin (100), streptomycin (10), sulfisoxazole (1000), sulfamethoxazole + trimethoprim (23.75/1.25), trimethoprim (5) and tetracycline (30). The interpretation of the categories of susceptible, intermediate or resistant was based on the CLSI guidelines [26]. For the purpose of analysis, all readings classified as intermediate were considered as resistant unless indicated.

E. coli ATCC 25922 was used as a quality control organism.

Statistical analysis

The data analysis method that fits to survey data as it is implemented in STATA version 12 was used to estimate prevalence of *Salmonella* and to investigate its association with pre-specified background characteristics. In animal level analysis, the probability of selecting a given animal from a given herd was considered as a weighting variable. Animal level prevalence of *Salmonella* was calculated as the weighted percentage of *Salmonella* culture-positive fecal samples among the total number of animals examined. Herd level prevalence of *Salmonella* was calculated as the percentage of herds with one or more *Salmonella* culture-positive fecal samples among the total number of herds sampled. Association of weighted animal level prevalence and selected background characteristics was assessed using pearson chi-square within survey command of STATA software. Association of herd level *Salmonella* positivity and pre-specified characteristics was assessed using pearson chi-square. The difference between mean numbers of antimicrobials to which isolates were resistant was compared using a student *t*-test. Results were reported as being statistically significant whenever the *p*-value was less than 0.05.

Ethics statement

Ethical clearance for the study was obtained from the National Research Ethics Review Committee, Ethiopia. Informed oral consent was obtained from the farm owners at the time of sample collection.

Results

Prevalence and risk factors

Weighted animal level *Salmonella* prevalence was 2.3 % and at least one *Salmonella* positive animal was detected in 7.6 % (10/132) of the herds examined. There was no significant difference in prevalence of *Salmonella* between male and female animals. Significant difference in the prevalence of *Salmonella* was observed across different age groups ($p = 0.023$) and the largest was observed in cattle less than 6 months old. Similarly, animal level prevalence of *Salmonella* among study sites was significantly different ($p < 0.001$): highest prevalence was 5 % in Adaa followed by 4.2 % in Addis Ababa and 2.0 % in Sebeta (Table 1). However, herd level prevalence of *Salmonella* was not significantly different among study sites (Table 2). Diarrhea was detected in 34 of 1203 animals. Three of the diarrheic animals were positive for *Salmonella* whereas 27 out of 1169 non-diarrheic animals were positive for *Salmonella.* These 3 *Salmonella* positive diarrheic animals were a 2 week old calf infected with *S.*Typhimurium var. Copenhagen on a farm in Adaa district, a 3 month old calf infected with *S.* kentucky in Addis Ababa and a 6 year old cow infected with *S.* Dublin in the Sebeta district. Detection of diarrhea in an animal was significantly associated with animal level *Salmonella* carriage ($p = 0.012$) (Table 1). Detection of diarrhea in one or more animals in a farm was also significantly associated with herd level prevalence of *Salmonella* ($p < 0.001$) (Table 2). Out of 255 fecal samples collected from 24 diarrheic herds, 22 were positive for *Salmonella* whereas, only 8 of the 948 fecal samples collected from 108 non-diarrheic herds were positive for *Salmonella*. Six of 24 (25 %) of diarrheic herds were positive for *Salmonella* while only 4 of 108 (3.7 %) of non-diarrheic herds were positive for *Salmonella* ($p < 0.001$).

Table 1 Animal level prevalence of *Salmonella* and its unadjusted association with selected characteristics

Characteristics	Categories	Number	Weighted [a]percent positive for *Salmonella*	*p*-value
Sex	Male	101	1.4	0.483
	Female	1102	2.4	
Age	<6 month	280	4.5	0.023
	6 months–2 years	162	0.0	
	2 years–5 years	143	2.9	
	5 years–8 years	496	1.6	
	≥8 years	122	2.5	
Study site	Sebeta	141	2.0	<0.001
	Addis Ababa	319	4.2	
	Adaa	184	5.0	
	Barake	151	0.4	
	Welmera	195	0.0	
	Sululta	213	0.2	
Herd Size	Small [5–20)	480	1.8	0.117
	Medium [20–50)	369	2.1	
	Large [50+	354	4.3	
Have diarrhoea	No	1169	2.1	0.012
	Yes	34	9.4	
Overall		1203	2.3	

[a]The result was weighted by the probability of selecting animals from its respective farm

There was no significant difference in animal level prevalence of *Salmonella* among animals from farms of different herd size ($p = 0.117$) (Table 1). However, herd level prevalence of *Salmonella* was significantly higher in farms with large herd size ($p = 0.047$) (Table 2). All 30 *Salmonella* isolates were obtained from herds that were kept completely indoors, while none was recovered from farms that allowed their animals to graze outside occasionally or those where cattle were totally outdoors.

Salmonella serotype distribution

Nine different serotypes were identified (Table 3). *S.* Typhimurium, grouped with its variant *S.* Typhimurium var. Copenhagen, was the most common (7/30, 23.3 %) and was isolated from seven animals on three farms in

Table 2 Herd level prevalence of *Salmonella* and its unadjusted association with selected farm level characteristics

Characteristics	Categories	Number of farms studied	Percent positive for *Salmonella*	*p*-value
Study site	Sebeta	20	5.0	0.372
	Addis Ababa	38	13.2	
	Adaa	12	16.7	
	Barake	19	5.3	
	Welmera	18	0.0	
	Sululta	25	4.0	
Herd Size	Small [5–20)	79	3.8	0.047
	Medium [20–50)	33	9.1	
	Large [50+	20	20.0	
Farm diarrhoea status	Diarrheic	24	25.0	<0.001
	Non-diarrheic	108	3.7	
Overall		132	7.6	

Table 3 *Salmonella* serotype distribution and number and percent of intermediate and resistant isolates to antimicrobial agents

Serotype	Number	No. of intermediately resistant and resistant isolates																									
		Amp		Amc		Cf		Cip		Gm		K		Tmp		Te		Su		S		Nitro		Na		N	
		I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R	I	R
Aberdeen	1	–	–	–	–	–	–	1	–	–	–	1	–	–	–	1	–	1	–	1	–	–	1	1	–	1	–
Dublin	3	–	–	–		2	–	–	–	–	–	–	–	–	–	–	–	1	–	3	–	–	–	–	–	–	–
I:6,7,14:–:I,w	1	–	1	–	1	–	1	1	–	–	–	–	–	–	–	1	–	–	–	1	–	–	1	1	–	–	–
Kentucky	5	–	5	2	3	–	5	–	5	–	5	4	–	–	1	–	5	–	5	–	5	4	1	–	5	–	2
LivingstoneVar.14+	1	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–
Mikawasima	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–		1	–	–	–	–	–
Saintpaul	6	1	–	–	–	1	–	–	–	–	–	4	–	–	–	4	–	3	2	5	1	2	3	–	–	3	–
Typhimurium	7	–	–	–		2	–	–	–	–	–	3	–	–	–	4	–	3	–	7	–	1	1	1	–	1	–
Virchow	5	–	3	3	–	–	3	1	–	1	1	1	–	–	–	–	1	1	1	1	2	1	3	–	2	–	–
Total	30	1	9	5	4	5	9	4	5	1	6	13	–	–	–	10	6	10	8	18	8	9	10	3	8	5	2
% R		3.3	30	16. 7	13.3	16. 7	30	13.3	16.7	3.3	20	43.3	–	–	3.3	33.3	20	33.3	26.7	60	26.7	30	33.3	10	26.7	16.7	6.7
% (I + R)		33.3		30		46.7		30		23.3		43.3		3.3		53.3		60		86.7		63.3		36.7		23.3	

Since all isolates were susceptible to Amikacin, Chloramphenicol, Cefoxitin, Ceftriaxone and Sulfamethoxazole + Trimethoprim, they were not included in the table

Amp Ampicillin, *Amc* Amoxicillin and clavulanic acid, Cf Cephalothin, *Cip* Ciprofloxacin, *Gm* Gentamicin, *K* Kanamycin, *Tmp* Trimethoprim, *Te* Tetracycline, *Su* Sulfisoxazole, *S* Streptomycin, *Nitro* Nitrofurantoin, *Na* Nalidixic acid, *N* Neomycin, *I* Intermediate, *R* Resistant

three study sites (Adaa, Sululta and Barake districts). *S.* Saintpaul (6, 20 %) was isolated only from a single farm in the Adaa district, *S.* Kentucky (5, 16.7 %), and *S.* Virchow (5, 16.7 %) were isolated from five animals each in two different farms in Addis Ababa, while *S.* Dublin (3, 10 %) and one isolate (1, 3.3 %) of *S.* Livingstone var.14 +, *S.* I: 6, 7, 14:-: I,w, *S.* Mikawasima and *S.* Aberdeen were isolated from one animal on five different farms. Two different serotypes were isolated from two farms in the present study (Table 4).

Antimicrobial resistance

The common antimicrobials used in the farms were oxytetracycline, penicillin + streptomycin, and sulfonamide in 94.6, 81.8 and 13.6 % of the farms, respectively. Resistance patterns of the isolates are shown in Table 4. All isolates were resistant to at least one of the 18 antimicrobials tested. Twenty-six (86.7 %), 19 (63.3 %), 18 (60 %), 16 (53.3 %) of the isolates were resistant to streptomycin, nitrofurantoin, sulfisoxazole and tetracycline, respectively. Resistance to two or more antimicrobials was recorded in 90 % of the isolates, while resistance to 3 or more antimicrobials was detected in 21 (70 %) of the isolates. MDR to 7 or more antimicrobials were detected in 11 (36.7 %) of the isolates. The five *S.* Kentucky isolates were resistant to 10–13 antimicrobials (Table 4). One isolate (*S.* Kentucky) from a farm in Addis Ababa was resistant to 13 out of 18 antimicrobials tested. All isolates were susceptible to amikacin, chloramphenicol, cefoxitin, ceftriaxone and sulfamethoxazole + trimethoprim.

There was a statistically significant difference in the rate of occurrence of MDR between isolates obtained from dairy farms in Addis Ababa and outside of the city limits of Addis Ababa. The mean ± standard error of mean (SEM) number of antimicrobials to which isolates obtained from Addis Ababa were resistant was 7.23 ± 1.32, while isolates obtained outside of Addis Ababa were resistant to 4 ± 0.62 antimicrobials ($p = 0.01$). Resistance to first line antimicrobial agents in human medicine for treatment of *Salmonella* like beta-lactam and quinolones was also more common in isolates obtained from Addis Ababa. The extent of MDR varied with the serotype, as the overall MDR was more common in *S.* Kentucky, *S.* Virchow and *S.* Saintpaul compared to strains from other serotypes. Interestingly, all of the 5 Kentucky strains were resistant to nalidixic acid and ciprofloxacin (Table 4).

Discussion

Food animals are the primary sources for transmitting non-typhoidal *Salmonella* to humans [27]. Outbreaks of salmonellosis in humans has been linked to improperly pasteurized dairy products, undercooked beef, water runoff from farms, and direct animal or fecal contact [28]. In the current study, farm level prevalence of *Salmonella* was 7.6 % and individual animal level prevalence was 2.3 %, which is much lower than the previous studies in the USA, where 31 % of dairy farms had at least one cow shedding *Salmonella* in feces and 7.3 % of individual animals were shedding [29]. It is also much lower than a previous study conducted in Addis Ababa [15] that reported farm level fecal prevalence of 47.8 % and individual animal level prevalence of 7.7 %. A study on slaughtered cattle in Addis Ababa recovered *Salmonella* from 7.1 % of apparently healthy animals [30]. Recent study in Jordan showed 23 % and 4 % of herd level and individual animal level prevalence of *Salmonella* in dairy farms, respectively, which is also higher than our finding [31]. This difference could be due to differences in the *Salmonella* isolation protocol employed in each study, seasonal variation in *Salmonella* shedding of animals as well as other factors such as herd size and age composition [28, 29]. Most of the farms in the current study had small herd size. Moreover, animals of all age groups in the farm were sampled in the current study unlike the other two studies [15, 29] which involved only lactating cows.

This study also showed that *Salmonella* shedding was common in farms that keep animals completely indoors while none was detected in those that occasionally graze outside or are totally outdoors. Similarly, higher prevalence of *Salmonella* was reported in swine kept indoors than those kept outdoors [32]. This probably is due to free cycling of *Salmonella* between animals in a limited host environment once the pathogen gets access to the farm in animals kept indoors. The fact that the use of processed feed is more common in animals kept indoors than those kept outdoors might also suggest the possibility of indoor kept animals being infected with *Salmonella* from contaminated animal feed. A previous study has also shown livestock waste generated by animals consuming a diet principally composed of grass were less likely to harbor *Salmonella* spp. [33].

In this study, the larger the herd size, the higher the probability of having *Salmonella* positive animals in the farm, which is in agreement with previous reports [34–37]. This could be due to overcrowding of animals in the larger herds, especially those housed indoors, increasing animal to animal contact which enhances transmission of pathogens within the herd. Moreover, the larger the number of animals in the herd, the higher the probability of having a few weak and stressed animals, which increases the likelihood of continuous shedding of *Salmonella* from these cattle. Asymptomatic carrier cattle have been reported to shed *Salmonella* for up to 18 months [38]. Additionally, in the absence of mechanized feeding and milking systems in

Table 4 *Salmonella* serotypes isolated from dairy cattle in various study sites and their antimicrobial resistance pattern

Number	Study site	Farm Code	Serotype	R-pattern	
				Intermediate	Resistant
1	Adaa	DZC −03	Aberdeen	CipKTeSuSNaN	NitroS
2	Adaa	DZC −03	Saintpaul	NitroSuS	–
3	Adaa	DZC −03	Saintpaul	TeSuS	Nitro
4	Adaa	DZC −03	Saintpaul	AmpCfKTeSNaN	SuNitro
5	Adaa	DZC −03	Saintpaul	KTeN	SuSNitro
6	Adaa	DZC −03	Saintpaul	KS	–
7	Adaa	DZC −03	Saintpaul	NitroKTeSuSN	
8	Adaa	DZC-06	Typhimurium var. copehagen PT 193	CfKS	–
9	Adaa	DZC-06	Typhimurium var. copehagen PT 193	CfTeSuS	–
10	Adaa	DZC-06	Typhimurium var. copehagen PT U285	KTeSuSNitroNaN	–
11	Adaa	DZC-06	Typhimurium var. copehagen PT193	TeS	–
12	Addis Ababa	AAC-25	I:6,7,14:-:I,w	CipTeSNa	AmpAmcCfNitro
13	Addis Ababa	AAC-38	Kentucky	KNitro	AmpAmcCfCipGmTeSuSNa
14	Addis Ababa	AAC-25	Kentucky	Nitro	AmpAmcCfCipGmTeSuSNa
15	Addis Ababa	AAC-38	Kentucky	KNitro	AmpAmcCfCipGmTeSuSNa
16	Addis Ababa	AAC-38	Kentucky	AmcKNitro	AmpCfCipGmTeSuSNaN
17	Addis Ababa	AAC-38	Kentucky	AmcK	AmpCfCipGmTmpTeSuSNitroNaN
18	Addis Ababa	AAC-25	Livingstone Var.14+	Cip	Na
19	Addis Ababa	AAC-09	Mikawasima	SuNitro	–
20	Addis Ababa	AAC-23	Virchow	Amc	AmpCf
21	Addis Ababa	AAC-23	Virchow	AmcK	AmpCfCipGmTeSuSNitroNa
22	Addis Ababa	AAC-23	Virchow	AmcSu	AmpCfSNitroNa
23	Addis Ababa	AAC-23	Virchow	GmSNitro	–
24	Addis Ababa	AAC-24	Virchow	–	Nitro
25	Barake	BAR- 18	Typhimurium PT Atypical	KSuS	Nitro
26	Barake	BAR- 18	Typhimurium PT 67	S	–
27	Sebeta	SC-04	Dublin	Cf,S	–
28	Sebeta	SC-04	Dublin	CfSuS	–
29	Sebeta	SC-04	Dublin	S	–
30	Sululta	Suc-07	Typhimurium var. copehagen PT Atypical	TeS	–

PT Phagetype, *Amp* Ampicillin, *Amc* Amoxicillin and clavulanic acid, Cf Cephalothin, *Cip* Ciprofloxacin, *Gm* Gentamicin, *K* Kanamycin, *Tmp* Trimethoprim, *Te* Tetracycline, *Su* Sulfisoxazole, *S* Streptomycin, *Nitro* Nitrofurantoin, *Na* Nalidixic acid, *N* Neomycin

Ethiopia, several animal attendants are involved in daily activities of the large farms with the possibility of serving as a source of dissemination among individual animals in a farm. Contrary to the above findings, another study has reported that there is no association of herd size and *Salmonella* shedding [29].

The strong association of individual animal and herd level prevalence of *Salmonella* with detection of diarrhea in one or more animals suggests that *Salmonella* is one of the causes of diarrhea in dairy cattle in the study population. Detection of more *Salmonella* from diarrheic as well as non-diarrheic cattle in farms with one or more diarrheic animal in the herd might be due to the presence of carrier animals shedding *Salmonella* to other animals without showing clinical manifestations post infection or after recovery from clinical salmonellosis [39].

The higher *Salmonella* recovery rate in young animals in the current study is presumably due to the lack of an adequate adaptive immune response in the young calves compared to adult animals. Also, co-infection with multiple enteric pathogens is common in calves and may compromise their immune system. In addition, relative lack of protective microflora in calves may also predispose them to pathogenic organisms [37]. A previous study has similarly reported an inverse relationship of calf age and the prevalence of *Salmonella* [40].

The dominant serotypes isolated from dairy cattle in the current study, *S.* Typhimurium, *S.* Saintpaul, *S.* Virchow and *S.* Kentucky, are among the common causes of non-typhoidal salmonellosis in humans [41–43]. There is no previous report in Ethiopia showing serotype distribution of *Salmonella* in dairy cattle. The top three serotypes in slaughtered cattle in Addis Ababa were *S.* Mishmarhaemek, *S.* Typhimurium and *S.* Enteritidis [30]. In another study conducted in north Ethiopia in slaughtered cattle, *S.* Typhimurium and *S.* Newport were the two dominant serotypes recovered [44].

The observed high resistance to streptomycin and tetracycline is not surprising since these antimicrobials are commonly used in most of the farms for management of bacterial infections. Similar high resistance rates were reported to streptomycin (77 %) and tetracycline (65.5 %) in *Salmonella* isolates obtained from different food animals from Ethiopia [45]. Another study also reported 75 and 46.9 % resistance to streptomycin and tetracycline in *Salmonella* isolated from different food items and personnel in Addis Ababa [46]. Though nitrofurantoin and sulfonamide were less commonly used in the farms during the study period, large proportion of isolates were resistant to these agents. This is probably due to the fact that these antimicrobials had been used in the animal health sector for a long time in the country and *Salmonella* had already developed resistance. A previous study conducted in Addis Ababa [15] showed 83 % of *Salmonella* isolates from dairy farms to be resistant to 2 or more antimicrobials out of 10 antimicrobials tested. Unlike the previous study [15] that reported 100 % resistance to ampicillin, in the current study, only 33.3 % of the isolates exhibited resistance to ampicillin. However, most of the isolates obtained from farms in Addis Ababa were resistant to ampicillin.

Resistance to ciprofloxacin was not reported in the previous study [15], but in the current study, 30 % of the isolates were resistant to ciprofloxacin. Despite detection of resistance to ciprofloxacin and nalidixic acid in the current study, from our interview with farm owners during sampling, none of the farms was using ciprofloxacin or other quinolone antimicrobials to treat their dairy cattle, and use of quinolones is not a regular practice in veterinary medicine in Ethiopia. However, a similar high percentage of resistance to ciprofloxacin was observed in *S.* Kentucky isolates carrying *Salmonella* Genomic Island K (SGI1-K) collected during 2000–2008 from meat of swine, cattle and poultry [45]. Also, recently isolated *S.* Kentucky strains from diarrheic patients in Addis Ababa were resistant to several antimicrobials including ciprofloxacin [47]. MDR *S.* Kentucky belonging to a single clone resistant to quinolones and carrying SGI1-K has been reported from European travelers returning from different African and Asian countries [48]. This occurrence of MDR *S.* Kentucky in both humans and animals in the region might be due to this specific clone widely circulating in Africa.

The high MDR in *Salmonella* from dairy cattle is alarming. The incidence of MDR in *Salmonella* has increased in the last few decades globally [49]. Infection of humans with MDR strains of *Salmonella* has been reported to be associated with increased burden of morbidity, extended hospitalization, increased risk of invasive illness and increased mortality, compared to those infected with susceptible strains [50–52]. In fact, the increase in MDR observed in *Salmonella* isolates from dairy farms in Addis Ababa compared to those out of Addis Ababa could be due to greater availability of antimicrobial agents and extensive use of antimicrobials in both animals and humans, fostered by a highly populated city where animals and humans live in close proximity.

Conclusion

The occurrence of MDR *Salmonella* serotypes that commonly cause human salmonellosis in dairy herds residing in close proximity with human populations warrants the need for strict biosecurity and intervention strategies to control these *Salmonella* isolates in dairy farms and to protect human and animal health. Paramount is the resistance to ciprofloxacin, which is of great concern as this antimicrobial is among the last options for treatment of complicated non-typhoidal salmonellosis in humans.

Abbreviations

BPW: buffered peptone water; MDR: multi-drug resistance; PT: phage type; RVB: Rappaport-Vassiliadis broth; SEM: standard error of the mean; TTB: tetrathionate broth; WHO: World Health Organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TE, EE, WG, JSG and DA, participated in conception of the study and review of the draft manuscript. TE was involved in sample collection laboratory investigation and preparation of the draft manuscript. HA participated in laboratory work. RPJ was involved in serotyping, and phage typing of the isolates as well as preparation of the manuscript. GM participated in data analysis. All authors read and approved the final manuscript.

Acknowledgments

This study was financially supported by The National Institutes of Health (NIH) Fogarty International Center (grant 043TW008650) to W.A.G./J.S.G. and WHO/AGISAR to T.E. We are grateful to Linda Cole, Shaun Kernaghan, Ketna Mistry, Ann Perets and Betty Wilkie of the Public Health Agency of Canada, National Microbiology Laboratory at Guelph for serotyping and phagetyping of the *Salmonella* isolates. Technical assistance of Mr. Nega Nigusie during sample collection is highly appreciated.

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Received: 20 July 2015 Accepted: 9 February 2016
Published online: 16 February 2016

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Paper 2

Eguale et al. BMC Infectious Diseases (2015) 15:497
DOI 10.1186/s12879-015-1235-y

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Non-typhoidal *Salmonella* serotypes, antimicrobial resistance and co-infection with parasites among patients with diarrhea and other gastrointestinal complaints in Addis Ababa, Ethiopia

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Abstract

Background: Non-typhoidal *Salmonella* (NTS) is an important public health problem worldwide. Consumption of animal-derived food products and direct and/or indirect contact with animals are the major routes of acquiring infection with NTS. Published information, particularly on the serotype distribution of NTS among human patients with gastroenteritis and associated risk factors, is scarce in Ethiopia. This study investigated the prevalence, risk factors, serotype distribution and antimicrobial susceptibility of *Salmonella* species among diarrheic out-patients attending health centers in Addis Ababa and patients with various gastrointestinal complaints at Tikur Anbessa Specialized Hospital (TASH).

Methods: Stool samples were cultured for *Salmonella* species according to the WHO Global Foodborne Infections Network laboratory protocol. *Salmonella* serotyping was conducted using slide agglutination and microplate agglutination techniques. Antibiotic susceptibility testing was performed using the disk diffusion method according to Clinical and Laboratory Standards Institute guidelines.

Results: A total of 59 (6.2 %) stool samples, out of 957 were culture positive for *Salmonella* species. Fifty-five (7.2 %) of 765 diarrheic patients from health centers and 4 (2.1 %) of 192 patients from TASH were culture positive for *Salmonella* species. Multivariable logistic regression analysis after adjusting for all other variables revealed statistically significant association of *Salmonella* infection with consumption of raw vegetables (OR = 1.91, 95 % CI = 1.29–2.83, $\chi^2 = 4.74$, $p = 0.025$) and symptom of watery diarrhea (OR = 3.3, 95 % CI = 1.23–8.88, $\chi^2 = 10.54$, $p = 0.005$). Eleven serotypes were detected, and the most prominent were *S.* Typhimurium (37.3 %), *S.* Virchow (34 %), and *S.* Kottbus (10.2 %). Other serotypes were *S.* Miami, *S.* Kentucky, *S.* Newport, *S.* Enteritidis, *S.* Braenderup, *S.* Saintpaul, *S.* Concord and *S.* V:ROUGH-O. Resistance to three or more antimicrobials was detected in 27 (40.3 %) of the isolates. Resistance to five or more antimicrobials was detected in 17 (25.4 %). Resistance to individual antimicrobials was found at varying proportions: streptomycin (50; 74.6 %), nitrofurantoin (27; 40.3 %), sulfisoxazole (26; 38.8 %), kanamycin (23; 34.3 %), cephalothin (12; 17.9 %), and ampicillin (11; 16.4 %) respectively. Two *S.* Kentucky, one *S.* Typhimurium and one *S.* Concord isolates were multi-drug resistant to more than 10 antimicrobials.
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Conclusions: The study demonstrated significant association of *Salmonella* infection with consumption of raw vegetables. There was no significant association of *Salmonella* infection with co-occurring parasites. The study also showed the dominance of *S.* Typhimurium and *S.* Virchow in primary health care units. Overall, prevalence of MDR was low compared to previous studies. Although their proportion was low, *S.* Kentucky and *S.* Concord demonstrated wider spectrum of MDR. Continuous monitoring of circulating serotypes, antimicrobial resistance profile and characterization on molecular resistance determinants is essential for proper treatment of patients and for identifying potential environmental origins of antimicrobial resistance.

Keywords: Non-typhoidal *Salmonella*, Antimicrobial resistance, Prevalence, Serotype

Background

Non-typhoidal *Salmonella* (NTS) is an important public health problem worldwide. Globally, NTS is estimated to be responsible for 93.8 million cases of gastroenteritis and 155,000 deaths annually [1]. Unlike typhoid fever, which is mainly limited to developing countries, NTS occurs worldwide. Despite global morbidity, mortality due to NTS infection primarily occurs in the developing world and is related to co-morbidities [2]. Consumption of animal-derived food products and direct and/or indirect contact with animals are the major routes of acquiring infection with NTS [3–6]. NTS can also be transmitted from person to person or from contact with pets such as cats, dogs, rodents, reptiles, or amphibians [3, 4, 7, 8]. Several recent outbreaks have also been associated with consumption of contaminated plant products such as sprouts, tomatoes, fruits, peanuts, and spinach [9–11].

NTS usually causes self-limiting gastroenteritis characterized by diarrhea, abdominal pain and vomiting in people of all ages [12]. However, in children, the elderly and immunocompromised patients, severe invasive disease with complicated extra-intestinal illness, bacteremia and meningitis can be observed [7, 13]. Generally, antibiotic treatment is not necessary for NTS gastroenteritis unless it is invasive salmonellosis or it affects immunocompromised patients [14, 15]. Chemotherapy is believed to prolong the duration of shedding of bacteria and contribute to the development of antimicrobial resistance [16].

The relative importance of major etiologic agents responsible for diarrhea is not well established in Ethiopia. A few studies conducted in different parts of the country have shown that *Campylobacter*, *Salmonella, Shigella* and rotavirus are some of the major bacterial and viral pathogens isolated from stool of diarrheic patients, most of them children under 5 years of age [17–20].

Globally, the incidence of *Salmonella* infection associated with multi-drug resistance (MDR) has increased in the last few decades [21–23]. In Ethiopia, although there are a few studies on prevalence of *Salmonella* and antimicrobial susceptibility in humans, animals, and food of animal origin, there is no integrated surveillance and monitoring to establish the major serotypes responsible for non-typhoidal salmonellosis in humans and the associated risk factors. Moreover, most of the studies conducted in humans involved pediatric diarrheic patients and the isolates recovered from these patients were not sereotyped [24, 25]. Those that conducted serotyping indicated that *Salmonella enterica* subspecies enterica serovar Concord (*S.* Concord) and *Salmonella enterica* subspecies enterica serovar Typhimurium (*S.* Typhimurium) were the dominant NTS serotypes isolated from patients with diarrheal illness in Ethiopia [20, 26].

Information on *Salmonella* serotypes circulating in a given geographic area and their antimicrobial susceptibility is essential for designing appropriate intervention strategy since the outcome of infection varies with serotypes involved and their antimicrobial resistance profile. The objectives of the current study were therefore to investigate the prevalence, serotype distribution and antimicrobial resistance pattern of NTS species among diarrheic patients in 10 primary health centers in Addis Ababa. In addition, patients with gastrointestinal complaints who submitted stool samples to Tikur Anbessa Specialized Hospital (TASH) for bacterial culturing, diagnosis of gastrointestinal parasites and detection of *Helicobacter pylori* antigen were also included in the study. TASH is national referral hospital giving services mainly to patients from all sub-cities in Addis Ababa and those from outside Addis Ababa. Various patient related variables such as consumption of raw meat, milk and vegetables; patients demographic data; stool consistency; and the occurrence of other pathogens were recorded to analyze for possible association with *Salmonella* isolation.

Methods

Study design and area

A cross-sectional study was conducted in Addis Ababa, Ethiopia, from May 2013 to January 2014. Addis Ababa is organized under 10 sub-cities and there are 36 government-owned primary health centers in the city. Ten representative government-owned health centers were randomly selected, one from each sub-city. In

addition, patients with various gastrointestinal complaints at TASH were also included.

Sample size determination and study subjects

Sample size ($n = 576$) was calculated based on the previous baseline study in Addis Ababa, with a prevalence of 6.4 % [25] using the formula $(Z0/2)/\Delta)2p(1\text{-}p)$ at 95 % CI, margin of error of 2 %. However, to increase the number of isolates, a total of 765 patients from health centers were included. From each health center, a minimum of 71 and a maximum of 82 diarrheic patients of any age referred for laboratory diagnosis based on stool examination were recruited ($n = 765$). Diarrhea was defined as the passage of three or more loose or liquid stools per day [27]. In addition, 192 patients who presented with various gastrointestinal complaints (diarrhea, abdominal pain and gastritis) and submitted stool samples to the parasitology and microbiology laboratory at TASH were also included. Only 98 (51 %) of the 192 stool samples from TASH were diarrheic.

Patients' history, demographic and laboratory data

Information on various potential risk factors was collected including patients' history such as consumption of raw meat, milk and vegetables during the last 2 weeks by interviewing the patients during sample collection from health centers. All demographic, clinical and laboratory data obtained from study participants at health facilities such as stool consistency and laboratory examination results of stool specimens for other pathogens were recorded. The stool consistency was determined in the health center and hospital laboratories immediately after samples were received according to the Bristol stool consistency scale (type 5, 6 and 7) defined as loose, mucoid and watery, respectively [28]. Analysis for association of *Salmonella* infection status and various risk factors was conducted only for patients from health centers.

Sample collection, handling and transportation

Stool samples were collected from each study participants in clean screw capped plastic containers and transported to the microbiology laboratory of Aklilu Lemma Institute of Pathobiology (ALIPB) in ice box within 3–4 h of collection for culture and identification of *Salmonella* species. Sampling was first conducted from the 10 health centers sequentially and finally from TASH.

Microscopic examination of stool specimens

Direct microscopic stool examination was performed for detection of ova and parasites by laboratory technicians in health centers and hospital laboratories where the samples were collected immediately prior to transportation to ALIPB and the laboratory result was recorded.

Culture and identification of *Salmonella* species

Isolation and identification of *Salmonella* species was conducted according to World Health Organization (WHO) Global Foodborne Infections Network laboratory protocol [29]. Briefly, 5 g of feces was suspended in 45 ml of buffered peptone water and incubated for 24 h at 37 °C. One hundred μl of this suspension was transferred to 10 ml of Rappaport-Vassiliadis enrichment Broth (RVB), (Oxoid, USA) and incubated for 24 h at 37 °C. One ml of suspension was also transferred to 10 ml of Tetrathionate broth (Oxoid, USA) and incubated for 24 h at 42 °C. The sample from these two broths was streaked on to Xylose Lysine tergitol 4 (XLT-4) selective media and the plates were incubated at 37 °C for 24 h. Presumptive *Salmonella* colonies were then further investigated biochemically using Triple Sugar Iron (TSI) agar, Urea, Citrate and Lysine Iron Agar (LIA) slants. Those colonies with typical *Salmonella* biochemical properties were then further confirmed by genus specific PCR [30]. *Salmonella* recovered from both RVB and TTB of a single patient were first considered as different strains until the isolates were tested for antimicrobial susceptibility. When differences in antimicrobial susceptibility were observed, both isolates were considered as different strains. On the other hand, if the isolates showed similar susceptibility pattern, only one isolate was considered for further analysis.

Salmonella serotyping and phage typing

Salmonella isolates were serotyped and phage-typed at the Public Health Agency of Canada, World Organization for Animal Health (OIÉ) Reference Laboratory for Salmonellosis, Guelph, Ontario, Canada. Briefly, the somatic (O) antigens were determined by slide agglutination tests [31] and flagellar antigens were determined using a microplate agglutination technique [32]. The antigenic formulae of Grimont [33] were used to identify and assign the serotypes of the isolates. Phage typing of *S.*Typhimurium isolates was performed by the methods developed by Callow [34] and extended by Anderson et al. [35] with reference phages obtained from the Public Health England, Gastrointestinal Bacteria Reference Unit, Colindale, England and the Public Health Agency of Canada, National Laboratory for Enteric Pathogens, Winnipeg, Canada. *Salmonella* isolates that reacted with the phages but did not conform to any recognized phage type were designated atypical (AT).

Antimicrobial susceptibility testing

Susceptibility of the isolates to a panel of 18 antimicrobials was determined using the Kirby-Bauer disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute [36]. The following antimicrobials (Sensi-Discs, Becton, Dickinson and

Company, Loveton, USA) and disc potencies (μg) were used: amikacin (30), amoxicillin + clavulanic acid (20/10), ampicillin (10), cefoxitin (30), ceftriaxone (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), nitrofurantoin (100), streptomycin (10), sulfisoxazole (1000), sulfamethoxazole + trimethoprim (23.75/1.25), trimethoprim (5) and tetracycline (30). The interpretation of the categories of susceptible, intermediate or resistant was based on the CLSI guidelines [36] and for the purpose of analysis, all readings classified as intermediate were considered as resistant where necessary. Reference strain of *Escherichia coli* ATCC 25922 was used as a quality control.

Ethical consideration

The study protocol was ethically approved by the Institutional Review Board of College of Health Sciences, Addis Ababa University and National Research Ethics Review Committee (Permit#3-10/474/05 dated 29–03–2015). Individual oral informed consent was obtained from all adult participants and the parents or guardians of all children participated in the study.

Statistical analysis

Data was analyzed using STATA software version 11. Prevalence of *Salmonella* was calculated as a percentage of *Salmonella* culture-positive stool samples among the total number of samples examined. Associations of putative risk factors as well as occurrence of protozoan pathogens with *Salmonella* infection in patients from health centers were assessed using both univariable and multivariable logistic regression, accounting for clustering of patients at the health center level by using cluster-robust standard errors. The variables used in multivariable logistic regression were sex, age, consumption of raw (meat, milk, vegetables), stool consistency, vomiting, and infection status with *Entamoeba histolytica* and *Giardia lamblia.* Results were reported statistically significant whenever the *p*-value was less than 5 %.

Results

Salmonella prevalence and association with patient feeding habits and other factors

All *Salmonella* isolates recovered from a single patient from both RVB and TTB, though in some cases (11.9 %), differed in antimicrobial resistance pattern; they all belonged to a single serotype. Therefore, only a single serotype per patient was considered for risk factor analysis and serotype distribution analysis, while all isolates ($n = 67$) were considered for antimicrobial resistance analysis. Seven hundred and sixty-five diarrheic patients from health centers (329 male and 436 female) and 192 patients (101 male and 89 female) from TASH were included in the study. Fifty-five (7.2 %) and 4 (2.1 %) patients from health centers and TASH were culture positive for *Salmonella*, respectively. The overall prevalence of *Salmonella* in the current study was 6.2 %. There was no difference in prevalence of *Salmonella* between male and female patients as well as among different age groups (Tables 1 and 2). Among eating habits of patients, 10.8 % of those consuming raw vegetables were *Salmonella* culture positive, whereas only 6.1 % of patients with no habit of eating raw vegetables were positive for *Salmonella.* Multivariable logistic regression analysis after adjusting for all pre-specified

Table 1 Frequency of *Salmonella* infection among diarrheic out-patients attending health centers in Addis Ababa with respect to selected factors

Factor	Total	No (%) *Salmonella* positive	*P*-value
Sex			
Male	329	24(7.3)	0.92
Female	436	31(7.1)	
Consumption of raw meat			
No	622	44(7.1)	0.80
Yes	143	11(7.7)	
Consumption of raw milk			
No	720	51(7.1)	0.19
Yes	45	4(8.9)	
Consumption of raw vegetables			
No	571	34(6.0)	0.025
Yes	194	21(10.8)	
Vomiting			
No	714	51(7.1)	0.85
Yes	51	4(7.8)	
Age			
0–5 years	160	10(6.3)	
6–18 years	171	12(7.0)	0.77
19–45	359	27(7.5)	
>45	75	6(8)	
Stool consistency			
Mucoid	283	10(3.5)	
Loose	117	9(7.7)	0.005
Watery	365	36(9.9)	
Entamoeba histolytica infection			
Positive	145	14(9.7)	0.058
Negative	620	41(6.6)	
Giardia lamblia infection			
Positive	62	3(4.8)	0.46
Negative	703	52(7.4)	

Table 2 Association of selected risk factors with *Salmonella* infection in diarrheic out-patients in Addis Ababa health centers

Variable	Crude odds Ratio and 95 % confidence interval		Adjusted odds Ratio and 95 % confidence interval	
Sex				
Male	1.03	0.61–1.74	1.02	0.57–1.85
Female				
Consumption of raw meat				
No	1.1	0.58–2.08	0.86	0.55–1.36
Yes				
Consumption of raw milk				
No	1.28	0.53–3.01	1.29	0.51–3.26
Yes				
Consumption of raw vegetable				
No	1.92	1.36–2.70	1.91	1.29–2.83
Yes				
Vomiting				
No	1.11	0.37–3.32	0.93	0.34–2.52
Yes				
Age				
0–5 years				
6–18 years	1.14	0.41–3.13	1.03	0.38–2.81
19–45	1.22	0.53–2.81	1.02	0.44–2.35
>45	1.32	0.32–5.57	1.27	0.33–4.93
Stool consistency				
Mucoid				
Loose	2.28	0.90–5.76	2.33	0.91–5.97
Watery	2.99	1.23–7.27	3.05	1.22–7.60
Entamoeba histolytica infection				
Positive	1.85	0.84–4.08	1.77	0.85–3.67
Negative				
Giardia lamblia infection				
Positive	0.64	0.25–1.60	0.64	0.26–1.56
Negative				

variables revealed statistically significant association of *Salmonella* infection status with consumption of raw vegetables (OR = 1.91, 95 % CI = 1.29–2.83, $\chi^2 = 4.74$, $p = 0.025$) (Table 2).

Although there was variability in the detection rate of *Salmonella* from patients attending different health centers, the difference was not statistically significant ($p = 0.29$) (Fig. 1). Having watery diarrhea (9.8 %) was significantly associated with *Salmonella* recovery compared to mucoid (7.7 %) and loose stool (3.5 %) using multivariable logistic regression analysis after adjusting for the other variables (OR = 3.3, 95 % CI = 1.23–8.88, $\chi^2 =$ 10.54, $p = 0.005$). Comparison of patients with symptoms of vomiting to those with no vomiting revealed no significant difference in *Salmonella* culture-positivity (Tables 1 and 2).

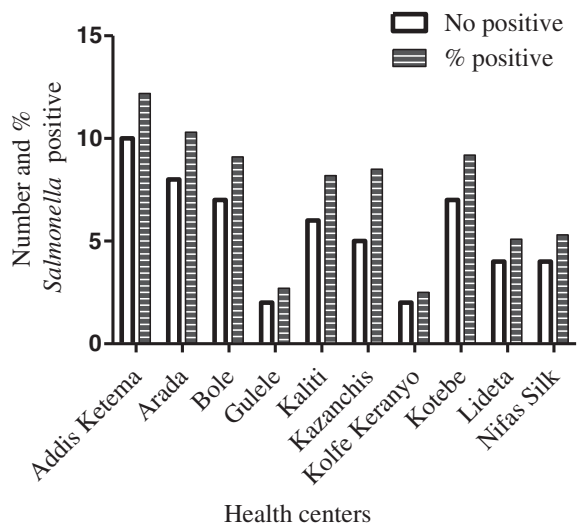

Fig. 1 Prevalence of *Salmonella* among out-patients attending primary health centers in Addis Ababa

Prevalence of ova and parasites and concomitant infection with *Salmonella*

The most common pathogens detected were *Entamoeba histolytica* (19 %), *Giardia lamblia* (8.1 %), egg of *Hymenolepis nanna* (0.9 %) and egg/larvae of *Strongyloides stercoralis* (0.7 %) among patients from health centers, whereas only *E. histolytica* (0.5 %), *G. lambia* (4.3 %), and egg of *Tanea* species (1.1 %) were detected in patients from TASH (Table 3). Although *Salmonella* was commonly detected in patients positive for *E. histolytica* (9.7 %) compared to negative ones (6.6 %) *Salmonella* infection was not significantly associated with any of the parasites (Table 2).

Table 3 Common pathogens detected from stool samples of the study participants

Pathogen infection status	Health Centers		TASH	
	Number examined	Number (%) positive	[a]Number examined	Number (%) positive
Entamoeba histolytica	765	145(19)	188	1(0.5)
Giardia lamblia	765	62(8.1)	188	8(4.3)
Hymenolepis nanna	765	7(0.9)	188	0(0)
Strongloid worm/eggs	765	5(0.7)	188	0(0)
Hook worm	765	3(0.4)	188	0(0)
Ascaris egg	765	2(0.3)	188	0(0)
Eggs of Tanea	765	0(0)	188	2(1.1)

[a]Only 188 of 192 samples at TASH were examined for ova and parasites

Table 4 Relative proportion of *Salmonella* serotypes isolated from diarrheic patients in Addis Ababa and their resistance profile

Serotype	No.	An	Amp	Amc	C	Cro	Cf	Cip	Fox	Gm	K	Sxt	Tmp	Te	Su	S	Nitro	Na	N
Braenderup	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
Concord	1	0	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	0
Enteritidis	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1
Kentucky	2	0	2	2	0	0	2	2	1	2	2	0	0	2	2	2	0	2	1
Kottbus	7	0	0	0	0	0	0	1	0	1	3	0	0	1	4	7	2	1	2
Miami	3	0	0	0	0	0	0	0	0	0	1	0	0	0	0	3	2	0	0
Newport	2	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0	1	0
Sainpaul	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0	1
Typhimurium	27	0	6	4	0	1	6	0	0	0	6	1	1	3	13	18	9	1	1
V:ROUGH-O;-:-	1	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0
Virchow	21	0	1	1	1	0	1	0	0	1	6	0	0	3	4	14	11	10	3
Total	67	0	11	8	2	2	12	3	2	5	23	3	3	9	26	50	27	16	9
(%)	100	0	16.4	11.9	3	3	17.9	4.5	3	7.5	34.3	4.5	4.5	13.4	38.9	74.6	40.3	23.9	13.4

An amikacin, *Amp* ampicillin, *Amc* amoxicillin and clavulanic acid, *Cf* cephalothin, *C*, chloramphenicol, *Cro* ceftriaxone, *Cip* ciprofloxacin, *Gm* gentamicin, *K* kanamycin, *Tmp* trimethoprim, *Sxt* sulfamethoxazole + trimethoprim, *Te* tetracycline, *Su*-sulfisoxazole, *Nitro* nitrofurantoin, *Na*- nalidixic acid, *N* neomycin

Salmonella Serotype distribution

Overall, 11 different serotypes were recovered, of which the majority were *S*. Typhimurium (22, 37.3 %) followed by *S*. Virchow (20, 33.9 %) and *S*. Kottbus (6, 10.2 %). Other serotypes such as *S*. Miami ($n = 2$), *S*. Kentucky ($n = 2$), *S*. Newport ($n = 2$), *S*. Enteritidis ($n = 1$), *S*. Braenderup ($n = 1$), *S*. Saintpaul ($n = 1$), *S*. Concord ($n = 1$), and *S*. V: ROUGH-O; – :- ($n = 1$) were also identified (Table 4).

Antimicrobial Susceptibility

At least one isolate was resistant to all tested antimicrobial agents except amikacin to which all isolates were susceptible. Fifty (74.6 %), 27 (40.3 %), 26 (38.8 %), 23 (34.3 %), 12 (17.9 %), and 11 (16.4 %) of the isolates were resistant to streptomycin, nitrofurantoin, sulfisoxazole, kanamycin, cefalothin, and ampicillin, respectively (Table 4). Nine (13.4 %) of the isolates were pan-susceptible to 18 antimicrobials tested. Six of these isolates were *S*. Typhimurium, while the remaining 3 were *S*. Virchow. Resistance to 2 or more antimicrobials was recorded in 64.2 % of the isolates, while resistance to 3 or more antimicrobials was detected in 40.3 % of the total isolates. MDR to 5 or more antimicrobials was detected in 17 (25.4 %) of the isolates. Two strains of *S*. Kentucky isolated from patients in two different health centers were resistant to 11 antimicrobials. Likewise, one *S*. Typhimurium phagetype 193 and one *S*. Concord recovered from two diarrheic children at TASH were resistant to 12 and 13 antimicrobials tested, respectively. In general, 36 different resistance patterns (R-Pattern) were detected among the 67 isolates examined which is an indication of phenotypic diversity of *Salmonella* strains circulating in the study area. The dominant R-pattern was resistance to a single antimicrobial agent: streptomycin ($n = 11$) followed by resistance to both streptomycin and nitrofurantoin (6 isolates) (Additional file 1: Table S1).

Association between serotype and antimicrobial resistance

Different serotypes appeared to exhibit differential resistance to some of the antimicrobials tested. For instance, resistance to ampicillin was noted in strains belonging to *S*. Concord, *S*. Kentucky, *S*. Typhimurium, *S*. V: ROUGH-O;-: and *S*. Virchow; while *S*. Kentucky and *S*. Kottbus were the only serotypes resistant to ciprofloxacin. In other cases, resistance to streptomycin was common among the serotypes (Table 4).

Discussion

In the current study, 7.2 % of patients from the health centers and 2.1 % from TASH were culture positive for *Salmonella* species. The possible explanation for low prevalence of *Salmonella* in patients at TASH could be due to health center treated patient referral to this hospital or because not all of the stool samples collected at TASH were from diarrheic patients. The observed heterogeneity of *Salmonella* infection across health centers goes with the known hygienic levels of residential areas served by these health centers. Addis Ketema and Arada health centers are located at the center of the old Addis Ababa with larger human population and lots of slum areas with poor hygiene compared to Kolfe Keranyo and Gulele health centers, which are located at periphery of the city with relatively better hygienic condition.

The overall prevalence of *Salmonella* (6.2 %) in the current study is in agreement with previous studies in Addis Ababa (5.3 %) [20] and Jimma hospitals (6.2 %)

[37] in pediatric patients. Previous studies in Addis Ababa in adult diarrheic out-patients showed a prevalence of 4.5 % [24] and 6.4 % [25]. Systematic review and meta-analysis of published works in Ethiopia over the period of 38 years has also shown a prevalence of 8.7 % in diarrheic children and 5.7 % in diarrheic adults [38].

Salmonella detection was more common in patients who consume raw vegetables. This suggests that raw vegetables could be one of the major vehicles for *Salmonella* infection in Addis Ababa. The common vegetables consumed by these patients were lettuce, tomatoes and green peppers. An accumulating body of evidence indicates that vegetables sold in markets could possibly be contaminated with *Salmonella* species. Indeed, studies conducted in Addis Ababa [39], Jimma (South–west Ethiopia) [40] and Mexico [41] showed that an appreciable percentage of vegetables sold in the respective markets are contaminated with *Salmonella* species. The use of fecally contaminated water for irrigation and washing of fresh produces can serve as a source of *Salmonella* contamination for raw vegetables in addition to cross-contamination in the kitchen [42]. More recently, *Salmonella* has been shown to colonize and be internalized into tomato during pre–and post–harvesting stages [43]. Special attention should, therefore, be paid to reducing the risk of infection of the public with *Salmonella* from raw vegetables. Appropriate pre–and post–harvest strategies of reducing contamination of vegetables by *Salmonella* and other enteric pathogens should be implemented.

The dominant pathogens other than *Salmonella* detected in the current study were *E. histolytica* followed by *G. lamblia.* However, no significant association of co-occurrence of parasites with *Salmonella* infection was detected.

S. Typhimurium followed by *S.* Virchow and *S.* Kottubus were the dominant serotypes in the current study unlike the reports by Beyene et al. [20] and Gebre-Yohannes et al. [44] where the dominant serotypes among NTS isolates were *S.* Concord followed by *S.* Typhimurium. In the present study, only one *S.* Concord was isolated from a one year old child at TASH. The difference between the studies could be attributed to differences in patient demography, season and place of collection. Furthermore, differences in dynamics of the sources of infection might have contributed to dominance of different serotypes in the respective studies. A previous study has shown that the epidemiology of NTS is characterized by the temporal dominance of certain successful clones followed by a decline and replacement with another clone [45]. The dominance of *S.* Typhimurium in the current study is in agreement with studies conducted in other sub-Saharan African countries including Kenya [46] and Congo [47].

Overall, rate of antimicrobial resistance is low in the current study compared to previous investigations. For instance Beyene et al. [20] reported 82.3 % resistance to ampicillin and 78.2 % to ceftriaxone, which is much higher than the current finding of 16.4 % and 3 %, respectively. Only 3 %, 7.5 %, 13.4 %, and 4.5 % of the isolates were resistant to chloramphenicol, gentamicin, tetracycline, and sulfamethoxazole + trimethoprim, respectively in the current study, while 81.4 %, 74.3 %, 39.8 % and 80.5 % of the isolates were resistant to the corresponding antibiotics, respectively, in the previously mentioned study. A separate study reported 59.7 %, 32.3 %, 61.7 %, 29 %, and 51.6 % resistance to ampicillin, cephalothin, chloramphenicol, tetracycline and Sxt, respectively in NTS isolates recovered from hospitalized patients at TASH in Addis Ababa [48]. The reason behind this discrepancy could be differences in serotype composition reported by the studies. As mentioned, the dominant serotype in the study by Beyene *et al.* [20] was *S.* Concord. However, only one isolate of this serotype was recovered in the current study. Though *S.* Concord was resistant to many antimicrobials in both studies, its rarity in the present study might have contributed to the relatively low levels of overall antimicrobial resistance that was observed. Furthermore, the previous studies were conducted in referral hospitals, where patients are admitted after being treated in health centers with various antimicrobials. Thus, patients could have been infected by clonal epidemic MDR strains of *S.* Concord or other serotypes. Some of the infections in the previous reports also might have been acquired from hospitals by MDR strains. Majority of the isolates in the current study were, however, collected from patients at primary health centers, with minimized prior exposure to antimicrobials. The fact that 2 of the 4 isolates from TSRH in the current study were resistant to 11 and 13 antimicrobials supports this assertion. Similar decrease in prevalence of resistance in NTS in rural district hospital has also been reported from Kenya [49].

Although we have no data on the antimicrobial use for diarrheic patients in the current study, the recent standard treatment guideline prepared by Drug Administration and Control Authority of Ethiopia for severe cases of infectious gastroenteritis including NTS recommends sulfamethoxazole + trimethoprim, ciprofloxacin and chloramphenicol [50]. However, resistance to these antimicrobials was low in *Salmonella* isolates in the current study probably due to the fact that these isolates were mainly obtained from patients attending primary health centers and were not exposed to prolonged selective pressure of antimicrobials. Drugs like streptomycin, nitrofurantoin, sulfonamides and ampicillin have long been used for management of various

infections in the country and high rate of resistance to these drugs might have developed as a consequence of this prolonged use.

Conclusions

There was no significant association of *Salmonella* infection with co-occurring parasites but there was significant association of *Salmonella* infection with consumption of raw vegetables. The dominant NTS serotypes at the primary health care units in Addis Ababa were *S.* Typhimurium, *S.* Virchow and *S.* Kottbus with variable antimicrobial resistance phenotypes. Although their proportion was low, *S.* Kentucky and *S.* Concord demonstrated extensive MDR. Further characterization on molecular resistance determinants and continuous monitoring of circulating serotypes and antimicrobial resistance profile is recommended. As the extent of MDR appears to be dependent on serotypes involved, it is vital to have at least one national *Salmonella* reference laboratory to conduct serotyping of *Salmonella* isolates from all regions of Ethiopia.

Additional file

Additional file 1: Table S1. Resistance pattern of *Salmonella* serotypes recovered from the study participants. (DOCX 15 kb)

Abbreviations

ALIPB: Aklilu Lemma Institute of Pathobiology; MDR: Multi-drug resistance; NTS: Non-typhoidal *Salmonella*; RVB: Rappaport-Vassiliadis Broth; TASH: Tikur Anbessa Specialized Hospital; TTB: Tetrathionate broth; WHO: World Health Organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TE, WAG, DA, JSG and EE, participated in conception of the study and review of the draft manuscript. TE was involved in sample collection laboratory investigation and preparation of the draft manuscript. HA participated in laboratory work. All authors read and approved the final manuscript.

Acknowledgments

This study was financially supported by The National Institutes of Health (NIH) Fogarty International Center (grant D43TW008650) to W.A.G./J.S.G., R56AI109002 to J.S.G. and WHO/AGISAR to T.E. We are grateful to Dr Roger P. Johnson and Ann Perets of the Laboratory of Foodborne Zoonoses, Public Health Agency of Canada for serotyping and phage typing of the *Salmonella* isolates. We are extremely grateful to Dr. Girmay Medhin for the technical contribution to the statistical analysis. We are thankful for cooperation of laboratory technicians at health centers and TASH during sample collection. Technical assistance of Mr. Ephrem Eguale in sample collection was greatly appreciated.

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Received: 12 April 2015 Accepted: 19 October 2015
Published online: 04 November 2015

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Paper 3

Comparative phenotypic and genotypic characterization of temporally related non-typhoidal *Salmonella* isolated from humans and food animals in central Ethiopia

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Abstract

Salmonella is one of the most commonly isolated pathogens and is consistently among the top cause of foodborne bacterial illnesses. The primary sources of human non-typhoidal *Salmonella* (NTS) infection are food producing animals such as cattle, poultry and swine. The objective of the current study was to characterize and compare temporally and spatially related *Salmonella* isolates from humans and food animals and to determine their phenotypic and genotypic relatedness. Isolation and identification of *Salmonella* species was conducted according to WHO Global Foodborne Infections Network laboratory protocol. *Salmonella* serotyping was conducted using slide agglutination and microplate agglutination techniques. Antibiotic susceptibility testing was performed using disk diffusion method according to Clinical and Laboratory Standards Institute guidelines. Genotyping of *Salmonella* isolates was conducted by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The dominant serovars frequently isolated from food animals and humans were *S.*Typhimurium, *S.* Virchow, *S.* Kentucky and *S.* Saintpaul. Frequency of resistance to tetracycline, ampicillin and cephalothin in *Salmonella* isolates from humans was relatively low compared to that seen in animals and the level of MDR was significantly higher in *Salmonella* isolates obtained from slaughtered cattle, dairy cattle and poultry compared to those obtained from Humans ($p < 0.05$). All *S.* Kentucky isolated from animals and humans were MDR with shared resistance phenotype (AmpAmcCfCipGmTeSuSNa). MLST analysis showed 3 novel allele types and 5 novel sequence types among 21 strains examined. Despite wide diversity, PFGE analysis revealed clonal or closely related genotypes of *S.* Virchow, *S.* Typhimurium, *S.* Kentucky, *S.* Braendurp and *S.* Miami circulating among humans and animals. Of particular concern is isolation of clonally related MDR *S.* Kentucky from dairy, slaughtered cattle, poultry and humans; MDR *S.* Virchow from dairy cattle, slaughtered cattle and humans. Detection of clonally related *Salmonella* isolates from humans and animals, the high MDR status of isolates from animals, and close proximity of farms and human residential area presents major public health problem. Therefore, integrated surveillance of *Salmonella* serovars in humans and animals and implementation of appropriate pathogen control strategy along critical points in food animal production from farm to bench is recommended.

Key words: Non-typhoidal *Salmonella*, resistance, serovar, genotyping

Introduction

Salmonella species are enteropathogenic bacteria capable of causing disease in a wide range of animals and humans. It is one of the most common pathogens associated with food of animal origin and is consistently at the top of the overall incidence of food-borne bacterial illnesses (Penteado and Leitao, 2004). The primary sources of human *Salmonella* infection are food-producing animals such as cattle, poultry and swine, mainly via contamination of carcass with the gastrointestinal content during slaughtering (Kagambèga et al., 2013). The sources and transmission routes of *Salmonella* in developing countries are poorly understood due to lack of coordinated national epidemiological surveillance systems (Kagambèga et al., 2013; Kariuki et al., 2006). As a result, the dominant serovars affecting humans and the relative contribution of different food animals as a source of *Salmonella* infection to humans is not clearly understood.

The development and accumulation of resistance to antimicrobials in foodborne pathogens are a major threat to public health globally. Drug resistant microorganisms or drug resistance genetic materials originating from food animals can reach humans through the environment, food products, and through direct contact with animals (Van Boeckel et al., 2015). Resistance acquired by microorganisms in food animals can directly be a threat to human health in case of zoonotic organisms such as *Salmonella* or can horizontally transfer their resistance genetic determinants from commensal microorganisms to human pathogens in gastrointestinal tract. Studies have shown strong association between the prevalence of antimicrobial resistant bacteria in animals and in humans (Fey et al., 2000; Vieira et al., 2011) as well as increase in occurrence of drug resistant bacteria in humans post consumption of specific drugs in livestock in the region (Hsueh et al., 2004). The levels of antimicrobial use in a population of food animals has also been shown to be correlated with the rate of occurrence of drug resistant microbes in humans (Aarestrup, 2005; Chantziaras et al., 2014) as well as rate of occurrence of drug resistance to commensal *E. coli* in animals (Chantziaras et al., 2014).

The global antimicrobial consumption in livestock in 2010 was estimated to be 63,151 tons and was proposed to rise by 67% by 2030. Ethiopia was among the group of countries estimated to use 6-7mg/Km^2 which was the fourth of ten category of antimicrobial consumption although the authors have acknowledged high uncertainty in their model prediction for antimicrobial consumption for Ethiopia (Van Boeckel et al., 2015). Although accurate data on the level of antimicrobial consumption in food animals is not available in Ethiopia, several reports have shown occurrence of MDR strains of NTS from various food animals and food animal products (Alemu and Zewde, 2012; Eguale et al., 2014; Molla et al., 2006).

Several serovars of *Salmonella* have been reported in various food animals, food products and humans in Ethiopia. In these isolates, a high level of drug resistance has been reported (Alemayehu et al., 2003; Beyene et al., 2011; Mache A, 1997; Molla et al., 2006). However, little is known on the phenotypic and genotypic relatedness of *Salmonella* isolates from humans and animals. Characterization of temporally and spatially related *Salmonella* serovars from humans and animals using phenotypic and genotypic techniques could give important information on the source of dominant serovars causing human salmonellosis among various food animals and could also provide information on the common drug resistance phenotypes shared among *Salmonella* isolates from humans and animals. The current study therefore aimed to characterize *Salmonella* isolates from food animals (dairy cattle, slaughtered cattle, poultry and swine) in Addis Ababa and nearby towns and those isolated from diarrheic clinical humans patients in Addis Ababa collected at the same time. Isolates were serotyped, phage typed, screened for antimicrobial susceptibility and selected representative isolates were also genotyped using PFGE and MLST.

Material and methods

Bacterial isolates

Salmonella isolates obtained from different sources (stool samples of diarrheic human patients, n= 68; feces of food animals (dairy cattle, n =30; slaughtered cattle, n= 20; poultry, n =26; pig, n=8) were used in the current study. *Salmonella* isolates were collected from April 2013 to March 2014. Isolation and identification of *Salmonella* species was conducted according to WHO Global Foodborne Infections Network Laboratory Protocol (WHO, 2010) and confirmed by genus specific PCR (Cohen et al., 1993).

Salmonella serotyping and phage typing

Salmonella isolates were serotyped and phage-typed at the Public Health Agency of Canada, World Organization for Animal Health (OIÉ) Reference Laboratory for Salmonellosis, Guelph, Ontario, Canada.

Briefly, the somatic (O) antigens were determined by slide agglutination tests (Ewing, 1986) and flagellar antigens were determined using a microplate agglutination technique (Shipp and Rowe, 1980). The antigenic formulae of Grimont and Weill (2007) were used to identify and assign the serovars of the isolates. Phage typing of *S.*Typhimurium isolates was performed by the methods developed by Callow (1959) and extended by Anderson et al (1977) with reference phages obtained from the Public Health England, Gastrointestinal Bacteria Reference Unit, Colindale, England and the Public Health Agency of Canada, National Laboratory for Enteric Pathogens, Winnipeg, Canada. *Salmonella* isolates that reacted with the phages but did not conform to any recognized phage type were designated atypical (AT).

Antimicrobial susceptibility testing

Susceptibility of the isolates to a panel of 18 antimicrobials was determined using the Kirby-Bauer disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). The following antimicrobials (Sensi-Discs, Becton, Dickinson and Company, Loveton, USA) and disc potencies (µg) were used: amikacin (30), amoxicillin + clavulanic acid (30), ampicillin (10), cefoxitin (30), ceftriaxone (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), nitrofurantoin (100), streptomycin (10), sulfisoxazole (1000), sulfamethoxazole + trimethoprim (30), trimethoprim (5) and tetracycline (30). The interpretation of the categories of susceptible, intermediate or resistant was based on the CLSI guidelines (CLSI, 2013) and for the purpose of analysis, all readings classified as intermediate were considered as resistant where necessary. Reference strain of *Escherichia coli* ATCC 25922 was used as a quality control.

Genotyping using pulsed-field gel electrophoresis

Representative isolates(n=47) were systematically selected and genotyped using PFGE to investigate genetic relatedness of *Salmonella* serovars obtained from food animals in Addis Ababa and surrounding districts and those obtained from diarrheic patients from Addis Ababa. Two out-group *Salmonella enterica* strains from Kenya and USA both isolated from swine were also included in this analysis. PFGE was performed according to Center for Disease Control and Prevention (CDC) PulseNet), as previously described (Ribot et al., 2006) using a contour clamped homogeneous electric field (CHEF)-Mapper (Bio-Rad Laboratories, Hercules, CA). Briefly, DNA digestion was performed using XbaI restriction enzyme. After staining with ethidium bromide, DNA fragments were visualized under UV trans-illumination (Gel Doc 2000, Bio-Rad Laboratories, Hercules, CA, USA). Gel images were photo documented using the Quantity one 1D analysis software (Bio-Rad Laboratories). PFGE gels were then analyzed using BIONUMERICS software V. 4.61 (Applied Maths NV, Keistraat, Belgium) using Dice coefficient similarity index and unweighted pair group average (UPGMA) cluster analysis. Image analysis was conducted based on 2.2% tolerance and 1.5% optimization. The plausible genetic threshold for clustering was 88%.

Multilocus sequence typing

Twenty-one representative isolates originating from human patients and different food animals were analyzed using multilocus sequence typing **(**MLST). Seven housekeeping genes *aroC* (chorismate synthase), *hemD* (uroporphyrinogen III cosynthase), *dnaN* (DNA polymerase III beta subunit), *hisD* (histidinol dehydrogenase), *sucA* (alphaketoglutarate dehydrogenase), *purE* (phosphoribosyl aminoimidazole carboxylase), *thrA* (aspartokinase+homogenize dehydrogenase) were amplified by PCR according to the recommendation of the MLST database for *Salmonella enterica* (http://web.mpiib-berlin.mpg.de/mlst). The list of primers and amplification protocols including PCR conditions indicated in the database were used for each PCR reaction. Amplification products were purified using Qiagen PCR purification kit and sequenced using recommended forward and reverse primers. The sequences

were assembled and analyzed using CLC Main Work Bench. The sequences were then trimmed to fit to the size of the MLST database requirement and submitted to the MLST database website (http://web.mpiib-berlin.mpg.de/mlst) and assigned existing or novel allele numbers and sequence types (STs). For selected isolates where whole genome sequence(WGS) was conducted using Alumina Miseq technology, raw reads (fastq files) of the sequence were uploaded to Centre for Genomic Epidemiology web server which analyzes the data and assign specific allele and sequence types(Larsen et al., 2012)..

Ethical consideration

The study protocol was ethically approved by the Institutional Review Board of College of Health Sciences, Addis Ababa University and National Research Ethics Review Committee (Permit#3-10/474/05 dated 29-03-2013). Individual oral informed consent was obtained from all adult participants and the parents or guardians of all children participated in the study.

Data Analysis

Difference in level of MDR occurrence in humans and animals was tested by student t-test and one way analysis of variance (ANOVA). The difference between the means was considered significant at $p<0.05$.

Results

Relative distribution of *Salmonella* serovars from food animals and diarrheic human patients

Overall, 152 *Salmonella* isolates from cattle, poultry, swine and human belonging to 20 serovars were characterized in this study. *S.*Typhimurium and *S.* Saintpaul were detected in all types of food animals and humans. The dominant serovars isolated from humans and animals were *S.*Typhimurium (27.6%), *S.* Saintpaul (21.7%), *S.* Virchow (18.4%), and *S.* Kentucky (6.6%). The frequency of *Salmonella* serovars according to host species is shown in Table 1. *S.* Typhimurium was the dominant serovar recovered from all host species representing 39.7% of the human infection and 17.9% of the total animal infection followed by *S.* Saintpaul representing 1.5% of human infection and 38.1% of animal infection in the current study. Among *S.* Typhimurium serovars, 10 different known phage types and 2 atypical phage types were identified. The dominant phage type was PT126 representing 19.1%, followed by PT1 (14.3%). Interestingly, all *S.*Typhimurium PT126 were isolated from human patients whereas other phage types were fairly distributed among other host species. *S.* Saintpaul, although was the first and the second most dominant serovar isolated from poultry and cattle, respectively, only one *S.* Saintpaul was detected from human. *S.* Virchow was the second dominant serovar isolated from humans and among animals it was recovered only from dairy cattle and slaughtered cattle. *S.* Kentucky though was of low proportion, it was detected from dairy cattle, slaughtered cattle, poultry as well as humans (Table 1).

Table 1 *Salmonella enterica* serovars isolated from cattle, poultry, swine and human in central Ethiopia

Serovar	Dairy	Slaughtered cattle	Poultry	Swine	Animal Total	Human	Total (%)
Typhimurium	7	4	3	1	15	27	42(27.6)
Saintpaul	6	4	20	2	32	1	33(21.7)
Virchow	5	2	-	-	7	21	28(18.4)
Kentucky	5	1	2	-	8	2	10(6.6)
Kottbus	-	1	-	-	1	7	8(5.26)
Miami	-	-	-	2	2	3	5(3.29)
Haifa	-	3	1	-	4	-	4(2.63)

Braendruep	-	2	-	-	2	1	3(1.97)
Dublin	3	-	-	-	3	-	3(1.97
Newport	-	-	-	-	-	2	2(1.32)
Milkawasima	1	2	-	-	2	-	2(1.32)
Livingstone var.14+	1	-	-	1	2	-	2(1.32)
Aberdeen	1	-	-	-	1	-	1(.66)
Concord	-	-	-	-	-	1	1(.66)
Agona	-	1	-	-	1	-	1(.66)
Entertidis	-	-	-	-	-	2	2(1.32)
Heildelberg	-	-	-	1	1	-	1(.66)
I:6;7,14,I,w	1	-	-	-	1	-	1(.66)
V:ROUGH-O;-:-	-	-	-	-	-	1	1(.66)
I,Rough-O:I:1,2	-	-	-	1	1	-	1(.66)
Total	**30**	**20**	**26**	**8**	**84**	**68**	**152**

Comparative antimicrobial susceptibility of *Salmonella* isolates from food animals and diarrheic human patients

On the whole, 140 (92.1%) of the 152 *Salmonella* isolates were resistant to one or more antimicrobials tested. These involved 50 (100%) of isolates from cattle, 24 (92.3%) of isolates from poultry, 59 (86.8%) of isolates from humans and 6 (75%) of isolates from swine (Table 2). Among all antimicrobials tested, frequency of resistance was more common to streptomycin (81.6%), nitrofurantoin (44.7%), kanamycin (44.7%), tetracycline (36.2%) and sulfisoxazole 53 (34.9%). The rate of occurrence of resistance to some antimicrobials is variable among the isolates collected from different sources. Frequency of resistance to tetracycline, ampicillin and cephalothin in *Salmonella* isolates from humans is relatively low compared to that seen in animals. For instance, resistance to tetracycline ranged from 34.6% - 70% in isolates obtained from food animals while only 13.4% of human isolates were resistant to tetracycline. Resistance to chloramphenicol was detected in 42.3% of isolates obtained from poultry, most of these isolates belonged to *S.* Saintpaul. On the other hand, all isolates obtained from other food animals were susceptible to chloramphenicol. Despite long history of use of chloramphenicol in humans in the country, only 2 (3%) of isolates from human patients were resistant to chloramphenicol in the current study, one of which was *S.* Concord isolated from diarrheic child in TASH. Overall, rate of occurrence of resistance to antimicrobials in human was less common compared to isolates obtained from food animals (Table 2).

Table 2. Antimicrobial resistance profile* of *Salmonella* isolates from different food animals and diarrheic human patients

Drug	**Source of *Salmonella* isolates**					
	Dairy cattle, (n=30)	Slaughtered cattle (n=20)	Poultry (n=26)	Swine, (n=8)	Human, (n=68)	Total (n=152)

	No.(% R)	No.(%R)	No.(%R)	No.(%R)	No.(%R)	No.(%R)
An	-	6(30)	-	-	-	6(3.9)
Amp	10(33.3)	5(25)	11(42.3)	1(12.5)	11(16.4)	38(25)
Amc	8(26. 7)	5(25)	12(46.2)	1(12.5)	8(11.9)	34(22.4)
Cf	14(46. 7)	8(40)	12(46.2)	1(12.5)	12(17.9)	47(30.9)
C	-	-	11(42.3)	-	2(3)	13(8.6)
Cro	-	-	1(3.9)	-	2(3)	3(2)
Fox	-	-	-	1(12.5)	2(3)	3(2)
Cip	9(30)	2(10)	5(19.2)	2(25)	3(4.5)	21(13.8)
Gm	7(23.3)	2(10)	2(7.7)	-	5(7.5)	16(10.5)
K	14(46. 7)	16(80)	12(46.2)	3(37.5)	23(34.3)	68(44.7)
Tmp	1(3.3)	2(10)	1(3.9)	-	3(4.5)	7(4.6)
Sxt	-	2(10)	1(3.9)	-	3(4.5)	6(3.9)
Te	16(53.3)	14(70)	9(34.6)	5(62.5)	9(13.4)	55(36.2)
Su	18(60)	17(85)	24(92.3)	-	26(38.9)	53(34.9)
S	26(86.7)	18(90)	24(92.3)	6(75)	50(74.6)	124(81.6)
Nitro	18(60)	15(75)	5(19.2)	3(37.5)	27(40.3)	68(44.7)
Na	9(30)	1(5)	5(19.2)	2(25)	16(23.9)	33(21.7)
N	5(16.7)	4(20)	3(11.5)	-	9(13.4)	21(13.8)

An, amikacin; Amp, ampicillin; Amc, amoxicillin and clavulanic acid; Cf, cephalothin; C, chloramphenicol; Cro, ceftriaxone; Cip, ciprofloxacin; Gm, gentamicin; K, kanamycin; Tmp, trimethoprim; Sxt, sulfamethoxazole + trimethoprim; Te, tetracycline, Su-sulfisoxazole; S, streptomycin; Nitro, nitrofurantoin; Na- nalidixic acid, N, neomycin

*All intermediately resistant isolates were considered resistant for this analysis

Mean±SEM number of drugs to which *Salmonella* isolates obtained from slaughtered cattle, dairy cattle, poultry, swine and human patients were resistant was 5.1±0.42, 5.33±0.7, 5.69±0.64, 2.88±0.74 and 3.1±0.35, respectively. The level of MDR was significantly higher in *Salmonella* isolates obtained from slaughtered cattle, dairy cattle and poultry compared to those obtained from humans ($p < 0.05$) while no significant difference was observed among isolates obtained from food animals. Fig.1 shows the mean number of drugs to which *Salmonella* isolates obtained from different sources.

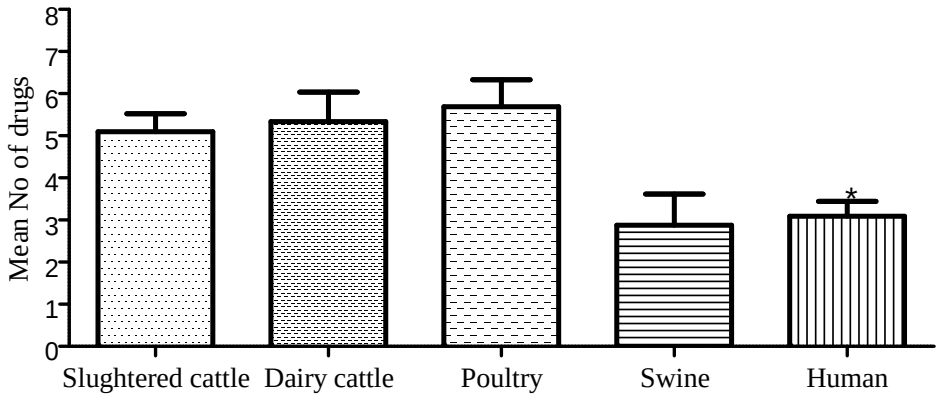

Fig.1. Level of multi-drug resistance among *Salmonella* isolates from different food animals in central Ethiopia and diarrheic human patients in Addis Ababa (Each bar represents mean ±SEM number of drugs to which isolates were resistant.

R-profile of *Salmonella* isolates from food animals and humans

Diverse phenotypic resistance profile was detected among *Salmonella* serovars isolated from food animals and humans. Complete sensitivity to all antimicrobial agents tested was more commonly detected in human isolates 9 (13.2%) than isolates from animals 3(3.6%). All *S.* Kentucky isolated from animals and humans were MDR to several drugs. All of them have shared resistance phenotype (AmpAmcCfCipGmTeSuSNa) (Table 3).

Table 3.*Salmonella* serovars isolated from cattle, poultry, swine and human feces and their antimicrobial resistance patterns

Serovar	**Source**					**Antimicrobial resistance pattern and host**	
	Cattle	Poultry	Swine	Human	Total	Intermediately resistant	Resistant
Aberdeen	1	-	-	-	1	CipKTeSuSNa(1C)	Nitro(1C)
Agona	1	-	-	-	1	GmKSNitro(1C)	SxtTmpTeSuNa(1C)
Braendrerup	2	-	-	1	3	AnCipKTeSuS(1C) CipTeSuS(1C) SuS(1H)	Nitro(1C)
Concord		-	-	1	1	Fox(1H)	AmpAmcCCroCfFoxGmKSxtTmpSuSNitro(1H)
Dublin	3	-	-	-	3	CfS(1C),S(1C), CfSuS(1C)	-
Haifa	3	1		-	4	CipKSuSNitro(1C), AnCipKSuSNitro(1C) KNitro(1C),CipKS(1P)	TeSu (1C) SxtTmpTeSuNitro Na(1P)
Enteritidis PT- AT	-	-	-	2	2	KS(1H)	
Heidelberg PT 2		-	1	-	1	AmcFoxTeS(1S)	AmpCf(1S)
I,Rough-O:I:1,2	-	-	1	-	1	KSNitro(1H)	-
I:6;7,14:-:I,w	1	-	-	-	1	CipTeSuSNitroNa(1C)	AmpAmcCf(1C)
Kentucky	6	2	-	2	10	Nitro(1C) KAmcSxt(1C) AmcKNitro(1C) KNitro(2C) AmcKNitro K(H) AmcKN(H) C(P)	AmpAmcCfCipTeSuSNa(1C) AmpCfCipGmTmpTeSuSNitroNaN(1C)AmpCfCipGmTeSuSNaN(C) AmpAmcCfCipGmTeSuSNa(C) AmpCfCipGmTeSuSNa(C) AmpAmcCfCipGmTeSuSNa (C) AmpAmcCfCipFoxGmTeSuSNa(H) AmpCfCipGmTeSuSNa(H) AmpAmcCfCipGmTeSuSNa(p) AmpAmcCfCipGmTeSuSNa(P)
Kottbus	1	-	-	7	8	CfKSuS(1C), S(1H), SNitro(1H), KSuS(1H), SuS((1H),CipGmKSuSNitro(1H), TeSuSNitro(1H), KS(1H)	TeNitro(1C)
Livingstone var.14+	1	-	1	-	2	Cip(1), CipTeS(1)	NA(2)
Miami	-	-	2	3	5	S(1H), KSNitro(1H), SNitro(1H),KTeSNitro(1S),CipKTeS(1S)	Nitro(1S)
Mikawasima	3	-	-	-	3	SuNitro(1), AnCipTe(1), CipTeSuSNitroN(1)	SuSNitro(1)

Newport				2	2	S(1), KSxt(1)	Tmp(1)
Saintpaul	10	20	2	1	33	AmcCf SuSNitro(1P) AmcCfKS(2P), AmcCfS(1P) AmcCipKSuS(1C), AmcCipKSuSNaN(1P) AmcCipSN(1P) AmpAmcCfCip SuSN(1C) AmpCfKTeS N(1C), AnkCipSuS(1C) CfKS Nitro(1P) CfKSuS(1H), CfS(2P) CipSuSNitroN(1P) KS(2C, 1P),KS(1P) KSNitro(1P), KSuS(1) KTeSN(1C) KTeSuSN(C) Sensitive (1S,1P) Su(1P)	CCroNa(1P) AmpCTeSu(1p), AmpCSu(1P), AmpCTe Su(1p) AmpCf Te(1C), AmpCCfNitro(1P) AmpC CfTeSu(1P) AmpCfTe(1C) SuNitro(1C), Nitro(1C) AmpAmcCSu(1P) AmpAmcCSu(2P) SuNitro(1C) Su(1P,) SuNitro(P) Nitro(1C) AmpAmcCCfTeSu(1P)
Typhimurium-PT - At	1	-	-	2	3	KS(1H),KSuSNitro(1C),SuS(1H)	
Typhimurium PT1	1	-	-	5	6	CfKSuSNitro(1H), Sensitive(2H), SNitro(1H), SuS(1P),S(1H)	
Typhimurium PT 2		1	-	3	4	KSuSNitro(1P),SuS(1H),S(1H),Sensitive(1H)	
Typhimurium PT 3	1	-	-	3	4	CipKNitroS(1C), KSuSNitro(1H) SuSNitro(1H), KSuSNitroNa(1H)	AmpAmcCf(1C), AmpAmcCf(1H), AmpCf(1H), AmpAmcCf(1H)
Typhimurium PT 4	1	-	-	-	1	CipKNitro	AmpAmcCf
Typhimurium PT 66	-	1	-	1	2	AmpNitro(1H),K(P)	TeSuS(1H, 1P)
Typhimurium PT 67	1	-	-	-	1	S(C)	-
Typhimurium PT 74	-	-	1	-	1	Sensitive(1C,1S)	
Typhimurium PT 126	-	-	-	8	8	SNitro(1H),SNa(1H),SuS(2H),Su(2H),S(2H),KS(1H)	Nitro(1H)
Typhimurium PT 193	1	-	-	3	4	AmcNitro(1H),Sensitive(2H)	AmpCfKSxtTmpTesuSN(1H), SxtTmpTeS(1C)
Typhimurium Var. Copenhagen PT 193	3	-	-	-	3	KS(1C),TeS(1C),TeSuS(1C)	
Typhimurium Var. Copenhagen PT At	1				1	CipTeS(1C),	
Typhimurium Var. Copenhagen PT U285	1				1	KTeSuSNitroN(1C)	
V:ROUGH-O;-:-	-	-	-	1	1	AmpCfKSNitro(1H)	KSNitro(1H)
Virchow	7	-	-	22	29	AmcCf(1C), AmcKNitro(1C), AmcSuNitro(1C) CipKTeSuSNitroN(1C), CS(1H), GmS(1H) GmSNitro(1C) K(1H), KNitro(1H) KSNitro(1H), KSuS Nitro(3H), KTeSuSN(1H) Nitro(1C,2H), SuSNitro(2H)SNitro(3H),S(4H) Sensitive(3H)	Amp(1C), AmpCfCipGmTeSuSNa(1C), AmpAmcCfS(1C) AmpAmcCf(1H)(Nitro(1C)
Total	**50**	**26**	**8**	**68**	**152**		

An, amikacin; Amp, ampicillin; Amc, amoxicillin and clavulanic acid; C, chloramphenicol; Cf, cephalothin; Cip, ciprofloxacin; Cro, ceftriaxone Gm, gentamicin; K, kanamycin; Na, nalidixic acid; Tmp, trimethoprim; Te, tetracycline, Su- sulfisoxazole; S-streptomycin; Nitro, nitrofurantoin; N, neomycin, Pt, Phage type; At, Atypical
H- human, C- cattle, S-swine, P-poultry

Genotyping using MLST

Among 21 isolates for which complete MLST data are available, 3 novel alleles (*aroC457*, *sucA432* and *thrA480)* and 5 new sequence types were found and deposited on the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). Both *S.* Kentucky from poultry and cattle were ST198 which is similar to the *S.* Kentucky strain commonly circulating in Africa and Asia. Most of *S.*Typhimurium from human, poultry and cattle belonged to the ST19, one *S.* Typhimurium vr. Copenhagen was ST313 while another isolate from human gave novel sequence type ST1936. But all *S.* Typhimurium isolates belonged to the same clonal complex. Among the 6 *S.* Saintpaul, all isolates from poultry and cattle had the same novel ST1934, while one isolate from human was ST49. *S.* Braenderup, *S.* Haifa, and *S.* Kottbus examined in the current study were all novel Sequence types (ST1932, ST1935 and ST1937), *S.* Virchow isolated from human gave ST303 (Table 4).

Table 4. MLST allele type, ST and ST-clonal complex for *Salmonella* serovars as assigned by the *Salmonella enterica* MLST database

Serovar	**No**	**Source**	***aroC***	***dnaN***	***hemD***	***hisD***	***purE***	***sucA***	***thrA***	***ST***	**St.CC**
Braenderup	1	Human	12	2	10	14	11	10	16	1937	-
Haifa	1	poultry	5	3	7	9	6	3	7	1932	-
Kentucky	1	cattle	76	14	3	77	64	64	67	198	56
Kentucky	1	poultry	76	14	3	77	64	64	67	198	56
Kottbus	2	Human	10	71	12	12	190	9	18	1935	-
Livingstone var.14+	1	cattle	117	135	18	12	162	162	38	543	-
Miami	1	swine	457	356	7	116	91	432	194	1933	-
Saintpaul	1	Human	5	14	21	9	6	12	17	49	14
Saintpaul	2	cattle	5	21	18	33	6	12	480	1934	14
Saintpaul	3	poultry	5	21	18	33	6	12	480	1934	14
Typhimurium PT 1	1	poultry	10	7	12	9	5	9	2	19	1
Typhimurium PT 1	1	Human	10	7	12	9	5	9	2	19	1
Typhimurium PT 193	1	Human	10	7	12	9	5	9	2	19	1
Typhimurium Var. Copenhagen PT193	1	cattle	10	7	12	9	391	9	2	19	1
Typhimurium PT 3	1	Human	10	7	3	9	5	19	2	1936	1
Typhimurium Var. Copenhagen PT Atypical	1	cattle	10	7	12		112	9	2	313	1
Virchow	1	Human	6	7	10		39	10	14	303	9

Genetic diversity of representative *Salmonella* isolates using PFGE

PFGE analysis in the current study showed large genotypic diversity with 11 genotypic clusters and 7 sporadic clones among 49 isolates (Fig. 2). The majority of the *Salmonella* isolates within a serovar are clonally related. All *S.* Virchow isolates from dairy cattle (n= 2), slaughtered cattle (n=1) and diarrheic human patients (n=5) in Addis Ababa formed a single cluster. This cluster is further sub-clustered into two. The first group consisted isolates from diarrheic patients (n=4) and dairy cattle (n=2) in Addis

Ababa with exactly identical PFGE profile while the second consisted of 1 isolate from diarrheic human patient and the other from slaughtered cattle.

Two of *S.* Kottbus obtained from two diarrheic patients from two separate health centers in Addis Ababa clustered together suggesting common source of infection while another *S.* Kottbus strain from slaughtered cattle in Addis Ababa was distantly related to these strains. The two *S.* Braenderup isolated from slaughtered cattle and diarchic patient in Addis Ababa also clustered together.

S. Kentucky isolated from slaughtered cattle in Addis Ababa abattoir (n=1), poultry from Adaa district (n=1), dairy cattle from Addis Ababa (n=1) and diarrheic human patient in Addis Ababa (n=1) clustered together indicating their clonality. In addition, all of these isolates were MDR to over 10 antimicrobials. One *S.* Kentucky isolate obtained from human was sporadic clone and clustered separately from other strains.

Six of the 8 *S.* Saintpaul examined in the current study isolated from poultry, dairy cattle, and swine clustered together while one *S.* Saintpaul from diarrheic patient and one from slaughtered cattle in Addis Ababa has shown different PFGE finger print with very distant genetic relationship. Those *S.* Saintpaul strains clustered together were from food animals in Adaa district except two isolates obtained from swine from Addis Ababa suggesting possibility of clonal spread across the district in different farms. S. Miami isolated from swine and human patient in Addis Ababa also showed identical PFGE profile indicating their clonality.

Strains of *S.*Typhimurium, the predominant serovar shared by food animals and humans, were grouped into 3 genotpypic clusters and 3 sporadic clones (with n=1). The first cluster involved only isolates from human, while the 2nd and 3rd cluster involved isolates from both humans and animals. Among isolates in the second cluster, strain BL-162(DT193) isolated from diarrheic child from TASH had shown completely identical PFGE profile with strain AA-306 (DT193) isolated from feces of slaughtered cattle at Addis Ababa slaughter house. However, strain from human patient was resistant to several drugs. Similarly in the third cluster, two (DT1) isolates from diarchic patients and one isolate from poultry(DT1) collected from Adaa district had shown closely related PFGE pattern indicating common source of infection.

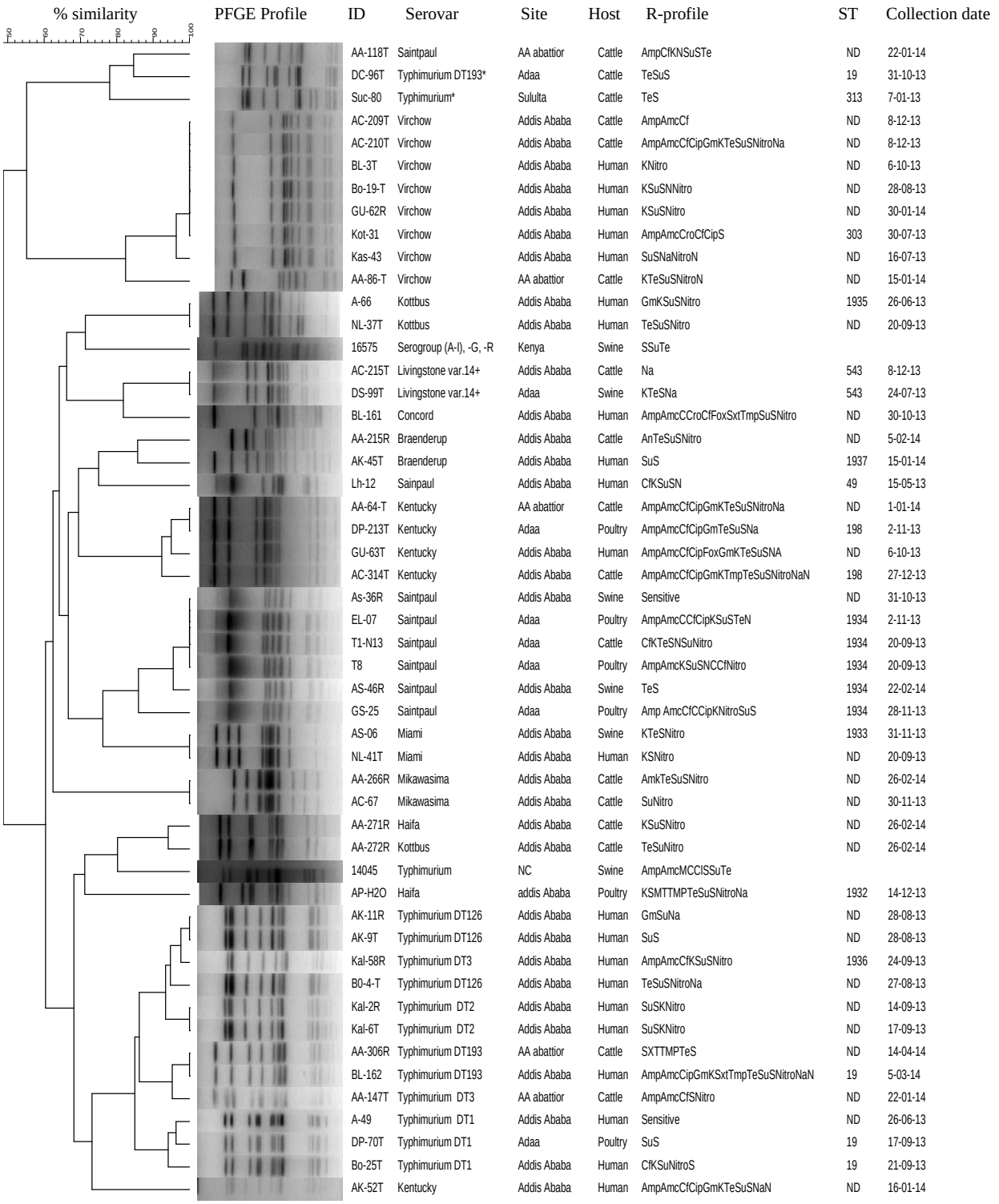

Fig 2. PFGE dendrogram showing genotypic similarity among *Salmonella* serovars isolated from humans, cattle, poultry and pigs in central Ethiopia

PFGE pulsed-field gel electrophoresis; AR, antimicrobial resistance. DT, Phagetype, Amk, amikacin; Amp, ampicillin; Amc, amoxicillin and clavulanic acid; C, chloramphenicol; Cf, cephalothin; Cip, ciprofloxacin; Cro, ceftriaxone Gm, gentamicin; K, kanamycin; Na, nalidixic acid; Tmp, trimethoprim; Te, tetracycline, Su- sulfisoxazole; S-streptomycin; Nitro, nitrofurantoin; N, neomycin

Discussion

In the current study, *Salmonella* serovars frequently isolated from clinical human patients such as *S.* Typhimurium, *S.* Virchow, *S.* Kottbus and *S.* Kentucky were also isolated from spatially and temporally related food animals. *S.* Saintpaul, although it was the most frequently isolated serovar from food animals, during the study period, only one *S.* Saintpaul was recovered from diarrheic patient in Addis Ababa. The probable reason why *S.* Saintpaul was not commonly detected in human patients despite its wide occurrence in animals could be due to less pathogenicity of strain of *S.* Saintpaul circulating in food animals to humans. The single human isolate might have acquired virulence factors useful for infecting human patients. The other possibility is as most of the strains of *S.* Saintpaul in the current study were isolated from poultry and dairy farms located in Adaa district, this strain might have been circulating only in this specific region and could not get access to the patients in Addis Ababa involved in the current study. Interestingly, genotyping by both MLST and PFGE revealed the single *S.* Saintpaul isolated from diarrheic human patient was distantly related to *S.* Saintpaul isolates obtained from food animals, suggesting other source of infection. *S.* Saintpaul was previously reported from camel (Molla et al., 2004) and minced beef (Zewdu and Cornelius, 2009) in the country. The strain of *S.* Saintpaul isolated from human patient in the current study was shown to be closely related to *S.* Typhimurium DT193 by PFGE than *S.* Saintpaul isolates from animals.

Diverse antimicrobial susceptibility phenotypes were observed among *Salmonella* serovars isolated from different sources. Three *S.* Typhimurium PT3 isolates from diarrheic human patients and one *S.*Typhimurium PT3 from cattle had common resistance profile (AmpCfNitroSuS) suggesting possibility of source of infection of human cases from cattle. One of the 4 *S.* Typhimurium Phagetype193 isolated from diarrheic child in TASH, Addis Ababa was MDR to 13 drugs tested unlike the other 2 human isolates from diarrheic patients at primary health centers which were pansusceptible to all drugs tested and one isolate from cattle resistant to only 4 drugs. Strain of MDR *S.* Typhimurium PT193 obtained from hospital might be due to nosocomial infection which acquired resistance due to frequent exposure to different drugs within the hospital. However, the PFGE profile of the MDR human isolate and that of cattle isolate were 100% identical indicating their clonality.

The second dominant serovar isolated from human patients in the current study, *S.* Virchow was also commonly detected in dairy cattle and slaughtered cattle collected during similar study period in Addis Ababa. Interestingly, all *S.*Virchow isolates in the current study were isolated from cattle and clinical diarrheic patients residing in Addis Ababa. Most of these strains have common antimicrobial resistance profile and representative isolates from different hosts has also shown identical PFGE finger print despite difference in geographic location, suggesting clonal spread of the strain in Addis Ababa. Similarly *S.* Virchow was reported to be the second dominant NTS serovar in human patients in Israel (Weinberger et al., 2006). It was frequently associated with blood stream infection(Mani et al., 1974). It is also among the top *Salmonella* serovars causing human salmonellosis in Europe (Bonalli et al., 2011). There is a need for appropriate control strategy to reduce spread of this pathogen in Addis Ababa and surrounding districts.

S. Kottbus was also one of the dominant serovars detected from human patients in Addis Ababa. Among food animals, only a single *S.* Kottbus was detected from feces of slaughtered cattle. Like *S.* Saintpaul mentioned above, this strain was not genotypically related to the two *S.* Kottbus strains isolated from diarrheic patients in Addis Ababa. *S.* Kottbus was previously reported from apparently healthy camels (Molla et al., 2004) and pork in Ethiopia (Zewdu and Cornelius, 2009) . Though we do not have data on previous occurrence of human *S.* Kottbus infection in the country, this serovar has been reported to cause serious multi-state outbreaks of human salmonellosis in other countries (Palmera-Suárez et al., 2007; Winthrop et al., 2003).

Occurrence of *S.* Kentucky in poultry, dairy cattle, slaughtered cattle as well as clinical human patients in Addis Ababa together with observed shared MDR phenotype and genotypic clonality of selected strains revealed by PFGE finger prints clearly indicates the clonal spread of this strain across various host species in the study area. Interestingly, all *S.* Kentucky strains in the current study were isolated from Addis Ababa city limit and were resistant to 9 antimicrobials in common (AmpAmcCfCipGmTeSuSNa). MDR *S.* Kentucky strains belonging to a single clone resistant to quinolones were previously reported from European travelers returning from different African and Asian countries (Le Hello *et al.*, 2011) and from beef, chicken and pork in Ethiopia (Molla et al., 2007). High level resistance to ciprofloxacin caused by mutation in QRDR in *in vitro* derived mutants of *S. enterica* was previously reported to be associated with fitness costs (O'Regan et al., 2010) which is contradictory to widespread occurrence of this MDR strain. Our previous study has shown that most of the MDR *Salmonella* isolates from Ethiopia including *S.* Kentucky were strong biofilm producers (Eguale et al., 2014) which could be the major contributing factor for persistence and clonal spread of this MDR strain. Circulation of such MDR and persistent strain in highly populated city like Addis Ababa is a threat to public health and requires serious attention.

S. Miami from swine and diarrheic human patients and *S.* Braendrup from cattle and diarrheic human patients were also clonally related indicating their common source. This recovery of similar serovars in humans and animals as well as the occurrence of related MDR resistance profile especially in *S.* Virchow and *S.* Kentucky indicates the transfer of *Salmonella* isolates and their drug resistance genetic markers from these food animals to humans or vise versa.

The overall frequency of resistance to most of the drugs and especially to tetracycline and sulfisoxazole was higher in *Salmonella* isolates from food animals compared to those obtained from clinical human patients. The possible reason for this could be due to frequent use of these drugs in farms favoring selection of resistant strains. Our previous study has also shown that drugs like oxytetracycline, streptomycin and sulfonamides are widely used in dairy farms in the study area (Paper 1). The significantly higher occurrence of MDR in *Salmonella* isolates from food animals compared to those from humans entails high risk of transmission of drug resistant isolates and resistance genetic markers to humans from these food animals.

Three novel alleles and 5 unique MLST sequence types were detected among only 21 isolates examined. Of the12 different sequence types within 9 serovars investigated in the current study 5 (41.7%) of them were unique sequence types reported for the first time. The diverse MLST allelic profile and sequence types compared to the results previously reported for *Salmonella* serovars in other regions of the world indicates that *Salmonella* isolates in Ethiopia are distinct from *Salmonella* isolates from other countries. Most of the available MLST data are mainly for isolates from western countries and there are very few previous *Salmonella* MLST data from Africa and specifically Ethiopia.

In general, despite wide diversity, similar serovars, resistance patterns and clonally related serovars of *Salmonella* were found in food animals and humans. There is clear indication that similar or closely related genotypes of *Salmonella* are circulating among humans and animals. Particularly, serovars of *S.* Virchow, *S.* Typhimurium, *S.* Kentucky, *S.* Braendurp and *S.* Miami were found to circulate among food animals and humans in the study area. Of particular concern is detection of clonally related MDR *S.* Kentucky in dairy, slaughtered cattle, poultry and humans; MDR *S.* Virchow in dairy cattle and slaughtered cattle and in humans. The fact that animals and humans live in close proximity in the study area cause a major public health problem. Therefore, integrated surveillance of *Salmonella* serovars in humans and animals and implementation of appropriate pathogen control strategy along critical points in food animal production from farm to bench is recommended.

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Paper 4

Molecular mechanisms of resistance to beta-lactam and quinolone antimicrobials in non-typhoidal *Salmonella* isolates from humans and animals in central Ethiopia

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Abstract

Beta-lactam and quinolone antimicrobial agents are commonly used for treatment of non-typhoidal *Salmonella* (NTS) and other pathogens. Resistance to these classes of antimicrobial agents has increased significantly in the recent years. However, little is known on the genetic basis of resistance to these drugs in *Salmonella* isolates from Ethiopia. The aim of this study was to investigate the prevalence and mechanism of resistance to beta-lactam and quinolone antimicrobials among NTS isolates from animals and humans. Isolates with reduced susceptibility to beta-lactams were tested for genes encoding for beta-lactamase enzymes, and those resistant to quinolones for mutation in quinolone resistance determining region (QRDR) and plasmid mediated quinolone resistance (PMQR) genes with PCR and sequencing. Out of 43 isolates resistant to one or more betalactam antimicrobial agents beta-lactamase genes *(bla*) were detected in 34 (79.1%) of the isolates. The dominant *bla* gene was *bla*TEM recovered from 33 (76.7%) of the isolates with reduced susceptibility to beta-lactam antimirobials. Majority of these were TEM-1 type 20 (60.6%) followed by TEM-57, 10 (30.3%) and 4 isolates with 2 novel *bla*TEM types with amino acid substitution at codon 6 (Gln6Ser n=3) and 13 (Phe13Leu n=1). None of the isolates carrying *bla*TEMs were resistant to 2nd and 3rd generation cephalosporins. The *bla*OXA10 and *bla*CTXM-15 were detected only in a single multidrug resistant (MDR) *S.* Concord of human isolate. Double mutations in *gyrA* (Ser83-Phe + Asp87-Gly) as well as *parC* (Thr57-Ser + Ser80-Ile) subunits of QRDR were detected in all *S.* Kentucky isolates fully resistant to both nalidixic acid and ciprofloxacin from animals (n=8) and humans (n=2). Single amino acid substitutions, Ser83-Phe (n=4) and Ser83-Tyr (n=1) were also detected. Double mutations (Val423-Gly + Asp459-His) were detected in *gyr*B in 2 isolates and a single mutation (Ser463-Ala) in 1 isolate. An isolate of *S.* Maimi susceptible to nalidixic acid but intermediately resistant to ciprofloxacin had Thr57-Ser and additional novel mutation Tyr83-Phe in *parC* gene**.** PMQR genes were not detected in any of the isolates. Although decreased susceptibility to ciprofloxacin and/or nalidixic acid according to the recent improved CLSI cut off point for resistance was observed in some isolates, neither mutations in QRDR nor PMQR genes were detected in these isolates suggesting other mechanism of reduced susceptibility. In general, *bla*TEM was the principal beta-lactamase enzyme and mutations within QRDR of *gyr*A and *par*C were the primary mechanism for resistance to quinolone drugs in NTS isolates from Ethiopia. Further study on extended spectrum beta-lactamase and quinolone resistance mechanisms in other gram negative pathogens in hospital and community setting is recommended.

Key words

Non-typhoidal *Salmonella*, quinolone, fluorouinolone, Beta-lactamase, resistance,

Introduction

Non-typhoidal *Salmonella* (NTS) is one of the leading causes of foodborne illnesses worldwide. Antimicrobial treatment is usually not recommended due to self-limiting nature of the disease. However, in invasive complicated salmonellosis associated with immunocopromised, young and elderly patients, treatment with beta-lactam antimicrobials such as ampicillin, ceftriaxone and quinolone drugs mainly ciprofloxacin are employed as a life saving agents (Hohmann, 2001). Resistance to beta-lactam antimicrobials and quinolones has increased

dramatically in NTS isolates from food animals as well as humans worldwide (Kruger et al., 2004; Olesen et al., 2004; Wadula et al., 2006; Wong et al., 2014). The common mechanism of resistance to beta-lactam antimicrobials is due to production of beta-lactamase enzymes with variable level of activity against different generations of beta-lactm antimicrobials. In addition to the first generation beta-lactamases: *bla*TEM1, *bla*SHV1, several extended spectrum *bla*TEM and *bla*SHV variants, other extended spectrum beta-lactamase enzymes such as *bla*CTX-M, *bla*CMY, *bla*OXA and AmpC have been reported in *Salmonella* serotypes from different parts of the world (Olesen et al., 2004; Carattoli, 2008; Li et al., 2007; Seiffert et al., 2014; Whichard et al., 2007).

Resistance to quinolones is mainly mediated by mutations in *gyrA* and *parC* genes in *Salmonella* and other gram negative organisms. Specifically, high level resistance to ciprofloxacin is mainly attributed to double mutations in the *gyrA* gene and single or double mutation in the *parC* gene (Redgrave et al., 2014). In addition to chromosomal mutation, other mechanisms such as activation of efflux pumps (multidrug efflux pump and quinolone specific plasmid mediated efflux pump encoded by *qep* genes), *Qnr* (plasmid-mediated quinolone resistance), porins, and quinolone-modifying enzyme (aac(6')-Ib-cr) have been associated with decreased susceptibility of quinolones (Redgrave et al., 2014). Of the particular concern is the occurrence of plasmid-mediated quinolone resistance encoded by several *qnr* genes, within the last few years in different parts of the world in various gram negative organisms including *Salmonella* (Harrois et al., 2014; Jacoby et al., 2014). These genes encode for pentapeptide proteins which protects bacterial topoisomerases from the effect of quinolones. They donot induce high level resistance but their presence leads to mutation in the QRDR (Robicsek et al., 2006). However, recent report from Senegal indicated presence of *qnrB1* together with quinolone modifying enzyme *aac(6')-Ib-cr* in *Salmonella* associated with full resistance to ciprofloxacin even in the absence of mutation in the QRDR (Harrois et al., 2014). In recent years, similar to beta-lactam antimicrobials, the rate of resistance to ciprofloxacin has increased considerably in both clinical and food isolates of *Salmonella* (Lin et al., 2015; Raveendran et al., 2008; Wong et al., 2014)

In Ethiopia reports revealed resistance to beta-lactam antimicrobials and quinolones, in *Salmonella* isolates from human patients and food of animal origin (Beyene et al., 2011; Zewdu and Cornelius, 2009). However, little data is available on the genetic basis of the observed phenotypic drug resistance. MDR *S.* Concord isolates obtained from children adopted from Ethiopia to different European countries and USA were reported to harbor *bla*CTXM-15, *bla*TEM1b, *bla*SHV-12 genes encoding resistance to third generation cephalosporins and *qnrA* and *qnrB* encoding reduced susceptibility to fluoroquinolones (Hendriksen et al., 2009; Kalender et al., 2009). The aim of this study was therefore to investigate the prevalence and mechanism of resistance to beta-lactam and quinolone antimicrobials among NTS isolates from humans and animals.

Methods

Bacterial isolates

Isolates investigated in the current study were obtained from a total of 152 NTS isolated from feces of food animals (dairy cattle=30, slaughtered cattle n=18, poultry, N=26, swine n=8) and diarrheic humans (n = 68) collected during 2013-2014 from Addis Ababa and surrounding districts were investigated in this study.

Antimicrobial susceptibility testing, serotyping and phage typing

Susceptibility of each isolate to 18 antimicrobials was determined using disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). The interpretation of the categories of susceptible, intermediate or resistant was based on the CLSI guidelines (CLSI, 2013) and for the purpose of analysis, all readings classified as intermediate were considered as resistant unless otherwise mentioned. *Escherichia coli* ATCC 25922 was used as a quality control. *Salmonella* isolates were serotyped and phage-typed at the Public Health Agency of Canada, World Organization for Animal Health (OIÉ), Reference Laboratory for Salmonellosis, Guelph, Ontario, Canada as described previously (Ewing, 1986; Shipp and Rowe, 1980; Grimont and Weill, 2007; Callow, 1959; Anderson et al., 1977). Isolates belonged to 20 serotypes (**Paper 3**).

Bacterial DNA extraction

Isolates were grown on LB agar (37°C, over night). A single colony was inoculated to 5ml of Luria-Bertani broth (LB) and grown in shaking incubator at 37°C for 16-18 h. Genomic DNA was then extracted using QIAGEN genomic DNA extraction kit according to the manufacturer's recommendation.

Detection and characterization of beta-lactamse enzymes

A total of 43 isolates (n=12) from humans and (n=31) from animal with reduced susceptibility to one or more of beta-lactam antimicrobials (Amp, Amc, Cf, Fox, Cro) were investigated for beta-lactamase enzymes. PCR and DNA sequencing were performed for the detection and characterization of beta-lactamase (*bla*) genes with oligonucleotide primers previously described for *bla*TEM, *bla*SHV, *bla*PER, blaPSE, *bla*OXA1, *bla*OXA4, *bla*OXA10, *blaCMY*, and *bla*CTX-M genes (Table 1). The PCR condition for all reactions involved initial denaturation for 3 minutes at 95°C followed by 30 cycles of (95°C for 30 s, specific annealing temperature for 1 m, and 72°C for 30 s) followed by final extension at 72°C for 5 m. Specific annealing temperature for each PCR reaction is shown in Table 1. Group specific primers were used to characterize *bla*CTX-M enzymes (Pitout et al., 2004). All amplicon sequences were translated to amino acid sequences using CLC Main Work Bench and compared with protein sequences in the Genbank database and classification of *bla*TEM enzymes was based on beta-lactamase classification database (http://www.lahey.org/Studies/temtable.asp Lahey).

Investigation of mechanism of quinolone resistance

Isolates with reduced susceptibility to nalidixic acid and/or ciprofloxacin (n=29), three human isolates and 26 animal isolates were examined for presence of known quinolne resistance determinants. Quinolone resistance determining region (QRDR): *gyrA, gyrB, parC and parE* genes were amplified using PCR. PCR was also used to examine for various plasmid mediated quinolone resistance genes: *qnrA, qnrB, qnrD, qnrS, qepA,* and *aac(6')-Ib-cr* as described previously(Table 2). Similar PCR conditions described previously were used with annealing temperature presented for each primer set presented in Table 2. PCR ampilicons were purified using QIAGEN gel extraction kit and PCR purification kit (QIAGEN, USA) and sequenced. Presence of mutation in the QRDR was examined by translating nucleotide sequences into proteins and aligning against reference sequence of *S.*Typhimurium strain LT2 on NCBI database (Accession Number AE006468). The lists of primers used for this investigation are shown in Table 2.

Table 1. List of primers used for detection and characterization of beta-lactamases

Gene/target	Primer	Sequence 5'-3'	Amplicon size	AT °C	Ref	Remark
BLA_{Tem} Gene	TEM-F1	ATGAGTATTCAACATTTCCG	862-bp	55	(Naiemi et al., 2005)	
	TEM-R1	GACAGTTACCAATGCTTAATCA				
	blaTEM-F2	TAA CCA TGAGTGATAACACT			(Naiemi et al., 2005)	sequencing
	blaTEM-R2	CCGATCGTT GTCAGAAGTAA				
BLA_{SHV} gene	Bla SHV-F1	CTTTACTCGCCTTTATCG	827-bp	56	Naiemi, 2005 #97]	
	Bla SHV-R1	TCCCGC AGATAAATCACCA				
	blaSHV-F2	ACTGCCTTTTTG CGCCAGAT			(Naiemi et al., 2005)	sequencing
	blaSHV-R2	CAGTTCCGTTTCCCAGCGGT				
Bla OXA-1	OXA-1-F	ATGAAAAACACAATACATATCAAC	755-bp	48	(Edelstein et al., 2004)	
	OXA-1-R	TTTCCTGTAAGTGCGGACAC				
Bla OXA -4	OXA-4-F	TCAACAGATATCTCTACTGGT	216bp	54	(Edelstein et al., 2004)	
	OXA-4-R	TTTATCCCATTTGAATATG				
Bla OXA-10	OXa 10-F	TCAACAAATCGCCAGAGAAG	277bp	57	(Edelstein et al., 2004)	
	Oxa-10-R	TCCCACACCAGAAAAACCA				
bla PER	Per1-F	AATTTGGGCTTAGGGCAGAA	925 bp	55	(Weldhagen et al., 2003)	
	Per1-R	ATGAATGTCATTATAAAAGC				
blaPSE	blaPSE-F	TGCTTCGCAACTATGACTAC			(Riano et al., 2006)	
	blaPSE-R	AGCCTGTGTTTGAGCTAGAT				
blaCYM	blaCMY2-F	TGGCCGTTGCCGTTATCTAC	868	57	(Chen et al., 2004)	
	blaCMY2-R	CCCGTTTATGCACCCATGA				
CTX-M group I	CTXM1-F3	GACGATGTCACTGGCTGAGC	499	55	(Pitout, Hossain et al. 2004	
	CTXM1-R2	AGCCGCCGACGCTAATACA				
CTX-M group II	TOHO1-2F	GCGACCTGGTTAACTACAATCC	351	55	(Pitout, Hossain et al. 2004)	
	TOHO1-1R	CGGTAGTATTGCCCTTAAGCC				
CTX-M group III	CTXM825F	CGCTTT GCCATGTGCAGCACC	307	55	(Pitout, Hossain et al. 2004)	
	CTXM825R	GCT CAGTACGATCGAGCC				
CTX-M group IV	CTXM914F	GCTGGAGAAAAGCAGCGGAG	474	62	(Pitout, Hossain et al. 2004)	
	CTXM914R	GTAAGCTGACGCAACGTCTG				

Table 2. List of primers used for detection of quinolone resistance mechanism

Gene	Primer name	Primer sequence (5′ to 3′)	Product size	AT in ºC	References
gyrA	GyrAFP	AAATCTGCCCGTGTCGTTGGT	344bp	58	(Fabrega et al., 2009)
	GyrARP	GCCATACCTACTGCGATACC			
gyrB	GyrB FP	GAATACCTGCTGGAAAACCCAT	446bp	57	(Fabrega et al., 2009)
	GyrB RP	CGGATGTGCGAGCCGTCGACGTCCGC			
parC	ParC FP	AAGCCGGTACAGCGCCGCATC	395bp	57	(Fabrega et al., 2009)
	ParC RP	GTGGTGCCGTTCAGCAGG			
ParE	ParE FP	TCTCTTCCGATGAAGTGCTG	240bp	55	(Eaves et al., 2004)
	ParE RP	ATACGGTATAGCGGCGGTAG			
qnrA	qnrA FP	ATTTCTCACGCCAGGATTTG	516bp	53	(Robicsek et al., 2006)
	qnrA RP	GATCGGCAAAGGTTAGGTCA			
qnrB	qnrB FP	GATCGTGAAAGCCAGAAAGG	469bp	53	(Robicsek et al., 2006)
	qnrB RP	ACGATGCCTGGTAGTTGTCC			
aac(6′)-Ib	aac(6′)-Ib FP	TTGCGATGCTCTATGAGTGGCTA	482-bp	55	(Park et al., 2006)
	aac(6')-Ib-RP	CTCGAATGCCTGGCGTGTTT			
	aac(6')-Ib-cr-seq	CGTCACTCCATACATTGCAA (for sequencing of aac(6′)-Ib-cr			
qepA	QepA FP	CGTGTTGCTGGAGTTCTTC	403bp	59	(Cattoir et al., 2008)
	QepA RP	CTGCAGGTACTGCGTCATG			
QnrD	QnrD FP	CGAGATCAATTTACGGGGAATA	565bp	53	(Cavaco et al., 2009)
	QnrD RP	AACAAGCTGAAGCGCCTG			
QnrS	QnrS FP	ACGACATTCGTCAACTGCAA	417bp	53	(Robicsek et al., 2006)
	QnrS RP	TAAATTGGCACCCTGTAGGC			

Results

Prevalence of resistance to beta-lactam antimicrobials and beta-lactamase genes in *Salmonella* isolates from animals and humans

Of 43 isolates resistant to one or more beta-lactam antimicrobial agents (Amp, Amc, Cf, Fox and Cro), *bla* genes were detected in 34/43 (79.1%) of the isolates. The dominant *bla* gene responsible for resistance to beta-lactam antimicrobial agents in majority of *Salmonella* isolates 33 (76.7%) was found to be variants of *bla*TEM gene. Majority of these were TEM-1 type 20 (60.6%) followed by TEM-57, 10 (30.3%) and 4 isolates with 2 novel *bla*TEM types with amino acid substitution at codon 6 (Gln6Ser n=3) and 13 (Phe13Leu n=1). Both phenotypic resistance to beta-lactam antibiotics and detection of *bla* genes was more common in isolates collected from poultry compared to isolates from other sources (Table 21). In one of the human isolates *S.* Concord, 2 *bla* genes (*blaOXA-10* and *blaCTX-M15)* were detected. Both these genes encode for the enzymes capable of extended spectrum beta-lactamase activity. In 8 (18.6%) of the isolates, none of the tested *bla* genes were detected (Table 3).

Of the 20 serotypes in the current study, resistance to at least one beta-lactam antimicrobials was detected in 9 serotypes and *bla* gene was detected only in isolates belonging to 6 serotypes. Among the dominant serotypes, 66.7%, 92.3%, 50% and 100% of *S.* Typhimurium, *S.* Saintpaul, *S.* Virchow and *S.* Kentucky; were positive for variants of *blaTEM* gene respectively. All of the 10 *S.* Kentucky isolates collected from cattle, poultry and human were resistant to ampicillin, cephalothin and ampicillin + clauvlanic acid and were all positive for *blaTEM* gene (Table 4).

Table 1. Occurrence of *bla* genes in *Salmonella* isolates from different sources with reduced susceptibility to beta-lactam antimicrobials

Source	Total no. of isolates	[+]Resistant to ≥one of beta-lactams(%)	*bla genes detected***			*Not detected*	*No.(%)positive for bla genes*
			blaTEM	*blaOXA 10*	*blaCTX-M*		
Dairy Cattle	30	11(36.7)	7	-	-	4	7(63.6)
Slaughtered cattle	18	5(27.8)	5			0	5(100)
Poultry	26	13(50)	12	-		1	12(92.3)
Swine	8	2(25)	1	-	-	1	1(50)
Human	68	12(17.7)	9	1	1	2	*10(83.3)
Total	**152**	**43(28.3)**	**34**	**1**	**1**	**8**	**34(79.1)**

[+]Ampicillin, Cephalothin, Cefoxitin, Ceftriaxone, amoxicillin and clavulanic acid
**Though all isolates were screened for *bla*TEM, *bla*SHV, *bla*OXA1, *bla*OXA4, *bla*PER, *bla*PSE and *bla*CMY2, none of them were positive for these genes
**blaOXA10* and *blaCTX-M15* were detected in a single isolate

Table 4. Occurrence of beta-lactamase genes among different serotypes

Serovar	Total	No. Resistant[+] (%)	*bla* positive	*bla* Type	Host
Typhimurium	42	9(21.4)^{Cf}	7(66.7)	*blaTEM*	5/6(H),(2C) 0/1(S),
Saintpaul	33	13(39.4)	13(100)	*blaTEM*	10/10(p),1/1(H), 2/2(C)
Virchow	28	*4(14.3)	1(25)	0	1/3(C),0/1 (H)
Kentucky	10	10(100)	10(100)	*blaTEM*	6/6(C), 2/2(P), 2/2(H)
Kottubus	8	-	-	-	-
Miami	5	-	-	-	-
Haifa	4	1(25)^{Amp}	0	0	0/1(P)
Braendruep	3	-	-	-	-
Dublin	3	2(66.7)^{Cf}	0	0	0/2(C)
Newport	2	-	-	-	-
Milkawasima	2	-	-	-	-
Livingstone var.14+	2	-	-	-	-
Aberdeen	1	-	-	-	-
Concord	1	1(100)	*1(100)*	*blaoXA10+blaCTXM*	1/1(H)
Agona	1	-	-	-	-
Entertidis	2	-	-	-	-
Heidelberg	1	1 **AmpCf** *Amc*	-	-	0/1(S)
I:6;7,14,I,w	1	1	*1*	*blaTEM*	1/1(C)
V:ROUGH-O;-:-	1	1 **AmpCf**	-	-	0/1(H)
I,Rough-O:I:1,2	1	-	-	-	-
Total	152	43	34		

*all the 3 Virchow isolates from cattle had **AmpCf***Amc* phenotype and only one was positive for TEM1, the single human isolate had **AmpAmcCf** phenotype
[+] Beta-lactam R-profile of isolates negative for *bla* gene (**Bold**=fully resistant, *Italics*=intermediate). C-cattle, H- human, P-poultry, S-swine

Interestingly, all 10 *bla*TEM-57 were recovered from *S.* Saintpaul isolated from poultry while those *S.* Saintpaul strains obtained from cattle and human were all TEM-1. In Addition, 2 different *bla*TEM types with novel amino acid sequences (Gln6-Ser, n=3) in two *S.* Kentucky and (Phe13-Leu, n=1) in *S.* Typhimurium were detected (Table 5). Despite change in amino-acid sequences, no difference in antimicrobial susceptibility to beta-lactam antimicrobial agents was seen among isolates carrying different variants of *bla*TEM enzymes.

Among 9 isolates for which none of the tested *bla* genes were detected, most of them were susceptible to major beta-lactams and were at the margin of susceptibility and intermediate only for cephalothin (n=4) for Serotype Dublin and Typhimurium and ampicillin (n=1), for *S.* Haifa(n=1). On the other hand 3 of *S.* Virchow and one *S.* V:ROUGH-O;-:- were completely resistant to Ampicillin and Cephalothin.

Table 5. Host distribution, beta-lactam R-profile and *bla* type based on amino acid sequence

Serotype	**No**	**Host type**	**R-profile**		**bla-Type**
			I	**R**	
Concord	1	Human	Fox	AmpAmcCroCf	*OXA-10* and *blaCTX-M15*
Dublin	2	Cattle	Cf	-	-
Haifa	1	Poultry	Amp	-	-
Heidelberg phagetype 2	1	Swine	Amc	AmpCf	TEM-1
I:6;7,14:-:I,w	1	Cattle	-	AmpAmcCf	TEM-1
Kentucky	2	Cattle		AmpAmcCf	TEM-1
Kentucky	2	Cattle	Amc	AmpCf	TEM-1
Kentucky	2	Cattle		AmpAmcCf	Novel(Gln6Ser)
Kentucky	2	Poultry		AmpAmcCf	TEM-1
Kentucky	1	Human	Fox	AmpAmcCf	TEM-1
Kentucky	1	Human	Amc	AmpCF	TEM-1
Saintpaul	1	Human	Amc	AmpCf	TEM-1
Saintpaul	2	Cattle	Amc	AmpCf	TEM-1
Saintpaul	4	Poultry	AmcCf	Amp	TEM-57
Saintpaul	1	Poultry		AmpAmcCf	TEM-57
Saintpaul	3	Poultry	Cf	AmpAmc	TEM-57
Saintpaul	2	Poultry	Amc	AmpCf	TEM-57
Typhimurium phagetype 1	1	Human	Cf		-
Typ*h*imurium phagetype 3	1	Human		AmpAmcCf	TEM-1
Typhimurium phagetype 3	1	Cattle		AmpAmcCf	Novel(Phe13Leu)
Typhimurium Phagetype 3	1	Human		AmpAmcCf	Novel(Gln6Ser)
Typhimurium phagetype 3	1	Human	Amc	AmpCf	TEM-1
Typhimurium phagetype 4	1	Cattle		AmpAmcCf	TEM-1
Typhimurium phagetype 66	1	Human	Amp		TEM-1
Typhimurium phagetype 74	1	Swine	Cf		-
Typhimurium phagetype 193	1	Human	Amc	AmpCf	TEM1
V:ROUGH-O;-:-	1	Human		AmpCf	-
Virchow	1	Cattle	Amc	AmpCf	TEM-1
Virchow	2	Cattle	Amc	AmpCf	-
Virchow	1	Human		AmpAmcCf	-

-= R-gene not detected

Mechanism of resistance to quinolone antimicrobials

Out of 29 *Salmonella* isolates with reduced sensitivity to quinolones, high level resistance to both nalidixic acid and ciprofloxacin was observed only in 10 *S.* Kentucky isolates (34.5%) (Table 6). Interestingly, over half of the quinolone resistant isolates 17(58.6%) in the current study were also resistant to at least one of the beta-lactam antimicrobials and all *S.* Kentucky isolates with high level nalidixic acid and ciprofloxacin resistance were MDR to several antimicrobials. All of *S.* Kentucky isolates had double mutations in *gyrA* (Ser83Phe + Asp87Gly) and *ParC* (Thr57Ser + Ser80Ile). Single mutation in *gyrA* (Ser83Phe) was observed in 4 isolates (*S.* Livingstone var.14+(2), *S.* Virchow (1), *S.* I:6;7,14:-:I,w(1). All these isolates were resistant to nalidixic acid and intermediately resistant to ciprofloxacin. Single mutation in *gyrA* (Ser83Tyr) was detected in one *S.* Haifa from poultry with R-phenotype of [resistant to nalidixic acid and intermediately resistant to ciprofloxacin]. Overall, double and single mutation in *gyrA* was detected in 15 (51.7%) of isolates. Double mutation in *parC* (Thr57Ser +Tyr83Phe) was detected in one *S.* Miami isolate obtained from swine. This strain was sensitive to naldixic acid and intermediately resistant to ciprofloxacin. The Tyr83-Phe is novel mutation. However, this additional mutation does not seem to contribute to decrease in sensitivity to nalidixic acid, rather this strain was susceptible with zone of inhibition of 22 mm but intermediately resistant to ciprofloxacin. A strain of *S.* Agona with only single mutation at Thr57Ser was intermediately resistant to nalidixic acid but sensitive to ciprofloxacin. Double substitution in *gyrB* gene (Val423Gly+Asp459His) was detected in two isolates; *S.* Mikawasima and *S.* Braenndrerup, the latter having additional substitution in *parC* gene (Thr57Ser) associated with intermediate susceptibility with both nalidixic acid and ciprofloxacin whereas the former with intermediate susceptibility only to naldixic acid respectively. A strain of serotype V:rough-O;-:- susceptible to nalidixic acid but intermediately resistant to ciprofloxacin had single mutation of Ser463Ala on *gyr*B gene. No mutation was detected in *parE* gene in all isolates examined in the current study. Nine isolates with reduced sensitivity to nalidixic acid and or ciprofloxacin had no mutation in any of the QRDR (Table 6).

Plasmid mediated quinolone resistance genes

None of the tested plasmid mediated quinolone resistance genes were detected in any of the isolates examined in the current study. Seven isolates belonging to serotypes Saintpaul, Typhimurium, Aberdeen,Virchow and Haifa were susceptible to naldixic acid but had shown reduced sensitivity to cirpfloxacin according to CLSI (2013) with zone of inhibition ranging from 25-28mm cut-off points. There appears to be other resistance mechanisms responsible for observed decreased sensitivity

Table 6. Susceptibility of isolates to quinolone drugs and mutation in QRDR

	Serotype	**zone of inhibition mm (susceptibility category)**		**Other R-pattern***	**Mutation in QRDR**		
		Na	Cip		*gyrA*	*gyrB*	*parC*
Cattle	Aberdeen	19[S]	27[I]	**Nitro***KTeSuS*	-	-	-
Cattle	Virchow	20[S]	25[I]	*KTeSuSNitroN*	-	-	-
Cattle	Typhimurium PT 3	21[S]	25[I]	**AmpAmcCf**K*SNitro*	-	-	-
Cattle	Typhimurium PT 4	22[S]	27[I]	**AmpAmcCf**K*Nitro*	-	-	-
Cattle	Haifa	21[S]	25[I]	*KSuSNitro*	-	-	-
poultry	Saintpaul	13[R]	31[S]	**C***AmcCf SuS Nitro*	-	-	-
Cattle	Saintpaul	17[I]	25[I]	**AmpAmcCCf** K**Nitro** *SuS N*	-	-	-
Cattle	Saintpaul	21[S]	27[I]	**Nitro***AnKSuS*	-	-	-
poultry	Saintpaul	20[S]	27[I]	*SuSNitroN*	-	-	-
Human	V:ROUGH-O;-:-	22[S]	28[I]	**AmpCf**KSNitro	-	Ser463Ala	-
Cattle	Mikawasima	20[S]	25[I]	**SuSNitro***AnTe*	-	Val423Gly+ Asp459His	-
Cattle	Agona	17[I]	31[S]	GmK**SxtTmpTeSuSNitro**	-	-	Thr57Ser
Cattle	Braenndrerup	17[I]	25[I]	**KTeSuS***AnNitro*	-	Val423Gly+ Asp459His	Thr57Ser
Swine	Miami	22[S]	27[I]	**Nitro***KTeS*	-	-	Thr57Ser+Tyr83Phe
poultry	Haifa	0[R]	25[I]	**Sxt TmpTeSuNitro***KS*	Ser83Tyr	-	-
Cattle	Virchow	0[R]	12(I)	**Amp**Amc**CfGm**K**TeSuS***Nitro*	Ser83Phe	-	-
Cattle	Livingstone var.14+	0[R]	24(I)	-	Ser83Phe	-	-
Cattle	I:6;7,14:-:I,w	7[R]	30(I)	**AmpAmcCf***TeSuSNitro*	Ser83Phe	-	-
Swine	Livingstone var.14+	0[R]	20[I]	*TeS*	Ser83Phe	-	-
Cattle	Kentucky	0[R]	14[R]	**AmpAmcfTeSuS***Nitro*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
Cattle	Kentucky	0[R]	12[R]	**AmpCfGmTmpTeSuSNitroN** *AmcKSxt*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
Cattle	Kentucky	0[R]	11[R]	**AmpCfGmTeSuSN***AmcKNitr o*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
Cattle	Kentucky	0[R]	9[R]	**AmpAmcCfGmTeSuS***KNitro*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
Cattle	Kentucky	0[R]	12[R]	**AmpCfGmTeSuS***AmcKNitro*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
Cattle	Kentucky	0[R]	10[R]	**AmpAmcCfGmTeSuS***KNitro*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
Human	Kentucky	0[R]	8[R]	**AmpAmcCfFoxGmTe SuS***K*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
Human	Kentucky	0[R]	10[R]	**AmpCfGmTeSuS***AmcKN*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
poultry	Kentucky	0[R]	9[R]	**AmpAmcCfGmTeSuS**	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile
poultry	Kentucky	0[R]	11[R]	**AmpAmcCfGmTeSuS***C*	Ser83Phe +Asp87Gly	-	Thr57Ser + Ser80Ile

*Resistance status, isolates were fully resistant to antimicrobials written in Bold and intermediately resistant to those written in italics

Discussion

Detection of higher resistance rate to beta-lactam antibmicrobials (50%) and *bla* genes more commonly in isolates from poultry could be due to the fact that drugs like ampicillin and amoxicillin are frequently employed in poultry farms in the country leading to selection pressure. The dominant beta-lactamase enzyme detected in

the current study was variants of *bla*TEM and the majority of them were *bla*TEM-1 which is in accordance with the observed spectrum of resistance to only ampicillin and first generation cephalosporin in most of the isolates. In Africa, *bla*TEM-1 has been reported from *Salmonella* isolated from poultry in Egypt (Ahmed and Shimamoto, 2012), *Salmonella* isolates from children adopted from Mali (Boisrame-Gastrin et al., 2011), and *S.* Enteritidies in Senegal (Sow et al., 2007).

All *S.* Saintpaul isolated from poultry in the current study carried *bla*TEM-57 while those from cattle and human carried *bla*TEM-1. This is probably due to mutation of *bla*TEM gene in a strain of *S.* Saintpaul in one of the poultry farms and clonal spread of strain carrying this mutant gene to farms in the area. All poultry *S.* Saintpaul were isolated from farms in Adaa district. Compared to TEM-1, TEM-57 has substitution of Gly to Asp at position 92 of amino acid sequences which was first reported from *Proteus mirabilis* (Bonnet et al., 1999) and latter on from *E. coli* in China (Yuan et al., 2009). To our knowledge, this is the first report of detection of *bla*TEM-57 in *Salmonella*. The two novel TEM enzymes detected in *S.* Kentucky and *S.* Typhimurium in the current study with mutation at position 6 (Gly to Ser) and 13 (Phe to Leu) were not associated with extended spectrum activity against second and third generation cephalosporins.

One of the human isolates *S.* Concord resistant to ampicillin, cephalothin, cefoxitin and ceftriaxone was shown to produce *bla*CTXM-15 and *bla*OXA-10. Previous studies have also shown *bla*CTXM-15 producing *S.* Concord isolated from children adopted from Ethiopia to different European countries and USA (Fabre et al., 2009; Hendriksen et al., 2010). Another study also showed that *bla*CTXM-15 isolated from *S.* Concord from Ethiopia was chromosomally encoded (Fabre et al., 2009). However, both previous studies have shown production of SHV-12 in most of *S.* Concord from Ethiopia and OXA-10 was not detected. This is presumably due to loss of plasmid encoding for SHV-12 and acquisition of OXA-10 in new isolate from Ethiopia since the previous study was conducted in isolates collected in 2008 or earlier. During the last few years, CTX-M-15 and other related CTX-M enzymes has been widely reported from various *Enterobacteriaceae* including *Salmonella* in different African countries from both hospital and community settings (Fam et al., 2011; Harrois et al., 2014; Kiiru et al., 2012; Rafai et al., 2015; Usha et al., 2008). Oxacilinases including OXA-10 have commonly been isolated from different enterobacteriaceae including *Salmonella* (Hendriksen et al., 2013). Interestingly, no isolate producing SHV variant of enzyme was detected in the current study implying the possible acquisition of SHV-12 enzyme by *S.* Concord in the previous studies after being imported to Europe or USA.

The possible reason for not detecting *bla* genes in a few isolates in the current study despite testing for most of the known *bla* reported in *Salmonella* could be due to poor sensitivity of phenotypic resistance detection methods. In two *S.* Typhimurium and 2 *S.* Dublin intermediately resistant only to cephalothin, one *S.* Haifa intermediately resistant to ampicillin, the reading was at the margin of intermediate and susceptible. However, all the 3 *S.* Virchow were fully resistant to ampicilin and cephalothin and intermediately resistant to amoxicillin + clauvlanic acid. For these isolates, other resistance mechanisms not investigated in this study such as alterations in the beta-lactam targets (PBPs) (Sun et al., 2014), absence or down regulation of the production of outer membrane porins (Delcour, 2009) over expressed efflux pump (Piddock, 2006) and different ampC betalactamases (Jacoby, 2009) for which we have not tested might be responsible for observed reduced susceptibility. In general, the rate of occurrence of extended spectrum beta-lactamases in *Salmonella* isolates in the current study is low. This could be due to the fact that most of the human isolates were obtained from primary health care centers and a use of 2nd and 3rd generation cephalosporin is not a common practice in

veterinary medicine. The single MDR *S.* Concord in the current study was isolated from hospitalized one year old child suggesting the possibility of nosocomial infection.

High level resistance to fluoroquinolones has been associated with substitution of amino acid at codon 83 and 87 of *gyr*A gene whereas resistance to only nalidixic acid is associated with single or double mutation in *par*C gene in Gram-negative organisms (Hsueh et al., 2004; Redgrave et al., 2014). Detection of two amino acid substitutions in the *gyrA* gene at codon 83 and 87 and *ParC* gene at codon 57 and 80 in all *S.* Kentucky isolates with high level resistance to both nalidixic acid and ciprofloxacin obtained from humans and animals suggests the possibility of clonal spread of *S.* Kentucky strain in the human and animal population in the study area. Similar mutations in *gyr*A and *par*C gene were reported from *S.* Kentucky from French travelers returning from northeast and eastern Africa (Weill et al., 2006). Study on *S.* Kentucky ST198 from different countries have also shown similar mutation in *gyr*A at position 83 (Ser83-Phe) for all isolates and substitution of Asp at codon 87 with asparagine, tyrosine or glycine residues. However, only single substitution in *par*C gene at codon 80 (Ser80-Ile) was reported previously and none of them had substitution at codon 57 of *par*C gene (Le Hello et al., 2011). *S.* Kentucky isolates in the current study also belonged to ST198 suggesting the clonal relatedness of our isolates to the internationally spreading clone of *S.* Kentucky (**Paper 3**). Additional substitution at codon 57 of *par*C gene in the Ethiopian isolates might have occurred separately. Contrary to these local and global spread of MDR fluoroquinolone resistant *S.* Kentucky, previous study on *S.* Typhimurium has shown that mutation based fluoroquinolone resistance is associated with fitness cost and resistant strains are less invasive (Fabrega et al., 2009). This suggests that this internationally spreading clone of *S.* Kentucky has unique mechanism probably encoded by plasmids. Furthermore we have previously shown that *S.* Kentucky strains from Ethiopia has strong biofilm forming ability which is one of the important trait for persistence of the organism in the host or the environment (Eguale et al., 2014) contributing to its dissemination.

Amino acid substitutions at similar positions in *Gyr*A gene has been reported in *Salmonella* and other Gram-negative pathogens associated with high level fluoroquinolone resistance (Baucheron et al., 2005; Eaves et al., 2004; Lin et al., 2009; Zurfluh et al., 2014). Four of the *Salmonella* isolates resistant to nalidixic acid and intermediately resistant to ciprofloxacin had only single substitution in *gyr*A, Ser83Phe whereas one isolate *S.* Haifa from poultry had Ser83-Tyr substitution. Previous studies have also shown that a single mutation in *gyr*A results only in complete resistance to nalidixic acid and not ciprofloxacin (Fabrega et al., 2009; Zurfluh et al., 2014). Although isolates with single mutation in *par*C gene resulted only with reduced susceptibility to nalidixic acid, *S.* Miami isolate with no mutation in *gyrA* gene but double substitution in *parC* gene (Thr57-Ser) and novel substitution (Tyr83-Phe) was fully susceptible to nalidixic acid and intermediately susceptible to ciprofloxacin. This suggests that the novel mutation at codon 83 of *parC* gene is in favor of the activity of nalidixic acid and against the activity of ciprofloxacin.

The observation of double substitution in *gyrB* gene (Val423Gly+Asp459His) associated with intermediate susceptibility only to nalidixic acid shows minor contribution of mutation in GyrB compared to gyrA. Interestingly, 9 isolates with reduced sensitivity to ciprofloxacin and some to nalidixic acid had no mutation in QRDR. We have also not detected any PMQR gene in any of the isolates. Other resistance mechanisms not tested in this study such as multidrug efflux pump and altered outer membrane porins might be involved (Redgrave et al., 2014).

Conclusion

Co-occurrence of beta-lactamases with ciprofloxacin resistant determinants in large proportion of isolates is a major threat. Occurrence of MDR *S.* Kentucky with high level fluoroquinolone resistance mediated by double mutations in *gyrA* and *parC* genes in cattle, poultry, human in the study area suggests clonal spread of this strain in the study area and needs strict pathogen control strategy to hamper spread of this pathogen. Further study on extended spectrum beta-lactamase and fluoroquinolone resistance mechanisms in other gram negative pathogens in hospital and community setting is recommended since the majority of the current isolates were from healthy animals at farm level and human patients from primary health care centers.

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Paper 5

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Association of multicellular behaviour and drug resistance in *Salmonella enterica* serovars isolated from animals and humans in Ethiopia

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Keywords
antimicrobials, biofilms, Ethiopia, integron, *Salmonella*.

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2014/0505: received 11 March 2014, revised 21 May 2014 and accepted 12 June 2014

doi:10.1111/jam.12579

Abstract

Aims: To determine the association between multicellular behaviour, integron status and antibiotic resistance among 87 Ethiopian *Salmonella enterica* isolates of animal and human origin.
Methods and Results: Isolates were characterized for their biofilm forming ability, antimicrobial susceptibility and the presence and characteristics of a class 1 integron and *Salmonella* genomic island 1 (SGI1). The majority of isolates grown at environmental temperatures (20°C) exhibited robust biofilm formation (72·4%) and displayed RDAR colony morphology on Congo red agar plates. The presence of a class 1 integron correlated with the extent of drug resistance and ability to exhibit multicellular behaviour.
Conclusions: Although cellulose production and RDAR morphology correlated with increased multicellular behaviour, neither was required for biofilm formation. Contrary to previous reports, colony morphology was generally consistent within a serovar. No integrons were detected in isolates deficient for multicellular behaviour, indicating a potential role of bacterial community formation in transfer of genetic elements among environmental isolates.
Significance and Impact of Study: Infection by *Salm. enterica* is a major public health problem worldwide. The dominance of multidrug resistance and multicellular behaviour in *Salmonella* isolates of Ethiopian origin highlights a need for integrated surveillance and further detailed phenotypic and molecular studies of isolates from this region.

Introduction

Bacterial biofilms are defined as a collection of aggregated microbial sessile communities that are bound to inert or living surfaces and embedded in a self-produced heteropolymeric matrix (Costerton *et al.* 1999). Bacteria exhibiting multicellular behaviour in a biofilm state are often highly resistant to antibacterial drugs, disinfectants and the host immune system (Hoyle and Costerton 1991; Fux *et al.* 2003). Multicellular behaviour in *Salmonella* is characterized by a transition from free, planktonic growth to a sessile, bacterial community surrounded by a dense extracellular matrix composed of macromolecules such as polysaccharides, proteins and nucleic acids (Flemming and Wingender 2010). The major components of the extracellular matrix (ECM) of *Salmonella* biofilms are thin aggregative fimbriae (curli) and cellulose (Romling 2005). Colony morphology on Congo red agar is

indicative of the types of ECM components present in a bacterial community and has been reported to be predictive of biofilm forming capability (Romling *et al.* 2000). Strains capable of multicellular behaviour and biofilm formation frequently form colonies on Congo red plates of a RDAR (red, dry and rough) morphotype associated with the co-expression of curli and cellulose, BDAR (brown dry and rough) associated with the production of curli but not cellulose or PDAR (pink dry and rough) that produce cellulose but not curli. Those strains that cannot produce biofilms are often characterized by a SAW (smooth and white) morphotype expressing none of these ECM components (Romling *et al.* 2000; Romling 2005; Malcova *et al.* 2008). Other exopolysaccharides involved in biofilm formation are capsular polysaccharides (Anriany *et al.* 2001; de Rezende *et al.* 2005) and colanic acid (Solano *et al.* 2002; de Rezende *et al.* 2005). Different serovars and strains of *Salmonella* have been reported to vary in biofilm forming ability, with strong ability (in part mediated by the ECM components) reported to contribute to persistence of the strains on food and food processing surfaces (Vestby *et al.* 2009).

An integron is a genetic unit capable of capturing and incorporating diverse resistance genes into the bacterial genome via site-specific recombination mediated by the *intI1*-encoded integrase (Hall and Stokes 1993). Class 1 integrons play an important role in the global spread of antibiotic resistance by aiding the transfer of cassettes encoding multidrug resistance throughout the Gram-negative-bacterial population (Fluit and Schmitz 1999). Class 1 integrons can be found within *Salmonella* genomic island 1 (SGI1), a 43-kilobasepair (kbp) genomic island of 44 open reading frames, many of which have homologies to genes of unknown function. The antibiotic resistance genes on this island have been localized to a 13-kbp segment of the SGI1 termed the MDR region (Briggs and Fratamico 1999). Exchange of mobile genetic elements, including these integron-associated cassettes, is dramatically increased by close sustained interactions among bacteria such as those observed in biofilm communities (Molin and Tolker-Nielsen 2003). Indeed, previous reports have indicated an association in *Salmonella enterica* between strong biofilm formation and the presence of a complex class I integron found within SGI1 (Malcova *et al.* 2008). Within a biofilm community, the densely packed, matrix-encased bacteria are afforded a generalized structural protection thought to increase antibiotic resistance, as well as an optimal environment for intra- and inter-species genetic exchange of specific antibiotic resistance genes (Molin and Tolker-Nielsen 2003). Additionally, numerous studies have indicated a high level of antibiotic resistance associated with strong biofilm formation in *Salm. enterica* and other organisms (Kim and Wei 2007; Kwon *et al.* 2008; He and Ahn 2011; Baugh *et al.* 2012). Indiscriminate use of antibiotics has been demonstrated not only to drive the formation of bacterial biofilms (Kaplan 2011) but to select for and fix the presence of integrons within a bacterial population (Gillings *et al.* 2008).

Studies conducted in Ethiopia have shown a high prevalence of *Salmonella* in various food animals and humans, with these strains possessing high levels of drug resistance (Molla *et al.* 2006; Beyene *et al.* 2011). A recent report by the WHO warned of increasing development of antimicrobial resistance and commented on the need for increased surveillance of the emergence of antimicrobial resistance in food animals (WHO 2014). Currently, studies investigating the correlation between integron-mediated antibiotic resistance, biofilm forming abilities and multicellular behaviour of *Salmonella* isolates from Ethiopia are lacking. The objective of this study is therefore to characterize biofilm forming ability, expression of multicellular morphotypes and ECM production of selected *Salm. enterica* serovars isolated from food animals and humans in Ethiopia. Additionally, the association of biofilm formation and drug resistance was assessed; particularly class 1 integrons and the SGI1, which contain mobile multidrug resistance genetic determinants.

Materials and methods

Bacterial isolates

A total of 87 epidemiologically unrelated *Salm. enterica* isolates collected in Ethiopia between 2000 and 2008 from swine ($n = 34$), cattle ($n = 20$), camel ($n = 15$), poultry ($n = 7$), sheep ($n = 6$), goats ($n = 1$) and humans ($n = 4$) were used. The isolates represented 20 serovars and were selected based on origin and serovar type. *Salmonella* isolates were previously serotyped at the Public Health Agency of Canada, Office International des Épizooties (OIÉ) Reference Laboratory for Salmonellosis, Guelph, Ontario, Canada. Antigenic formulae (O and H antigens) were determined on the basis of slide agglutination and used to assign *Salmonella* serovar designations to the isolates (Popoff 2001). Bacterial isolates were stored in Luria-Bertani broth supplemented with 20% glycerol at −80°C. Prior to experimentation, isolates were grown on LB agar (37°C, overnight) and a single colony was selected for inoculation of cultures and agar plates. Experiments were conducted at 37°C or 20°C (±1°C) as indicated.

Screening of the isolates for multicellular behaviour

Biofilm forming ability of the isolates was assessed using the crystal violet staining method on polystyrene

microtiter plates (Djordjevic *et al.* 2002; Malcova *et al.* 2008) at 20 and 37°C in tryptic soy broth 20 times diluted (TSB 1 : 20). Bacterial colony morphology was assessed after 6 days at 20°C on Luria-Bertani plates without salt (LBNS) agar supplemented with Congo red (40 μg ml^{-1}) and Coomassie Brilliant Blue dyes (20 μg ml^{-1}) (White *et al.* 2006).

Cellulose production (Table 1) was assessed on LBNS agar containing 20 μg ml^{-1} calcofluor (Fluorescent Brightener 28, Sigma-Aldrich, St. Louis, MO). After 6 days of growth at 20°C, the plates were visualized under UV light at 366 nm (White *et al.* 2006). In order to quantify the level of cellulose production by different strains, a modified broth calcofluor-binding assay was used. Briefly, overnight cultures were normalized by optical density and diluted 1 : 100 in LBNS liquid medium containing calcofluor (20 μg ml^{-1}) in black 96-well microtiter plates (Corning Costar®, Cambridge, MA) and incubated at 20°C for 6 days. The optical density (600 nm) and fluorescence (excitation = 366 nm, emission = 565 nm) of bacteria in each well were then measured using a Spectra Max M2 Plate reader. Wild-type *Salmonella* serotype Typhimurium (JSG210 = ATCC 14028s) and *ΔbcsA*::kan (cellulose mutant, JSG1748, (Prouty and Gunn 2003)) strains were used as positive and negative controls, respectively. All fluorescence readings were normalized to optical density and compared to controls. Readings less than that of JSG1748 were considered negative for the production of cellulose. The ability of liquid cultures to form a pellicle at the air–liquid interface was assessed in glass test tubes at 20°C following 6 days of growth in a static standing position (Solano *et al.* 2002).

Antimicrobial susceptibility screening

Susceptibility of the isolates to a panel of 18 antimicrobials was determined using the Kirby-Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute 2007). The following antimicrobials (Sensi-Discs, Becton, Dickinson and Company, Sparks, MD) and disc potencies (μg) were used: amikacin (30), amoxicillin + clavulanic acid (30/10), ampicillin (10), cefoxitin (30), ceftriaxone (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), nitrofurantoin (100), streptomycin (10), sulfisoxazole (1000), sulfamethoxazole+ trimethoprim (23·75/1·25), trimethoprim (5) and tetracycline (30). The interpretation of the categories of susceptible, intermediate or resistant was based on the CLSI guidelines (Clinical and Laboratory Standards Institute 2007, 2013) and for the purpose of analysis, all readings classified as intermediate were considered as resistant unless otherwise noted. *Escherichia coli* ATCC 25922 was used as a quality control.

Detection of class 1 integrons and *Salmonella* genomic island 1 (SGI1)

Identification of class 1 integrons and drug resistance cassettes was conducted via colony PCR employing primers directed at *intI1* and conserved flanking segments (CS) as previously described (Leverstein-Van Hall *et al.* 2002) (Levesque *et al.* 1995). PCR products were visualized in agarose gels, and those isolates determined to be *IntI1* positive and 5′-CS and 3′-CS PCR negative were gel purified and submitted for sequencing at The Ohio State University Plant Microbe Genomics Facility. All integron-positive *Salmonella* isolates (n = 29) were analysed for the presence of the SGI1 left and right junction and subsequently tested to determine the order of antimicrobial resistance genes as described elsewhere (Levesque *et al.* 1995; Leverstein-Van Hall *et al.* 2002; Doublet *et al.* 2008). PCR product sizes and sequences were analysed using BLAST and GenBank (accession number AF261825).

Conjugation assays

To determine whether the integrons of the *Salmonella* isolates were on conjugative DNA elements, all class 1 integron-positive isolates were conjugated with rifampicin-resistant (Rif^R) and sulfamethoxazole-susceptible (Sul^S) *E. coli* (MG1655). Both donor (*intI1*-positive sulfamethoxazole-resistant *Salmonella* isolates) and recipient bacteria were cultured in LB broth (OD_{600} = 0·6). The mating of donor (sulfamethoxazole-resistant isolates) and recipient was conducted by mixing in a 1 : 9 ratio (v/v) in 2 ml of LB broth followed by incubation overnight in a water bath at 37°C followed by transconjugant selection on MacConkey agar containing rifampicin (50 μg ml^{-1}) and sulfamethoxazole (512 μg ml^{-1}). Transconjugant (Rif^R/Sul^R) colonies were patched and further tested for their antimicrobial susceptibility patterns and the presence of class 1 integrons (Vo *et al.* 2010).

Data analysis

To compare biofilm formation and cellulose production of isolates with different morphotypes and of different serovars, the data from grouped isolates were pooled and compared by one-way analysis of variance (ANOVA). Association of drug resistance determinants and biofilm formation/multicellular behaviour was examined using the

chi-square test. The difference between the means was considered significant at $P < 0{\cdot}05$.

Results

Morphotypes, biofilm formation, cellulose production and pellicle formation

The morphologies exhibited by the 87 *Salmonella* isolates grown on Congo red agar (6 days, 20°C) were RDAR ($n = 63$, 72·4%), BSAM ($n = 19$, 21·8%), SAW ($n = 3$, 3·5%) and BDAR ($n = 2$, 2·3%). In spite of their differing host origins, most of the isolates belonging to a given serovar produced a similar morphotype. The exceptions were serovars Anatum and Blockley, which demonstrated two different morphotypes. All isolates, regardless of serovar, expressed the SAW morphotype when grown at 37°C. Representative images of the observed colony morphotypes for the isolates are shown in Fig. 1.

A statistically significant difference in the level of biofilm formation was detected among morphotypes at 20°C ($P = 0{\cdot}0002$). Isolates that expressed the RDAR morphotype at 20°C produced more biofilm as compared to those that expressed other morphotypes (Fig. 2). At 37°C, most isolates formed robust biofilms although they all displayed the SAW morphotype (no cellulose or curli) on Congo red agar. However, biofilm formation of most of the isolates was higher at 20°C than at 37°C (Table 2). Pellicle formation at the air–liquid interface, thought to be a biofilm indicator, was also examined for all isolates. The majority of isolates ($n = 84$, 96·6%) produced a pellicle at 20°C except for two isolates of serovar Dublin and one Hadar. All of the isolates that produced RDAR morphotypes produced thick and stable pellicles that were not dispersed by agitation, while pellicles produced by isolates with BSAM and BDAR morphotypes were fragile and easily dispersed when agitated. A cellulose-deficient control strain of *Salm.* Typhimurium (JSG1748, Δ*bcsA*::kan) formed a fragile pellicle in contrast to a curli mutant of *Salm.* Typhimurium (JSG3132, Δ*csgG*) which produced no pellicle at all.

In an agar-based calcofluor-binding assay at 20°C, all isolates that produced the RDAR morphotype showed calcofluor binding, while all isolates that produced other morphotypes did not demonstrate appreciable levels of calcofluor binding. A broth culture calcofluor-binding assay was developed to better quantify presumed cellulose production. There was a strong correlation between qualitative and quantitative assessment methods of cellulose production at 20°C (Table 1). Isolates that exhibited the RDAR morphotype produced significantly higher levels of cellulose compared to others ($P < 0{\cdot}0001$) at 20°C (Fig. 3). Cellulose production at 37°C was minimal, with no significant difference in calcofluor binding among the field isolates, wild-type JSG210 *Salm.* Typhimurium and the *Salm.* Typhimurium Δ*bcsA*::kan mutant strain (data not shown). All isolates that failed to produce a pellicle ($n = 3$, 3·5%) demonstrated SAW or BSAM morphotypes and showed no cellulose production at 20°C. As described above, within specific serovars, the colony morphotypes were quite uniform, as was pellicle formation at 20°C. A noteworthy exception was one of the 12 serovar Hadar isolates which was the only serovar Hadar isolate that did not produce a pellicle (Table 2). Although cellulose production appeared to be important for biofilm formation in isolates that expressed the RDAR morphotype, the level of biofilm formation was not directly related to that of cellulose production. For example, some of the serovars such as Hadar and I:6·8:- produced no detectable cellulose but formed robust biofilms. Alternatively, some serovars that were the top cellulose producers (such as

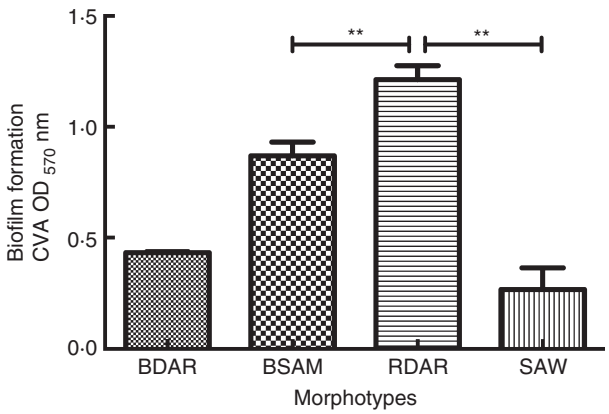


Figure 2 Association of biofilm formation and expression of different morphotypes on Congo red agar (6 days of growth at 20°C, $**P < 0{\cdot}005$). BDAR associations could not be determined due to the low number of isolates in this class.

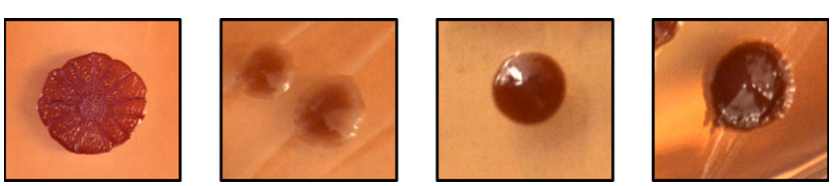
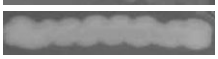


Figure 1 Representative images of *Salmonella* colony morphologies observed following 6 days of growth on LBNS Congo red agar (at 20°C). (a) Red dry and rough (RDAR). (b) Smooth and white (SAW). (c) Brown smooth and mucoid (BSAM). (d) Brown dry and rough (BDAR).

Table 1 Representative qualitative and quantitative measurement of calcofluor binding in solid and liquid medium

	Qualitative*		
Serovar	Visual	Degree of binding	Quantitative†
S. Dublin		–	−97·0
S. Kentucky		+	951·7
S. Newport		++	1576·1
S. Typhimurium		+++	3495·6

*UV fluorescence of bacterial colonies following 6 days incubation at 20°C on LBNS agar supplemented with 20 μg ml^{-1} calcofluor.
†96-well microplate UV fluorescence reading of bacterial cultures following 6 days incubation at 20°C in LBNS broth supplemented with 20 μg ml^{-1} calcofluor. Fluorescence readings (RFU/A_{600}) were adjusted to cellulose-deficient *S.* Typhimurium JSG1748 *ΔbcsA*::kan.

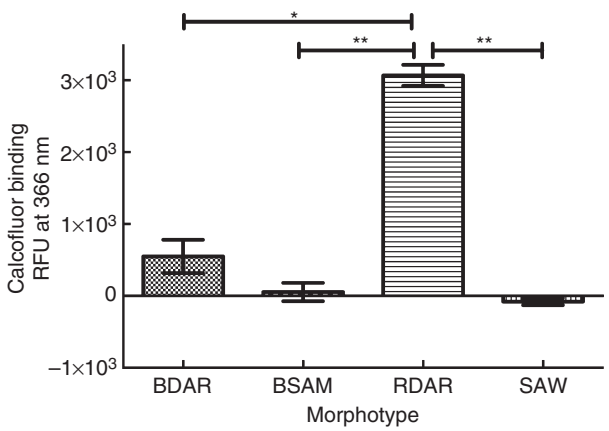


Figure 3 Level of cellulose production by strains expressing different morphotypes. Bacteria were cultured in LBNS medium with 20 μg ml^{-1} of calcofluor for 6 days at 20°C and fluorescence was read (**$P < 0{\cdot}001$, *$P < 0{\cdot}01$).

serovar Enteritidis) were not the best biofilm producers (Table 2).

Antimicrobial resistance phenotypes (R-type)

The majority of isolates (n = 74, 85·1%) exhibited multidrug resistance (resistance to ≥3 tested drugs) to the activity of between 3 and 11 of the 18 antibiotics tested. Only six isolates (7%) were susceptible to all antibiotics tested. Of these susceptible isolates, 3 (50%) failed to exhibit multicellular behaviour, demonstrated a SAW morphotype, showed no or little pellicle formation and exhibited minimal biofilm production. Out of the 87 *Salmonella* isolates, resistance was detected to streptomycin (77%), tetracycline (65·5%), sulfisoxazole (52·9%), neomycin (41·4%), nalidixic acid (41·4%), nitrofurantoin (39·1%), cephalothin (33·3%), ampicillin (31%), sulfamethoxazole and trimethoprim (24·1%), amoxicillin + clavulanic acid (20·7%). Only one isolate (1·2%) was resistant to ceftriaxone while all isolates tested were susceptible to amikacin and cefoxitin. Recent modifications to the CLSI interpretative criteria for *Salmonella* resistance to ciprofloxacin have been implemented to facilitate detection of *Salmonella* with clinically relevant decreases in susceptibility to fluoroquinolones (Humphries *et al.* 2012; Clinical and Laboratory Standards Institute 2013). Using these revised standards, only 25 of the tested isolates (28·7%) were sensitive to ciprofloxacin, while 60 (68·9%) exhibited intermediate resistance or resistance (46 = intermediate, 14 = resistant). Analysis of the occurrence of resistance to selected antimicrobials by strains expressing different morphotypes showed that resistance to ampicillin, cephalothin, and sulfisoxazole, was more common in strains with the RDAR morphotype than the BDAR/BSAM morphotypes. On the other hand, resistance to gentamicin, streptomycin, kanamycin and nitrofurantoin was more common in isolates expressing BDAR/BSAM morphotypes (Table 3).

Class 1 integrons and SGI1

Because of the high level of antimicrobial resistance detected among the *Salmonella* isolates, the presence of a class 1 integron, which often carries resistance determinants, was examined. Integron-bearing isolates were then further tested to determine whether the detected class 1 integron was located within SGI1. A class 1 integron was detected in 29 (33·3%) of the 87 isolates examined, being found in serovars Braenderup (6/6), Kentucky (14/15), Typhimurium (7/11), I: 6,7:e,h:- (1/1) and Newport (1/11). Table 4 shows the size and antimicrobial resistance gene cassette profiles of the integrons identified. *Salmonella* serovar Typhimurium was the only serovar in which two distinct integrons were detected. There was a positive correlation between the presence of a class 1 integron and number of antimicrobial agents to which isolates were resistant ($X^2 = 44{\cdot}2$, $P < 0{\cdot}0001$) (Fig. 4). Eight out of the 29 (27·6%) class 1 integron-positive isolates were able to transfer their antimicrobial resistance genes to *E. coli* by conjugation. Thus, a substantial portion of the integron-positive isolates demonstrated the capacity for horizontal transfer of antibiotic resistance among enteric pathogens. However, all SGI1-positive isolates and two of the *Salm.* Typhimurium isolates bearing a non-SGI1 integron were unable to transfer their integron to *E. coli.* This demonstrates that for most of the

Table 2 Biofilm formation, expression of morphotypes, cellulose production and pellicle formation of different *Salmonella enterica* serovars

Serovar	No	Biofilm formation (Mean crystal violet A_{570} ± SEM)		Cellulose (Mean UV fluorescence ± SEM)	Morphotype		Pellicle formation*	
		20°C	37°C	20°C	20°C	37°C	+/−	Type
Anatum	4	0·85 ± 0·02	0·72 ± 0·04	2127 ± 759·9	RDAR/BSAM(3/1)	SAW	+	Intact/fragile
Blockley	5	0·42 ± 0·02	0·49 ± 0·02	168·5 ± 28·26	BDAR/BSAM(2/3)	SAW	+	Fragile
Bovismorbificans	1	0·68 ± 0	0·51 ± 0	3024 ± 0	RDAR	SAW	+	Intact
Braenderup	6	0·63 ± 0·11	0·47 ± 0·11	2629 ± 183	RDAR	SAW	+	Intact
Dublin	2	0·21 ± 0·14	0·32 ± 0·11	−58·53 ± 38·4	SAW	SAW	−	–
Eastborne	2	0·97 ± 0·07	0·73 ± 0·02	1249 ± 229·4	RDAR	SAW	+	Intact
Enteritidis	4	1·02 ± 0·03	0·53 ± 0·02	4315 ± 45·11	RDAR	SAW	+	Intact
Hadar	12	0·92 ± 0·08	0·71 ± 0·04	−181·5 ± 16·9	BSAM	SAW	11 + (1−)	Fragile
Haifa	2	0·59 ± 0·04	0·47 ± 0·08	3146 ± 93·25	RDAR	SAW	+	Intact
Heidelberg	1	1·73 ± 0	0·52 ± 0	2832 ± 0	RDAR	SAW	+	Intact
Infantis	2	1·46 ± 0·02	0·53 ± 0·14	2937 ± 102·2	RDAR	SAW	+	Intact
Kentucky	15	1·12 ± 0·07	0·55 ± 0·02	2869 ± 148	RDAR	SAW	+	Intact
Newport	11	1·27 ± 0·07	0·59 ± 0·04	2114 ± 152·7	RDAR	SAW	+	Intact
Reading	1	1·08 ± 0	0·31 ± 0	364·7 ± 0	RDAR	SAW	+	Intact
Saintpaul	1	2·36 ± 0	0·60 ± 0	28 730	RDAR	SAW	+	Intact
Typhimurium	11	1·90 ± 0·11	0·48 ± 0·04	3253 ± 188·8	RDAR	SAW	+	Intact
Uganda	1	0·38 ± 0	0·35 ± 0	−51·07 ± 0	SAW	SAW	+	Fragile
I:6,7:e,h:-	1	0·48 ± 0	0·39 ± 0	2609 ± 0	RDAR	SAW	+	Intact
I:6,8:-	3	0·97 ± 0·01	0·78 ± 0·05	−188·7 ± 32·9	BSAM	SAW	+	Fragile
I:9,12:-:-	2	1·36 ± 0·5	0·81 ± 0·1	3474 ± 348·1	RDAR	SAW	+	Intact

*Pellicle formation was determined at 20°C.

Table 3 Association of morphotypes and resistance to selected antimicrobial agents

Antimicrobials	Morphotype*	Total No. of isolates	% Resistant	X^2	*P*-value
Ampicillin	BDAR(2)/BSAM (19)	21	4·7	9·62	0·0019
	RDAR	63	41·3		
Gentamicin	BDAR/BSAM	21	81	26·5	<0·0001
	RDAR	63	20·6		
Streptomycin	BDAR/BSAM	21	100	7·1	0·008
	RDAR	63	73		
Nitrofurantoin	BDAR/BSAM	21	85·7	23·78	<0·0001
	RDAR	63	25·4		
Sulfisoxazole	BDAR/BSAM	21	14·3	18·52	<0·0001
	RDAR	63	68·3		
Cephalothin	BDAR/BSAM	21	4·8	10·97	0·0009
	RDAR	63	44·4		
Kanamycin	BDAR/BSAM	21	23·8	8·8	0·003
	RDAR	63	3·2		
Tetracycline	BDAR/BSAM	21	90·5	6·58	0·01
	RDAR	63	60·3		

*Morphotypes observed: RDAR (n = 63), BSAM (n = 19), BDAR (n = 2).

isolates, the class 1 integron was located on the chromosome; either incorporated in the SGI1, or found separately, and was not mobilizable.

Of the 29 isolates positive for a class 1 integron, 19 (65·5%) were positive for SGI1 or its variants. Classical SGI1 conferring resistance to ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfisoxazole, tetracycline) is associated with invasive disease (Varma *et al.* 2005). This multidrug resistance pattern was detected in four serovar Typhimurium isolates: Typhimurium DT 104 (n = 2), Typhimurium var. Copenhagen (n = 1) and Typhimurium (n = 1).

Table 4 Class 1 integron profile, SGI1 and its variants

Size of variable region(s) (bp)	Gene cassette(s)	*n*	Type of SGI1	Serovar
1600	*aadA1 + dfrA1*	1	–	I:6,7:e,h:-
1600	*aadA1 + dfrA1*	6	–	*S.* Braenderup
1600	*aac(3)-Id+aadA7*	14	SGI1-K	*S.* Kentucky
750	*dfrVII*	1	–	*S.* Newport
1000 + 1200	*aadA2, blaPSE-1*	2	SGI1	*S.* Typhimurium DT 104
1000 + 1200	*aadA2, blaPSE-1*	1	SGI1	*S.* Typhimurium
1000 + 1200	*aadA2, blaPSE-1*	1	SGI1	*S.* Typhimurium var. Copenhagen
1000	*aadA2*	1	SGI1-C	*S.* Typhimurium DT 104
1600 + 2000	*aadB+catB3, blaOXA-30 + aadA1*	1	–	*S.* Typhimurium (pT 193)
500	*dfrA5*	1	–	*S.* Typhimurium (pT 193)

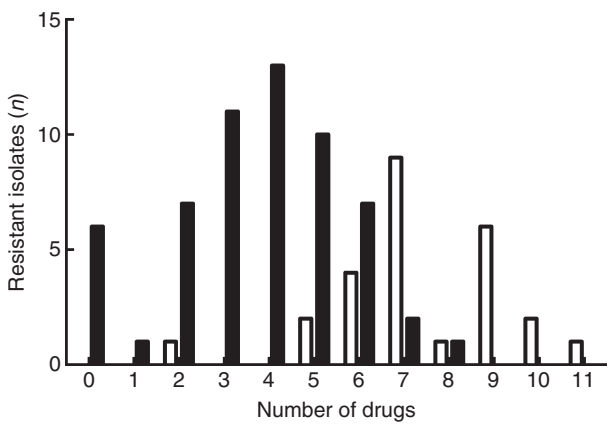


Figure 4 Association between the presence of class 1 integrons and multidrug resistance ($X^2 = 44{\cdot}2$, $P < 0{\cdot}0001$). Black bars represent integron-negative isolates, while white bars represent integron-positive isolates.

All serovar Kentucky isolates ($n = 14$) carried SGI1-K and one Typhimurium isolate carried an incomplete SGI1 (SGI1-C) conferring resistance only to streptomycin and sulfisoxazole (Table 4).

All class 1 integron- and SGI1-positive isolates exhibited a RDAR morphotype when grown on Congo red agar. None of the isolates characterized by a BSAM, SAW or BDAR morphotype were positive for a class 1 integron and SGI1. In order to assess the relationship of a class 1 integron and the SGI1 to biofilm forming capacity of the isolates, we compared mean biomass (as determined by crystal violet assay) of biofilms formed by 11 *Salm.* Typhimurium isolates, which were +/+, +/− and −/− for a class 1 integron and the SGI, respectively. At environmental temperature (20°C), isolates carrying a class 1 integron in SGI1 (+/+) and those with a non-SGI1 integron (+/−) formed significantly more biofilm than isolates carrying no integrons (−/−). There was no difference in biofilm forming capacity between (+/+) and (+/−) isolates. Collectively, the results indicated that isolates carrying a class 1 integron (SGI1 or otherwise) formed approximately 50% more biofilm than those lacking an integron.

Discussion

Salmonella are capable of colonizing a broad range of human and animal hosts and one of the most frequent causes of bacterial foodborne illness (Majowicz *et al.* 2010). As a zoonotic pathogen, the possibility that antimicrobial use in livestock could be driving the development and spread of multidrug resistance is of particular concern to the health of both humans and food animals worldwide. Globally, Ethiopia is among the 10 largest producers of livestock. The industry accounts for 12–19% of the country's annual GDP and the livelihood of over 65% of the country's rural population is directly dependent on livestock (Food and Agriculture Organization of the United Nations 2004; Pagel and Gautier 2012). There is limited restriction on the sale or administration of antibiotics, multidrug combinatorial treatments are common (which can be helpful or harmful, depending upon the usage), and prophylactic, subtherapeutic concentrations of antibiotics are known to be used in some food animal farms (Drug Administration and Control Authority of Ethiopia 2009; Bedada and Zewde 2012). Although formal studies on patterns of veterinary pharmaceutical usage in Ethiopia are few, annual use of veterinary antibiotics is estimated to be over 100 tons, the most common of which are reported to be tetracycline, sulfonamides and streptomycin (Syit 2011; Mekuria *et al.* 2013; Embassy of The Federal Demo-

cratic Republic of Ethiopia 2013). Accordingly, these drugs accounted for the three most commonly identified resistance phenotypes among the 87 isolates examined. Thus, lack of prudent use of antibiotics in both human and veterinary medicine in Ethiopia may have contributed to the observed resistance in these isolates, however further studies to better understand the patterns of antimicrobial use in the country are needed.

Ciprofloxacin is a critical antimicrobial therapy, which is widely used for empirical treatment of Gram-negative infections in humans and has become a mainstay of treatment for salmonellosis over the past decade (Parry *et al.* 2010). Recent reports have highlighted the emergence of *Salmonella* with reduced susceptibility to fluoroquinolones, which is associated with treatment failures and poor outcomes in human infections (Rickert-Hartman and Folster 2014; WHO 2014). In the light of these reports, the high incidence of intermediate resistance and resistance to ciprofloxacin observed among the tested isolates is of particular concern. Due to the concern over acquisition of resistance, fluoroquinolones are recommended for use in Ethiopian livestock animals only as a therapeutic treatment (Drug Administration and Control Authority of Ethiopia 2006). However, it is common practice in the global poultry industry to treat entire flocks through the addition of fluoroquinolones such as Enrofloxacin to water (Nelson *et al.* 2004), and previous studies have found a high prevalence of fluoroquinolone resistance in Ethiopian *Salmonella* isolates (Beyene *et al.* 2011). Therefore, it will be of critical importance to conduct future studies to understand the patterns of microbial resistance and fluoroquinolone usage in Ethiopia.

Formation of RDAR colony morphology on Congo red agar is indicative of the production of biofilm ECM components curli and cellulose (Romling 2005; White *et al.* 2006). In such colonies, it is thought that the ECM forms a highly hydrophobic network with tightly packed cells aligned in parallel within a rigid matrix that enhances biofilm formation on abiotic surfaces (Jain and Chen 2007). In agreement with previous findings, the majority of Ethiopian *Salmonella* isolates (72·4%) expressed the RDAR morphotype at 20°C (Solomon *et al.* 2005). Isolates characterized by the RDAR morphotype produced significantly greater biofilms than isolates exhibiting BDAR, BSAM or SAW morphotypes. Our work has demonstrated that although the majority of isolates produced a RDAR morphology correlating with strong biofilm formation, some non-RDAR serovars were similarly able to form strong biofilms.

Although previous studies on *Enterobacteriaceae* have reported that co-expression of curli and cellulose (evidenced by the RDAR colony morphology) is responsible for formation of a stable biofilm matrix (Zogaj *et al.* 2001; Garcia *et al.* 2004), in this work several isolates of the BSAM morphology exhibiting minimal cellulose production were similarly able to form stable biofilms. Previous studies of *Salm.* Typhimurium (Malcova *et al.* 2008), Enteritidis (Solano *et al.* 2002) and Kentucky (de Rezende *et al.* 2005) have indicated that the major ECM in biofilms of strains characterized by this BSAM morphotype was reported to be an overproduced capsular polysaccharide (de Rezende *et al.* 2005; Malcova *et al.* 2008). Thus, while an ECM (typically including curli and cellulose) is important for biofilm development, the exact ECM constituents that allow efficient biofilms to occur are not specific.

Unlike previous reports, where isolates of the same serovar produced various morphotypes (White *et al.* 2006; Vestby *et al.* 2009; Turki *et al.* 2012), in the current study, we found that colony morphotypes observed on Congo red agar at 20°C were serovar-specific for most of the isolates. For example, despite differences in host origin, all Enteritidis, Kentucky, Newport and Typhimurim exhibited the RDAR morphotype and produced cellulose, while all serovar Hadar isolates formed the BSAM morphotype and did not produce cellulose. Solomon *et al.* also reported that Hadar isolates examined were negative for cellulose production (Solomon *et al.* 2005).

Isolates characterized by a BDAR or BSAM morphotype formed fragile and easily dispersed pellicles, while those exhibiting the RDAR morphotype produced thick and stable pellicles. *Salmonella* serotype Typhimurium lacking either cellulose (JSG1748, Δ*bcsA*::kan), or curli (JSG3132, Δ*csgG)* formed BDAR and PDAR morphotypes on Congo red agar, respectively. The cellulose-deficient strain (JSG1748) formed a fragile pellicle at the liquid–air interface whereas the curli mutant (JSG3132) produced no pellicle at all. This suggests that the tight and relatively strong aggregation of the bacteria in the pellicle produced by RDAR isolates and laboratory reference strains is associated primarily with the expression of curli but that formation of a wild-type pellicle requires co-expression of both curli and cellulose.

Although cellulose has been widely reported to be a critical structural component of the biofilm matrix, our study has shown that cellulose is not a basic requirement for biofilm formation at either 20 or 37°C, but it is important for expression of the RDAR morphotype and formation of an intact pellicle. Furthermore, we have demonstrated a strong correlation between results obtained by measuring the fluorescence of calcofluor binding in liquid medium with the established qualitative calcofluor-binding technique on agar plates. Thus, the liquid assay described here provides a new, objective method to quantify the level of cellulose production by bacteria grown in broth culture.

In the present study, class 1 integrons were detected in over 30% of tested isolates of which a further 27% demonstrated the potential for horizontal transfer to *E. coli*. Of note is the finding that class 1 integrons were found exclusively in serovars that expressed the RDAR morphotype and formed a stable and intact pellicle. Multicellular behaviour was strongly associated with the presence of a class 1 integron which may indicate a synergistic relationship in which biofilm formation facilitates the transfer of class 1 integrons, which then function to increase antimicrobial resistance. Based on the positive association of biofilm forming ability, the presence of class 1 integrons and SGI1, and antibiotic resistance, we speculate that the imprudent use of antibiotics in Ethiopia (in humans and animals) may be selecting for both biofilm formation and antibiotic resistant strains. The close association of bacteria in the biofilm is known to aid DNA transfer, facilitating the spread of antibiotic determinants such as the class 1 integron. On the other hand, acquisition of class 1 integrons and/or the SGI1 may provide encoded factors that affect biofilm formation in a manner unrelated to antibiotic resistance, as Golding *et al.*(Golding *et al.* 2007) reported that the acquisition of SGI1 led to the increased expression of *sthE*, a putative major fimbrial subunit in *Salm.* Typhimurium LT2, which may be associated with biofilm formation. Other reports that have also observed a correlation between multidrug resistance and biofilm production showed that loss or inhibition of one or more multidrug resistance efflux pumps in *Salm.* Typhimurium resulted in impaired biofilm formation, further demonstrating an association of drug resistance and biofilm formation (Kim and Wei 2007; Kwon *et al.* 2008; Baugh *et al.* 2012).

In summary, *Salm. enterica* serovars from Ethiopia are dominated by multicellular behaviour and multidrug resistance. Both traits likely enable the bacteria to withstand environmental insults and may advance the dissemination of these isolates in this endemic region. Although it is widely known that overuse of antibiotics can select for increasingly resistant strains, the implication that increased antimicrobial resistance may be intimately linked to more robust biofilm formation warrants further consideration. If antibiotic resistant serovars also tend to be robust biofilm formers, this may mean that indiscriminate antimicrobial use is selecting not only for more resistant strains but more persistent ones as well. This demands special emphasis on prudent use of antimicrobials in both the veterinary and human health sectors in this country and suggests the need for integrated and sustainable surveillance on multicellular behaviour and drug resistance in *Salm. enterica* of animal and human origin in the region. Further detailed studies on the multicellular behaviour of *Salm. enterica* and association of biofilm formation and multidrug resistance, concurrent with the development of new antimicrobials and methods to counteract *Salmonella* biofilm formation, are worthy areas of future study.

Acknowledgements

This research was financially supported by The National Institutes of Health (NIH) Fogarty International Center (grant 043TW008650) to W.A.G./J.S.G. and a NIH (grant AI066208) to J.S.G.

Conflict of interest

No conflict of interest exists.

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Paper 6

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Inhibition of *Salmonella enterica* Biofilm Formation Using Small-Molecule Adenosine Mimetics

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Biofilms have been widely implicated in chronic infections and environmental persistence of *Salmonella enterica*, facilitating enhanced colonization of surfaces and increasing the ability of the bacteria to be transmitted to new hosts. *Salmonella enterica* serovar Typhi biofilm formation on gallstones from humans and mice enhances gallbladder colonization and bacterial shedding, while *Salmonella enterica* serovar Typhimurium biofilms facilitate long-term persistence in a number of environments important to food, medical, and farming industries. *Salmonella* regulates expression of many virulence- and biofilm-related processes using kinase-driven pathways. Kinases play pivotal roles in phosphorylation and energy transfer in cellular processes and possess an ATP-binding pocket required for their functions. Many other cellular proteins also require ATP for their activity. Here we test the hypothesis that pharmacological interference with ATP-requiring enzymes utilizing adenosine mimetic compounds would decrease or inhibit bacterial biofilm formation. Through the screening of a 3,000-member ATP mimetic library, we identified a single compound (compound 7955004) capable of significantly reducing biofilm formation by *S.* Typhimurium and *S.* Typhi. The compound was not bactericidal or bacteriostatic toward *S.* Typhimurium or cytotoxic to mammalian cells. An ATP-Sepharose affinity matrix technique was used to discover potential protein-binding targets of the compound and identified GroEL and DeoD. Compound 7955004 was screened against other known biofilm-forming bacterial species and was found to potently inhibit biofilms of *Acinetobacter baumannii* as well. The identification of a lead compound with biofilm-inhibiting capabilities toward *Salmonella* provides a potential new avenue of therapeutic intervention against *Salmonella* biofilm formation, with applicability to biofilms of other bacterial pathogens.

Salmonella enterica serovar Typhi and *Salmonella enterica* serovar Typhimurium are among the most commonly encountered foodborne and waterborne pathogens worldwide. Although their respective disease states differ, both organisms are capable of forming bacterial biofilms in mammalian and/or environmental niches. Biofilms are aggregated mixtures of sessile bacteria that are implicated in many chronic infections and are known to facilitate bacterial persistence by increasing antimicrobial resistance and interfering with the host immune response (1, 2). Biofilms are encased within a mixture of secreted and cell wall-associated polysaccharides, glycoproteins, and glycolipids, as well as extracellular DNA, known collectively as extracellular polymeric substances (EPS) (1, 3). This process of surface adherence, self-aggregation, and matrix envelopment serves a critical step in the continued cycle of infection for both *S.* Typhi and *S.* Typhimurium, by increasing the duration of bacterial persistence and shedding, providing resistance to host and environmental stressors, and ultimately enhancing the likelihood of colonizing a new host.

S. Typhi is a human-specific pathogen and the primary etiological agent of typhoid fever (4). Although different levels of availability and efficacy of diagnostic tests in regions in which the disease is endemic can complicate estimations of disease incidence (5), current data indicate that there are over 20 million new cases of typhoid fever each year, resulting in over 200,000 deaths worldwide (6). Following ingestion of viable bacteria, acute infection with *S.* Typhi is initiated by bacterial translocation across the intestinal epithelium and subsequent uptake by local phagocytic cells, permitting systemic dissemination associated with clinical manifestations of acute disease (7). Mortality rates range from 10 to 15% without treatment, due to fatal complications from dissemination to the lung, liver, spleen, and central nervous system (8), compared to <1% with appropriate treatment (9). Regardless of treatment, 3 to 5% of patients infected with *S.* Typhi recover from the stage of acute disease without fully eliminating the bacteria; instead, they become asymptomatic chronic carriers (10). Chronic carriage is characterized by colonization of the human gallbladder, a unique niche for microbial infection (11), from which bacteria can spread by means of fecal shedding (12). We previously described a mechanism of chronic carriage involving bacterial biofilm formation on cholesterol gallstones present in the gallbladder (13). To date, cholecystectomy in tandem with antibiotic treatment is the primary effective treatment option for chronic infection (14); however, such invasive treatment is poorly suited to the patient care settings in many regions in which typhoid is endemic.

Surface adherence, bacterial aggregation, and biofilm formation are similarly important in the persistence and dissemination of gastroenteritis-causing nontyphoidal *Salmonella* serovars. Biofilm aggregations of *S.* Typhimurium are involved in persistent

Received 23 May 2014 **Returned for modification** 27 June 2014
Accepted 6 October 2014

Accepted manuscript posted online 13 October 2014

Citation Koopman JA, Marshall JM, Bhatiya A, Eguale T, Kwiek JJ, Gunn JS. 2015. Inhibition of *Salmonella enterica* biofilm formation using small-molecule adenosine mimetics. Antimicrob Agents Chemother 59:76–84. doi:10.1128/AAC.03407-14.

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doi:10.1128/AAC.03407-14

colonization of swine and poultry as well as cross-contamination in food preparation and farm environments (15, 16). In spite of efforts to improve detection and prevention, the incidence of food-borne salmonellosis in the United States has changed little in recent years, remaining a major economic and public health burden. In light of the importance of biofilm formation in continued disease spread, the identification of novel compounds that interfere with this process would greatly increase our ability to combat biofilm-mediated infections.

Bacterial kinases participate in the regulation of cellular processes known to be important for biofilm formation, such as quorum sensing (17, 18) and production of extracellular matrix components (19, 20). In recent years, kinases have been assigned a high priority as candidate drug targets due to their endogenous small-molecule-binding motifs and their importance in cellular functions (21). Due to the reliance of several biofilm-associated processes on bacterial kinases and other ATP-requiring proteins, we sought to pursue drug development through interference with ATP-dependent pathways using small-molecule ATP mimetic compounds. Screening of 3,000 putative ATP mimetic compounds for potential biofilm-inhibiting effects resulted in the identification of a promising lead compound (compound 7955004), which was able to inhibit biofilm formation by both *Salmonella* and *Acinetobacter* species in a manner that did not alter bacterial growth or viability. Subsequent testing of compound 7955004 revealed no evidence of mammalian cellular cytotoxicity, even at high concentrations (6-fold greater than the 50% effective concentration [EC_{50}]). Taken together, these results indicate that compound 7955004 is a promising candidate drug for further optimization as a potential antibiofilm therapeutic agent.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemical inhibitors. The *Salmonella* strains used in this study were wild-type *S.* Typhimurium 14028s (strain JSG210) and *S.* Typhi TY2 (strain JSG624). Bacteria were stored at −80°C in Luria-Bertani (LB) broth containing 20% glycerol. Overnight cultures were grown in LB broth, tryptic soy broth (TSB), or superoptimal broth (SOB) at 37°C, with aeration. Growth for a 24-h biofilm assay involved normalizing cultures to an optical density at 600 nm (OD_{600}) of 0.8, diluting the cultures 1:100 in TSB diluted 1:20 or in undiluted SOB for *S.* Typhi, and growing the cells at 30°C under static conditions. *Acinetobacter baumannii* 19606 (strain WLS2401), *Staphylococcus aureus* (strain USA300), and *Pseudomonas aeruginosa* (strain PAO1) 24-h biofilm assays were carried out in a similar manner using TSB.

Compound 7955004 [3-(2-furylmethyl)-2-[[(5-hydroxy-1*H*-pyrazol-3-yl)methyl]thio]-3,5,6,7-tetrahydro-4*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidin-4-on] was purchased from ChemBridge (San Diego, CA). Compounds were prepared at 5 mM concentrations in dimethyl sulfoxide (DMSO) and were stored in the dark at 4°C. The original high-throughput screen was conducted using 5 μM drug concentrations and a Biomek 2000 robot, with dilutions of the drug being made in 1× phosphate-buffered saline (PBS). Controls were treated with 0.1% DMSO (final concentration) and PBS alone. Polystyrene 96-well plates were made by Corning Inc. (Corning, NY). Each plate contained 80 drugs, leaving 16 wells for controls. Cultures and drugs were added simultaneously, and the Biomek 2000 system carried out the assay in a manner similar to that for the rapid attachment biofilm assay described below.

Rapid attachment biofilm assay, EC_{50} determination, and biofilm dispersion assay. To determine biofilm inhibition by the compounds, overnight cultures of the desired bacterial strains were grown in TSB at 37°C on a roller drum or shaker. Cultures were normalized to an OD_{600} of 0.8, and 1:100 dilutions of the normalized cultures in TSB (diluted 1:20) were incubated at 30°C under static conditions, to promote biofilm formation. The OD_{600} was read at 24 h, to observe the growth of planktonic bacteria. Plates were washed in double-distilled water and heat-fixed at 60°C for 1 h. Biofilms were stained for visualization with 33% gentian violet solution (10 ml gentian violet, 18 ml 1× PBS, 1 ml methanol, and 1 ml isopropanol) for 5 min, followed by release of the stain with 33% acetic acid (33 ml glacial acetic acid and 67 ml 1× PBS). The released dye was measured at OD_{570}. The half-maximal effective concentration (EC_{50}) of each compound was determined by finding the concentration corresponding to 50% of the maximum biofilm inhibition achieved using various concentrations of compound 7955004 (0.125 μM to 50 μM). Biofilm dispersion assays were carried out in a similar manner, except that 30 μM compound 7955004 was added after a 24-h biofilm was established and the cells were incubated at 30°C for another 24 h, under static conditions. Biofilms were quantified by gentian violet staining and dye release.

Determination of bacterial viability following drug exposure. In order to establish the potential bactericidal or bacteriostatic effects of compound 7955004, cultures were grown in the presence or absence of the drug and plated for viability. Overnight cultures were grown as described above, back-diluted 1:100, and incubated in TSB for 24 h at 37°C, with aeration, in the presence or absence of 30 μM compound 7955004. Following 0 h, 2 h, 4 h, 8 h, 16 h, and 24 h of incubation, 10-μl aliquots were removed, serially diluted, and plated for viability.

Eukaryotic cellular cytotoxicity indicated by lactate dehydrogenase release. Effects on eukaryotic cells were quantitatively assessed by 12-h and 24-h lactate dehydrogenase (LDH) release experiments using an LDH cytotoxicity detection kit (Clontech, Mountain View, CA). The HepG2 hepatocyte cell line was grown in modified essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine and was incubated in a humidified 5% CO_2 incubator at 37°C. Cells were seeded at 1×10^4 cells ml^{-1} in a polystyrene 96-well tissue culture plate and were allowed to adhere overnight. Adherent HepG2 cells were subjected to 12 or 24 h of drug exposure (at 30 μM or 50 μM) in triplicate. Positive and negative controls consisted of equal volumes of 0.1% DMSO (final concentration) and 2% Triton X-100, respectively. LDH release was measured by a colorimetric assay at 490 nm.

ATP-binding proteins captured by ATP-Sepharose affinity matrix and visualized by silver staining. In order to determine potential ATP-binding protein targets of compound 7955004, bacterial lysates were prepared from wild-type *S.* Typhimurium cultures grown under biofilm-inducing conditions (3 ml of TSB diluted 1:20, with growth at 30°C with aeration). Cells were centrifuged, resuspended in 1 ml of lysis buffer (50 mM HEPES [pH 7.4], 120 mM NaCl, 20 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.1% Triton X-100, and 1× complete protease inhibitors), and prepared with a FastPrep system and bacterial lysis column B, containing 0.1-mm silica spheres, for extraction of DNA, RNA, and proteins. The lysate was run through ATP-Sepharose resin (22, 23) (graciously provided by Timothy A. J. Haystead, Duke University) and allowed to bind for 1 h. Low-salt (120 mM, 2 times), high-salt (300 mM, 2 times), and low-salt (120 mM, 2 times) rinses were used to wash off nonspecifically binding proteins. Washes were optimized such that no proteins were present in the eluent when analyzed on silver-stained gels. The column was subsequently washed with a solution of 500 μM compound 7955004 to compete protein targets off the column, followed by a wash with 10 μM ATP to clear the column of remaining ATP-bound proteins. Proteins were electrophoresed on a 10% SDS-PAGE gel and stained with silver nitrate (24) to determine the optimal number of wash steps and to identify protein bands of interest. Column elution fractions following the wash with compound 7955004 were subsequently electrophoresed (10% SDS-PAGE) and stained with Sypro Red dye. These protein bands were excised and submitted to the Ohio State University Mass Spectrometry and Proteomics Facility. Identified peptides were analyzed using Mascot Deamon v2.2.1 (Boston, MA) and compared with the NIH/NCBI database.

Data analysis. All experiments were conducted in triplicate, and data represent a minimum of 3 biological replicates. All data analysis, statistical

significance testing, and EC_{50} calculations were performed using GraphPad Prism 6 software. Results from treated and control samples were analyzed for significance using Student's *t* tests or one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison posttest. The EC_{50} values, defined here as half-maximal observed inhibitory concentrations, were calculated by plotting the percent biomass in treated wells relative to that in untreated control wells. Data were plotted as a function of the $\log_{10}$ compound concentration, and standard sigmoidal dose-response curves were fit to the data; 95% confidence intervals (CIs) are reported to indicate the error of each EC_{50} determination. *P* values of <0.05 were considered significant.

RESULTS

A candidate adenosine mimetic compound is identified in a high-throughput assay of *S.* Typhimurium biofilm inhibition. A high-throughput screening assay was developed to identify compounds that would inhibit biofilm formation by wild-type *S.* Typhimurium (strain JSG210). A library of 3,000 putative kinase inhibitors was assembled by ChemBridge Corporation from a 450,000-compound library based on the predicted probability of binding to the ATP-binding region of proteins and allosteric sites of kinase targets. ChemBridge selected compounds to include in the library based on the statistical significance of chemical similarity to ATP, on the basis of Tanimoto scores (25). Low-energy conformations of 5′-*O*-methyladenosine were used to query library compounds for structures mimicking the adenosine portions of ATP, without providing energy for cellular processes. Biofilm formation by wild-type *S.* Typhimurium 14028s was quantified using a rapid attachment biofilm assay adapted from previously described methods (26), with an automated Biomek robot platform. Bacteria and drug (5 μM) were added together at the beginning of the experiment. Biofilm formation was quantitated following 24 h of growth, and candidate drugs were selected based on demonstration of ≥30% reduction in the overall biomass, relative to untreated controls. Initial automated screening resulted in identification of 34 compounds exhibiting ≥30% reduction in biofilm formation, 5 compounds exhibiting ≥40% reduction in biofilm formation, and 3 compounds exhibiting ≥50% reduction in biofilm formation. Manual testing of these compounds using the rapid attachment biofilm assay in 3 separate experiments eliminated all except one compound, i.e., compound 7955004 [3-(2-furylmethyl)-2-[[(5-hydroxy-1*H*-pyrazol-3-yl)methyl]thio]-3,5,6,7-tetrahydro-4*H*-cyclopenta[4,5]thieno[2,3-*d*]pyrimidin-4-on] (Fig. 1), which reliably demonstrated ~55% reduction of *S.* Typhimurium biofilm formation, relative to untreated controls, at a concentration of 30 μM.

Compound 7955004 inhibits *S.* Typhimurium biofilm formation in a dose-dependent manner, which is not due to bactericidal or bacteriostatic activity. Biofilms were grown as described above, in the presence of various concentrations of compound 7955004 (0.625 μM to 50 μM). Biofilm formation was inhibited in a dose-dependent manner, reaching a maximum of approximately 55% inhibition at a concentration of 30 μM ($P < 0.0001$) (Fig. 2A), which corresponded to visual observations. Prior to crystal violet quantitation of the biofilms, plates were read at OD_{600} to monitor planktonic growth (Fig. 2B), which showed no significant difference in mean OD_{600} values in the presence versus the absence of compound 7955004 ($P = 0.8241$). We further verified the lack of bactericidal/bacteriostatic activity through bacterial CFU enumeration in the presence or absence of 30 μM drug (Fig. 2C), and no significant differences between the regression curves fit to treated versus untreated control data points were found ($P = 0.3525$). The dose-response curve was used to plot the observed activity of compound 7955004, such that 0% corresponds to the minimal observed biofilm inhibition by compound 7955004 and 100% corresponds to the greatest inhibition, permitting calculation of an EC_{50} of 7.27 μM (95% confidence interval [CI], 5.46 μM to 9.68 μM) (Fig. 2D).

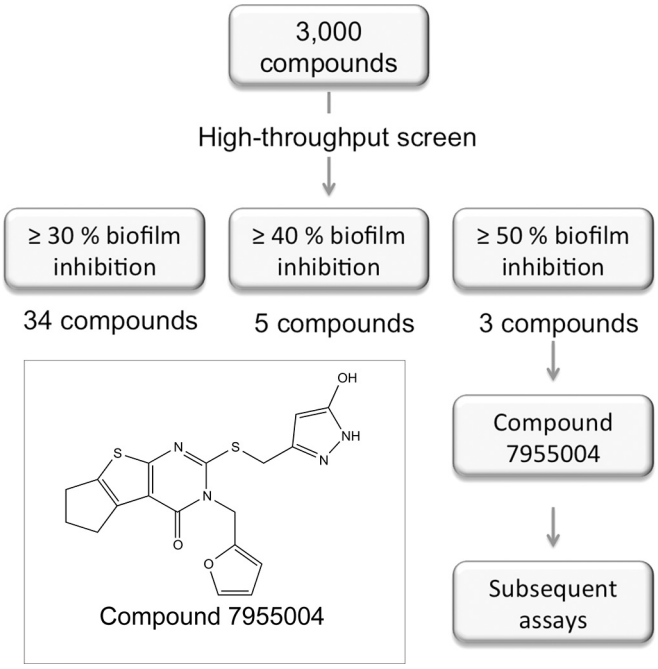

FIG 1 High-throughput screen of the adenosine mimetic library against *S.* Typhimurium biofilm formation. Compounds that inhibited biofilm formation were identified with their putative interacting proteins by using ATP-Sepharose resin and were correlated with their phenotypic effects. The target protein interactome will be analyzed and derivatization of the lead compounds will be carried out in order to optimize inhibitory effects. The initial library was composed of 3,000 putative ATP mimetic compounds. Preliminary high-throughput screening was used to select compounds of interest based on observation of ≥30% biofilm inhibition in the presence of the drugs. Compound 7955004 showed ≥40% biofilm inhibition in our secondary screen and was prioritized as the lead compound of interest. The chemical structure of the adenosine mimetic compound 7955004 is shown.

Compound 7955004 was subsequently tested for its ability to inhibit biofilm formation by *S.* Typhi. Although *S.* Typhi forms robust biofilms *in vivo*, biofilm formation *in vitro* is more variable and progresses more slowly than for *S.* Typhimurium. The rapid attachment assay was adapted to promote *S.* Typhi biofilm formation by growth in SOB for an extended period (60 h), followed by biofilm quantitation and data analysis as described above. The results indicated that biofilm inhibition by *S.* Typhi was more modest than that observed for *S.* Typhimurium, reaching a maximum of 20% reduction in the overall biomass, relative to untreated controls, which was achieved with 50 μM drug ($P < 0.0001$) (data not shown). Compound 7955004 was similarly tested for its ability to disperse preexisting 24-h biofilms. Biofilms of *S.* Typhimurium and *S.* Typhi were grown as described, and 30 μM drug was added at 24 h. After 48 h, biofilms were quantified and compared to a biofilm grown without drug. No significant decrease in biofilm presence was observed ($P = 0.8988$) (data not shown).

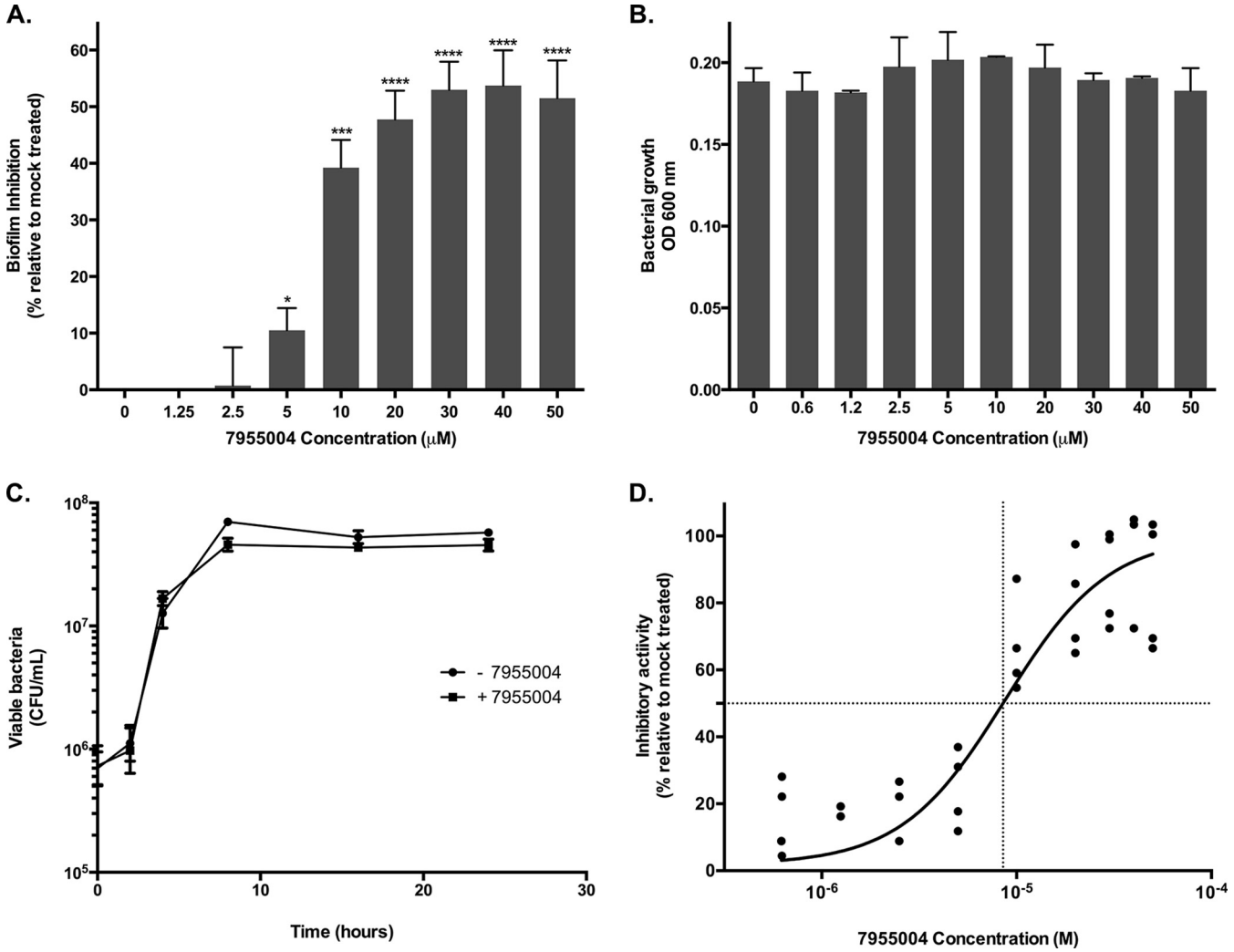

FIG 2 Compound 7955004 inhibits *S.* Typhimurium biofilm formation in a dose-dependent manner, which is not due to bacterial killing. (A) Quantitation of *S.* Typhimurium biofilm formation via crystal violet staining of the bacterial biomass. Biofilm formation was quantified in the presence of various concentrations of compound 7955004, demonstrating a dose-dependent response reaching a maximum of approximately 55% inhibition at a concentration of 30 μM, compared to biofilms formed in the presence of solvent alone. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. (B) Densitometric quantitation of 24-h bacterial growth in the presence of various concentrations of compound 7955004, demonstrating no significant difference among mean OD_{600} values ($P = 0.824$). (C) Bacterial viability determined in the presence or absence of 30 μM compound 7955004 at various time points (0, 2, 4, 8, 16, and 24 h) by serial dilution and enumeration to calculate CFU/ml, confirming that growth of planktonic bacteria remains unaffected by the presence of the compound ($P = 0.8241$). (D) EC_{50} (7.27 μM [95% CI, 5.46 μM to 9.68 μM]) calculated by plotting percent biofilm inhibition, relative to wild-type values, as a function of $\log_{10}$ compound concentrations and fitting the data using GraphPad Prism 6.

Compound 7955004 inhibits *Acinetobacter baumannii* biofilm formation in a dose-dependent manner, which is not due to bacterial killing. To determine whether the biofilm-inhibiting effects of compound 7955004 extended beyond *Salmonella* spp., the compound was tested against 3 other biofilm-forming microbial pathogens, i.e., *Staphylococcus aureus* (strain USA300), *Pseudomonas aeruginosa* (strain PAO1), and *Acinetobacter baumannii* (strain 19606). No effect on biofilm formation was observed with *P. aeruginosa*, and only limited effects were detected with *S. aureus* biofilms (data not shown). However, the testing of compound 7955004 against A. *baumannii* resulted in a dose-response curve similar to that observed for *S.* Typhimurium, with the exception that the reduction in the overall biomass, relative to untreated controls, was greater for *A. baumannii* than for *S.* Typhimurium, with up to 80% biofilm inhibition in the presence of drug concentrations of 30 μM to 50 μM (>5 μM; $P < 0.0001$) (Fig. 3A). As with *S.* Typhimurium, no difference in *A. baumannii* bacterial growth (OD_{600}) following 24 h of incubation in the presence of the drug was detected, implying that the inhibition of biofilm formation was not due to bactericidal or bacteriostatic effects ($P = 0.8478$) (Fig. 3B). An EC_{50} of 6.05 μM (95% CI, 3.36 μM to 10.86 μM) was determined as described above.

Compound 7955004 does not exhibit host cell toxicity. ATP-binding proteins are common in eukaryotic cells, which raises concerns about potential off-target effects when ATP mimetics are used to treat human disease. To determine the potential cytotoxic effects of compound 7955004, lactate dehydrogenase (LDH) assays were performed with HepG2 cells that had been grown in the

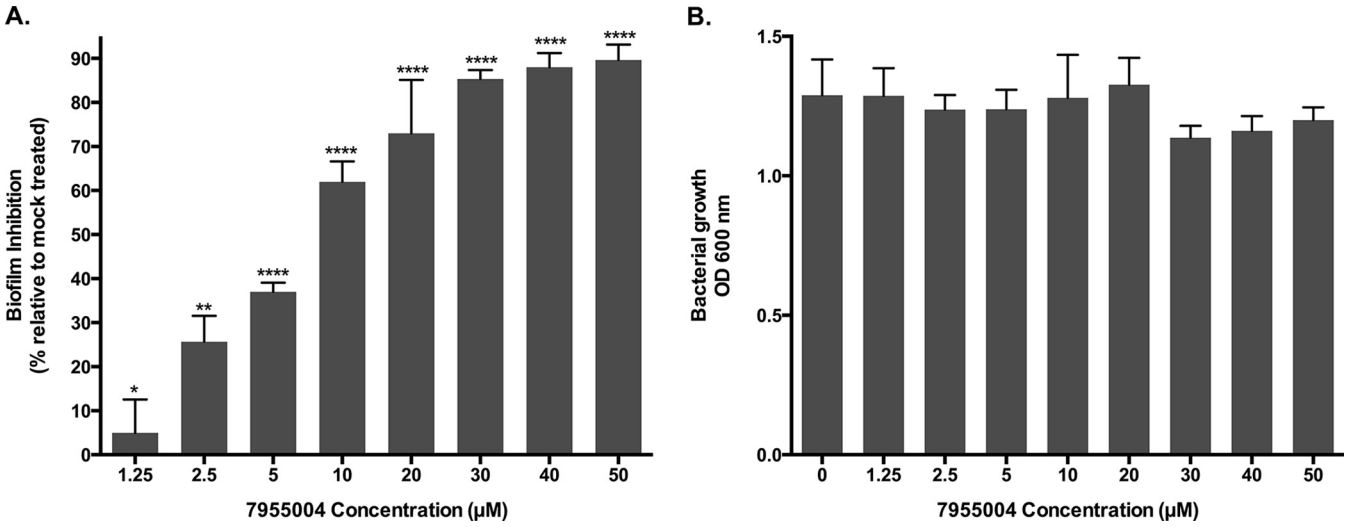

FIG 3 Compound 7955004 inhibits *A. baumannii* biofilm formation in a dose-dependent manner, which is not due to bacterial killing. (A) Quantitation of *A. baumannii* biofilm formation via crystal violet staining of bacterial biomass. Biofilm formation was quantified in the presence of various concentrations of compound 7955004, demonstrating a dose-dependent response reaching a maximum of approximately 80% inhibition at a concentration of 30 μM, compared to biofilms formed in the presence of solvent alone. *, $P < 0.05$; **, $P < 0.01$; ****, $P \leq 0.0001$. The EC_{50} is 6.05 μM (95% CI, 3.36 μM to 10.86 μM). (B) Evidence that planktonic growth is unaffected by the compound at 24 h (OD_{600}) ($P = 0.8478$).

presence of 50 μM, 30 μM, or 0 μM compound 7955004 or Triton X-100 (positive control) for 12 h or 24 h. Following treatment, LDH release was quantified, revealing that no significant difference in mean LDH release was detected for HepG2 cells treated with various concentrations of compound 7955004 versus untreated controls ($P > 0.55$), although all were significantly different from the Triton X-100-treated cells ($P < 0.001$), indicating no acute cellular cytotoxicity at the tested concentrations (Fig. 4A and B). This result was confirmed with a trypan blue exclusion assay (Fig. 4C). Thus, compound 7955004 showed no evidence of acute toxicity for human hepatocytes at a concentration of 50 μM, which was over 6-fold greater than the established EC_{50}.

Putative 7955004 protein targets are identified through ATP-Sepharose affinity chromatography. To determine potential interacting partners for compound 7955004 in the bacterial proteome, wild-type *S.* Typhimurium was grown overnight and bacterial lysates were prepared and run through an ATP-Sepharose displacement affinity matrix (Fig. 5A). In this assay, ATP-binding proteins are immobilized on matrix beads and retained on the column until they are displaced by a binding partner with greater or similar affinity or through allosteric interactions (22). Following exhaustive washes with salt buffer to remove nonspecifically bound proteins, compound 7955004 was added to the column and eluate fractions were collected and electrophoresed on an SDS-PAGE gel. ATP was added after these fractions were collected, to release all remaining bound proteins. After 3 biological replicates of this procedure, two bands (with molecular masses of 60 kDa and 25 kDa) were consistently eluted by com-

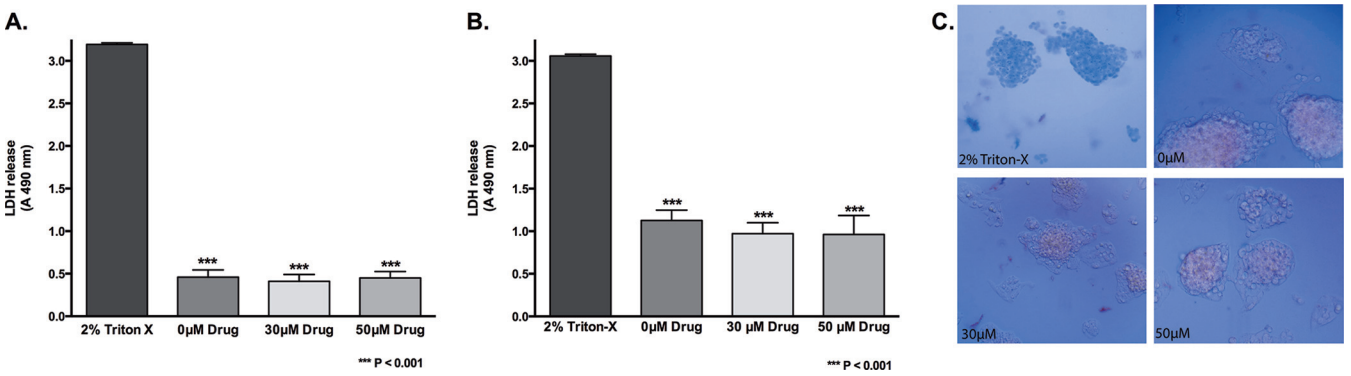

FIG 4 Eukaryotic Hep-G2 cells do not exhibit evidence of acute cytotoxicity in the presence of compound 7955004. (A and B) Twelve-hour (A) and 24-h (B) lactate dehydrogenase (LDH) release assays were performed to evaluate the potential cytotoxicity of various concentrations of compound 7955004 on HepG2 hepatocytes. LDH release was compared among adherent HepG2 cells exposed to 2% Triton X-100 (complete lysis), tissue culture medium alone, or 30 μM or 50 μM drug. Colorimetric quantitation of LDH release into the culture medium was conducted, demonstrating that even high concentrations of compound 7955004, at the upper boundary of the experimental dosage, do not exhibit LDH release above levels detected in untreated cells. ($P < 0.001$ relative to Triton X-100 versus $P = 0.55$ relative to untreated controls). (C) Images of HepG2 cells were obtained with an inverted microscope after 24-h drug treatments as described in panel A. Images demonstrate exclusion of trypan blue from the cytosol of cells treated with 0 μM, 30 μM, or 50 μM drug, in contrast to Triton X-100-treated cells, indicating that the tested concentrations of compound 7955004 do not result in compromise of cellular membrane integrity.

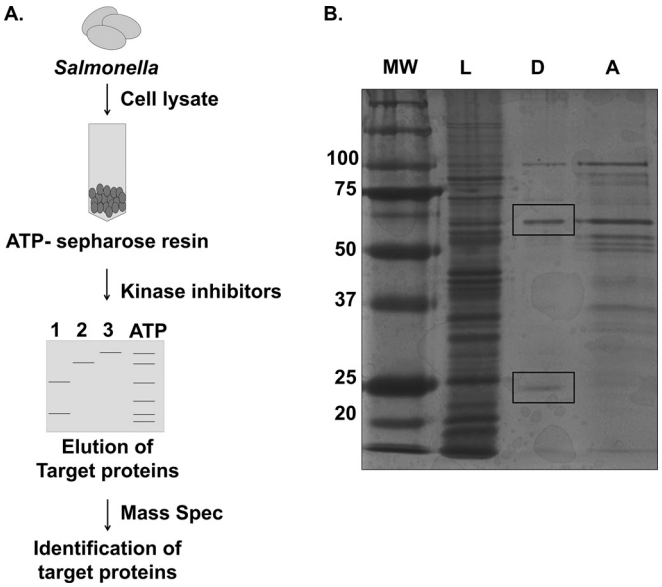

FIG 5 ATP-Sepharose resin was utilized to determine candidate targets of compound 7955004. (A) Lysates from *S.* Typhimurium grown under biofilm-inducing conditions were added to ATP-Sepharose resin and incubated on ice for 1 h to permit the interaction of bacterial ATP-binding proteins with the resin. Columns were then washed extensively to remove nonspecifically bound proteins and incubated with compound 7955004 (500 μM), followed by an ATP wash (10 μM) to compete off proteins bound to the column. (B) Eluted proteins were electrophoresed on a 10% SDS-PAGE gel and visualized via silver staining to determine potential target proteins. Lane MW, protein molecular weight markers, with values indicated in thousands; lane L, whole-cell lysate flowthrough; lane D, 500 μM 7955004; lane A, 10 μM ATP. Protein bands identified in lane D were subsequently submitted for mass spectrometry and were identified as GroEL (upper band, with molecular weight of 60,000; Hsp60) and DeoD (lower band, with molecular weight of 25,000; purine nucleoside phosphorylase).

pound 7955004. These protein bands were cut from the gels, submitted for mass spectrometry, and identified as *S.* Typhimurium GroEL (Hsp60) and purine nucleoside phosphorylase (PNP) (DeoD), respectively (Fig. 5B).

DISCUSSION

Bacterial biofilms were first reported in 1978, when they were observed both in the environment and under conditions associated with chronic human infections (27). Decades of research have now demonstrated that these multicellular community structures can greatly enhance bacterial resistance to antimicrobials and host immune molecules (27, 28). Biofilms are a unique and dynamic bacterial growth environment associated with altered gene expression profiles and increased horizontal transmission of resistance elements, facilitating adaptation to hostile environments. Such functions enhance bacterial growth in difficult host niches and aid the establishment of chronic infections. Numerous studies have demonstrated connections between biofilm formation by nontyphoidal *Salmonella* strains, antimicrobial resistance, and persistence in food animals and throughout the food chain. Our laboratory has demonstrated that biofilm formation in the gallbladder facilitates chronic carriage of *S.* Typhi (13, 29). In light of the importance of these multicellular structures in the infectious cycles of *S.* Typhimurium and *S.* Typhi, we sought to disrupt the formation of bacterial biofilms using small-molecule kinase inhibitors. It is thought that therapies that function through bactericidal activity present selective pressures for the development of antibiotic resistance (30); therefore, we focused on biofilm formation, a nonessential bacterial process known to facilitate persistent infections.

Reports of multidrug-resistant *Salmonella* strains are now common, and drug discovery is a high-priority research area in an effort to find novel ways to fight infections and to cure disease (21, 31). Small-molecule inhibitors are of great interest and are being explored for their ability to interrupt intracellular signal transduction and protein-protein interactions, as a method to disrupt processes necessary for virulence (32). Recently, small-molecule inhibition of *toxT* expression has been described for *Vibrio cholerae*, and Geske et al. showed that nonnative *N*-acyl homoserine lactone (AHL) signal molecules are able to inhibit quorum sensing and biofilm formation in *P. aeruginosa* (33, 34). Our screen targeted the complex but highly conserved cellular process of biofilm formation, employing an automated, high-throughput, preliminary screening method to assess potential drug targets. Previous research clearly demonstrated a plethora of biofilm-associated proteins and regulatory systems affecting biofilm formation (35); therefore, we employed a top-down approach whereby we began our screen by looking for the desired outcome (significant inhibition of biofilm formation), rather than targeting a particular system. Subsequent efforts were directed at identifying the drug target, in order to begin to investigate the mechanism by which the compound inhibited biofilm formation. Our preliminary screen included 3,000 compounds, from which we identified a single promising compound for further study, validation, and derivatization. Employing an automated preliminary screen permitted us to narrow the candidate compounds to 43, a number that could be tested manually in subsequent assays. The preliminary screen was conducted only once; therefore, it is certainly possible that further replicates of this process or changes in the designated 30% inhibitory threshold would yield other promising candidates from this library.

Our results demonstrate that targeting bacterial kinases/ATP-utilizing enzymes to inhibit biofilm formation could be a promising therapeutic approach for the prevention of *Salmonella enterica* biofilm formation. Compound 7955004 exhibits a dose-dependent response and demonstrates efficacy against biofilm formation at concentrations ranging from 0.625 μM to 50 μM. Inhibition of biofilm formation by compound 7955004 is not a result of bactericidal or bacteriostatic activity, as bacterial viability is unaltered after drug exposure. Compound 7955004 did not disperse existing biofilms, suggesting specific effects on early events in the process of attachment or biofilm microcolony formation. It was demonstrated previously that the process of biofilm formation results in increased antibiotic tolerance, which is not exhibited by genetically identical cells in the planktonic phase of growth (28, 36, 37). Therefore, the possibility that compound 7955004 may act by shifting cells out of microcolonies and into the planktonic phase, or maintaining them in a planktonic phase, is of great interest with regard to future studies investigating the potential for use of this compound in tandem with antibiotic treatment.

The core genomes of *S.* Typhi and *S.* Typhimurium share >98% sequence identity (38); however, *S.* Typhimurium is more amenable to laboratory study, genetic manipulation, and *in vivo* animal modeling (13). *S.* Typhimurium forms biofilms in numerous *in vitro* settings with greater speed and less variability than *S.*

Typhi, perhaps due to the former's ability to thrive in diverse host and environmental settings, compared with the latter's stringent host restrictions. Preliminary testing was conducted in *S.* Typhimurium in order to enable subsequent studies of identified lead compounds using available libraries of defined genetic mutations and murine modeling of chronic carriage. However, in spite of the more robust biofilm formation observed in *S.* Typhimurium and the similarity of the genomes of the two organisms, the observed biofilm-inhibiting effect of compound 7955004 was more modest with *S.* Typhi (20% inhibition) than with *S.* Typhimurium (55% inhibition). This could be due to the differing assay conditions required for measurable *in vitro* biofilm formation by *S.* Typhi. Additionally, studies aimed at identifying the interacting partners of compound 7955004 indicated a member of the RpoS regulon, DeoD, as a possible target. DeoD is positively regulated by RpoS (39, 40). Although it is widely used as a laboratory reference strain, *S.* Typhi Ty2 produces a nonfunctional RpoS protein (41), which potentially could diminish the effects of a compound targeting positively regulated components of the RpoS regulon. In addition to further lead compound optimization, additional testing will be conducted using clinical isolates carrying wild-type *rpoS*.

Although functional processes related to biofilm formation may be conserved widely among bacterial species, our laboratory and others have demonstrated that gene expression in *Salmonella* biofilms can be quite variable. Biofilm genes and surface antigens may be heterogeneously expressed in response to different substrata or growth conditions and even in different regions of an individual bacterial community (13, 42–44). As this work involved biofilms grown on a polystyrene surface, further studies will need to be conducted to investigate the potential of this compound to reduce biofilm formation on additional substrata and in different media. *In vivo* studies employing the murine model of chronic gallbladder carriage of *Salmonella* will be of particular interest (12).

Compound 7955004 showed cross-genus activity against *A. baumannii* biofilm formation. *A. baumannii* is a Gram-negative opportunistic pathogen that is frequently reported as a cause of nosocomial infections. Several genes and cellular processes involved in attachment and extracellular matrix production are shared among biofilm-forming bacterial pathogens (45); among these are the production of cellulose, type IV pili, and biofilm-associated protein (Bap) (35). Nonmicrobicidal approaches for *A. baumannii* have shown interference in biofilm formation with the addition of compounds with binding capacities in many domains of response regulators (46). Two-component systems have become a therapeutic target in recent years, due to their tight regulation of many virulence genes in *A. baumannii* and other pathogenic bacteria (47, 48). *Salmonella* and *Acinetobacter* are both able to form biofilms on biotic and abiotic surfaces, and they share biofilm regulatory genes such as *envZ/ompR* and *clpX* (4, 12, 49–52), supporting further investigation of the use of kinase inhibitors against biofilm formation by both genera.

Protein targets elucidated from the ATP-Sepharose studies included GroEL (Hsp60) and a purine nucleoside phosphorylase (DeoD). GroEL is a chaperone protein capable of interacting with and assisting in the proper folding of many proteins in eukaryotes, bacteria, and archaea (53, 54). In *Salmonella*, functional GroEL activity requires interactions with the cochaperonin protein GroES (55). Both GroEL and GroES rely on ATP binding and hydrolysis for their joint functions in cells. GroEL was found to be essential for biofilm formation in *Mycobacterium smegmatis* and more recently was identified in the extracellular matrix of nontypeable *Haemophilus influenzae* (56, 57). GroEL has also been implicated in bacterial adherence to eukaryotic cells, including intestinal epithelial cells with *Clostridium*, *Lactobacillus*, and *S.* Typhi (58–60). Although mutants of GroEL have been found to be lethal in *Escherichia coli*, previously described mechanisms of GroEL/GroES function reveal that ATP binding occurs at multiple steps, including substrate binding, GroEL/GroES interaction, and enzymatic functionality. Therefore, it is possible that the effects of compound 7955004 reduced the functionality of GroEL but less completely than genetic deletion of *groEL*.

The second major protein target eluted from the ATP-Sepharose column was a purine nucleoside phosphorylase (PNP), DeoD. DeoD is responsible for scavenging and breaking down nucleotides, producing free purine bases and a free sugar that can be utilized as a carbon source. Bacterial DeoD has been greatly studied as a potential drug target because of marked differences in subunit and active site interactions between human and bacterial PNP protein structures (61, 62). DeoD also has been previously implicated in bacterial multicellular behavior, having been found to be significantly increased in biofilms of *E. coli* lacking the biofilm-repressing *ycfR* (63). Additionally development of rugose colony morphology in *Vibrio* (64) and early attachment and biofilm formation in *Streptococcus* have been shown to be dependent on DeoD activity (65). Subsequent studies will focus on employing heterologous deletions in these putative target proteins to establish whether they are important in *Salmonella* biofilm formation and to better understand how compound 7955004 is working in the early stages of biofilm formation.

The data presented demonstrate the promise of synthetic small molecules to generally prevent *Salmonella* biofilm formation or to improve treatment methods for chronic *Salmonella* infections in mammalian hosts. Future work will involve optimization of compound 7955004 with the goals of increasing its potency and broadening its efficacy to additional biofilm-forming pathogens. Efficient targeting of these infectious foci has great potential to facilitate eradication of chronic infections, resulting in decreased global disease burdens.

ACKNOWLEDGMENTS

This work was supported by NIH/NIAID grants AI066208 (to J.S.G.), AI090644 (to J.J.K.), and 1-T32-AI065411 (an NRSA training grant administered by the Center for Microbial Interface Biology at The Ohio State University).

We thank members of Microbial Infection and Immunity, The Ohio State University, for assistance and scientific feedback, P. Hill and D. J. Wozniak for non-*Salmonella* bacterial strains and technical assistance with biofilm assays, C. A. Rappleye for provision of the HepG2 cells, M. E. Drew for Biomek 2000 technical assistance, and K. A Werbovetz for assistance in obtaining the ATP mimetic library. We also thank the Ohio State Mass Spectrometry and Proteomics Facility for assistance in protein identification and data interpretation (NIH grant P30-CA016058).

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Annex 1: Qquestionnaire for Surveillance of Salmonella drug resistance in animals

General information gathered at each farm site.

Commodity type: Poultry: ☐ Swine: ☐ cattle ☐

Slaughter house: ☐

Production system: Commercial intensive farm _____ Backyard Production _____

Farm/Owner name_____

Address region_____District:_____Kebele:_____

Number of animals in a farm _____

Type of feed:

Grass only:_____Grass and commercial processed feed:_____processed feed only:_____

History of antimicrobial use

Do you use antimicrobial drugs in the farm? _____ If yes for what purpose?

a. Treatment,
b. Prophylaxis,
c. As feed additive.

If you use for prophylaxis, at what frequency do you use?

a. Every 3 month
b. Every 6 months
c. Once per year
d. Other: specify_____

What are the common antibiotic drugs used in the farm? _____

Use of drugs

Drug class	Dosage	Duration of administration	Route of administration			Frequency/year
			Oral	Oral with feed or water	Injection	

Feed additives

Drug or other chemicals	Concentration in PPM	Frequency	Remarks

Type of disinfectant & method of application on the farm

Class of Disinfectant	Trade Name of Disinfectant (if applicable)	Method of Application		
		Pressure washer	Backpack	Other (describe)

General information about specific animal sampled

Species_____ sex_____ age_____

Typical clinical sign if the animal is sick:_____

Recent history of antibiotic treatment (Yes/ No)

Name of antibiotic drug if yes_____

Describe any additional important observations/ remarks

Annex 2: Questionnaire for investigation of use of antibiotics and its impact on prevalence of drug resistance *Salmonella* in humans

Patient Information

Serial number_____ Sex_____ Age_____ date_____

Site/place of collection: _____ region_____ woreda_____

Kebele_____

Association with farms

Is there any farm close to your residence?_____ How far?_____

Do you work in farm? _____ If yes what farm? Dairy, Poultry, Swine, other

What is your specific job in the farm (cleaning barn, milking, animal attendant, other)

Do you eat or drink raw animal products? _____

Have you ever got Diarrhea? _____ If yes when_____

Have you been treated with antibiotics? _____ If yes which antibiotic? _____

History of antibiotic treatment for non *Salmonella* cases within the last 1 year_____

If yes how many times_____ and which antibiotic_____ for how long did you take_____

How do you get antibiotics physician Prescription _____Self medication _____

Do you currently have diarrhea? _____

Annex 3: Participants information sheet

Background information of the study

Multidrug resistant non-typhoidal *Salmonella* (NTS) is one of the major health problem in Ethiopia and elsewhere in the world. However the major serovars affecting humans and animals, genetic determinants of resistance and contribution of use of drugs in animals to development of resistance in humans is not established. Therefore, this study is designed to investigate the major serovars of *Salmonella* in humans and animals in Ethiopia, prevalence of drug resistance, the genetic determinants of drug resistance and association of use of antibiotics in animals and drug resistance in humans. This study will be conducted in and around Addis Ababa at a radius of 150 km. The study has been ethically approved by Institutional Review Board (IRB) of School of Pharmacy, IRB of College of health Science, Addis Ababa University.

Therefore you and your family are voluntarily requested to participate in the study after understanding the objectives of the study, the risk and discomfort, benefits and confidentiality. Your parent's (in case of individuals under 18 years) and your choice not to participate in the study will have no effect on the care you will be provided from the health facility and hence you still receive the same treatment as others without any prejudice.

Objectives of the study

The main objective of the study is to investigate the major serovars of *Salmonella* in humans and animals in Ethiopia, prevalence of drug resistance, the genetic determinants of drug resistance and association of use of antibiotics in animals and drug resistance in humans

Study participants

In this study 87 people in close contact with animals and 87 individuals attending health stations with clinical signs of diarrhea will participate. Individuals with all age group and both sex will participate in the study.

Samples required and examinations/Research

If you and/or your parent (in case individuals under 18 yrs) agree to participate in the study, you will provide stool samples for isolation of *Salmonella.*

Risk and discomfort

We do not expect a definitive risk as a result of sample collection or participating in the study.

Benefits

Whether you participated or not you will get the routine health services rendered by health facility you visited. This means there is no direct benefit as a result of your participation in this study.

Incentives

You will not be given any incentives to participate in the study.

Confidentiality

Information related to your name will be treated strictly confidential. Names and identifiers will be coded and deleted after data collection and therefore data will be treated anonymously for communication of results. Informed consent forms and questionnaires will be kept in a locked cabinet and will be destroyed after a year following termination of data collection. The results of your laboratory examination will not be shown to anyone except the research team and your doctor.

Annex 4: <u>ጥናቱን በተመለከተ ለተሳታፊዎች የሚሰጥ መግለጫ</u>

መድኃኒቶችን መቋቋም የሚችል ሳልሞኔላ የሚባል የአንጀት ጥገኛ ባክተርያ በሃገራችን ከዋና የጤና ችግሮች አንዱ ነዉ :: ነገር ግን በሰዉ እና በእንስሳት ላይ በሽታዉን የምያስከትሉ ዋና ዋና ዝርያዎች ተለይተዉ የማይታወቁ ሲሆን በተጨማሪም የመድሃኒት መላመድ መንስኤ በወል ተለይቶ አይታወቅም :: ስለዚህ የዚህ ጥናት ዓላማ በኢትዮጵያ የሚገኙ የሳልሞኔላ ዝርያ ለማወቅ አንዲሁም የመድሃኒት መቋቋም ባሕሪን ለማጥናት እና መድሃኒት ለእንስሳት መጠቀም በሰዉ ላይ የምያስከተለዉን ተፅእኖ ለመፈተሽ ነዉ ::

ይህ ጥናት በአዲስ አበባ እና በዙርያዉ ባሉ አካባቢዎች እስከ መቶ ሃምሳ ኪሎ ሜትር ርቀት ድረስ ይሸፍናል :: የዚህ ጥናት አስፈላጊነቱ ታምኖበት በአዲስ አበባ ዩኒቨርሲቲ ፋርማሲ ትምህርት ክፍል እና በጤና ሳይንስ ኮሌጅ የጥናትና ምርምር ስነምግባር ኮሚቴ ፈቃድ አግኝቷል። ስለዚህ እርስዎ ወይም ቤተሰብዎ (እድሜያቸዉ ከ15 ዓመት በታች ለሆኑት) በጥናቱ እንዲሳተፉ ፈቃድዎን እየጠየቅን በምርምሩ ለመሳተፍ ከመወሰንዎ በፊት የሚከተሉት ዝርዝር መረጃ ማለትም የጥናቱ ተሳታፊዎች የሚሰጡት ናሙና ፣ የሚደረገዉ ምረመራ በጥናቱ በመሳተፍዎ ሊከሰቱ የሚችሉ ችግሮች ፣ጥናቱ የሚሰጠዉ ጥቅም ፣ የሚስጥር አጠባበቅን በተመለከተ በማንበብ ወይም እንዲነበብሎት በማድረግና ጥያቄ ካልዎት በመጠየቅ በቂ ግንዛቤ እንዲወስዱ ይጠየቃሉ :: በዚህ ጥናት መሳተፍ ሙሉ በሙሉ ፈቃደኝንት ላይ የተመሰረተ ሲሆን ምንም አይነት ምክንያት መስጠት ሳያስፈልግዎት በጥናቱ የሚኖርዎትን ተሳትፎ በማንኛዉም ጊዜ ማቆም የችላሉ:: ባለመሳተፍዎ ምክንያት ከጤና ተቋም ልያገኙ የሚችሉትን የጤና አግልግሎት አሁንም ሆነ ወደፊት ያለምንም አድልዎ ይሰጥዎታል::

<u>የጥናቱ አላማ</u>

የጥናቱ ዋና አላማ ጥናቱ በሚካሄድበት አካባቢ ባሉ ሰዎችና እንስሳት ላይ የሰልሞኔላን ስርጭት ፣ የመድሃንቶች መላመድና መንስኤን መፈተሽ ነዉ ::

<u>የጥናቱ ተሳታፊዎች</u>

በዚህ ጥናት ከእንስሳት ጋር ቅርበት ያላቸዉ 87 ሰዎች እንዲሁም በተቅማጥ ህመም በጤና ተቁአማት ሕክምና እየተከታተሉ ያሉ 87 ሰዎች ይሳተፋሉ:: በማንኛዉም እድሜ ክልል ያሉ ወንዶችም ሴቶችም ሊሳተፉ ይችላሉ::

<u>ከጥናቱ ተሳታፊዎች የሚወሰድ ናሙና</u>

እርስዎ ወይም ቤተሰብዎ በጥናቱ ለመሳተፍ ፈቃደኛ ከሆኑ የሰገራ ናሙና የሰጣሉ:: ይህም ናሙና የተለያዩ የሰልሞኔላ ዝርያዎች መኖር ያለመኖራቸዉን ለማወቅ ይመረመራል::

<u>በጥናቱ መሳተፍ ሊያስከትል የሚችላቸው ችግሮች</u>

በጥናቱ በመሳተፍ እና ናሙና በመስጠት ጋር በተያያዘ ሊከሰቱ የሚችሉ ችግሮች አይኖሩም ::

<u>በጥናቱ መሳተፍ ሊያስገኝ የሚችለዉ ጥቅም</u>

በጥናቱ ቢሳተፉም ባይሳተፉም ከጤና ተቋም የሚያገኙት የህክምና አገልግሎት እንደ ተጠበቀ ይሆናል :: በጥናቱ በመሳተፍዎ የሚያገኙት ቀጥተኛ የሆነ ጥቅም አይኖርም ::

<u>ማበረታቻ</u>

በጥናቱ እንዲሳተፉ ተብሎ ልዩ የገንዘብ ማበረታቻ አይደረግም ::

ምስጢር መጠበቅ

ከእርስዎ ወይም ከቤተሰብዎ የተሰበሰበ ማንኛዉም መረጃ በምስጢር ይያዛል:: እርስዎን ወይም ቤተሰብዎን ገላጭ የሆኑ ስምን ጨምሮ በምስጢር ቁጥር በመተካት የጥናቱ ዉጤት ይታተማል:: የስምምነትና የመጠየቅያ ቅፆች ጥናቱ ከተጠናቀቀ ከአንድ ዓመት በኃላ የሚቃጠል ሲሆን እስከዝያዉም ድረስ በሳጥን በመቆለፍ በምስጢር ይያዛል:: የላቦራቶሪ ዉጤትዎ ከሀክምዎና ከተመራማሪዎች ዉጭ ለሌላ ሰዉ ትላልፎ አይሰጥም::

Annex 5: Study participant consent form

Name of participant_____ age _____ sex_____

Code_____ study site/ health facility_____

I confirm that I and my parent (in case of individuals under 18 yrs) have been given adequate information about the research project "Study on drug resistance salmonella in central Ethiopia"

I, and my parent(s)(in case of individuals under 18 yrs) have been requested to provide stool sample. The researchers informed me that there is no major risk associated with participating in the study or providing the requested samples. I and /or my parents have also understood that the results of laboratory diagnosis will be used for research purposes and the information related to myself/my family will be kept strictly confidential. I and /or my parents are well informed that participation in the study is fully voluntary and I /or my family can withdraw anytime without giving any reason. Moreover, I am and/or my family is fully aware that non-participation in this project will not subject me or my family to any health service denial from the health facility either now or in the times to come. I and /or my parent confirm that all the information provided to me is clear and has been conveyed by the language that I fully understand. Finally, I and/or my parent declare that I have been given enough time to deliberate before I and/or my parent agree to participate in the study, I and/or my parent(in case individuals under 18 yrs) signed this informed consent.

Name of participant_____signature_____ Date_____

Name of parent/guardian_____Signature_____Date_____

Name of researcher obtaining consent_____ signature_____ Date_____

Name and signature of witnesses

1. _____
2. _____
3. _____

Name and address of contact person
Tadesse Eguale
Tele: +251911435759,
School of Pharmacy, Addis Ababa University, Ethiopia

Annex 6: Study participant consent form for underage

Name of participant_____ age _____ sex_____

Name of the parent or legal guardian_____

Code_____ study site/ health facility_____

I confirm that I and my child (under 18 yrs) have been given adequate information about the research project "Study on drug resistance salmonella in central Ethiopia"

My child(s) (under 18 yrs) have been requested to provide stool sample. The researchers informed me that there is no major risk associated with participating in the study or providing the requested samples. I and /or my child have also understood that the results of laboratory diagnosis will be used for research purposes and the information related to myself/my family will be kept strictly confidential. I and /or my child are well informed that participation in the study is fully voluntary and I /or my family can withdraw anytime without giving any reason. Moreover, I am and/or my family is fully aware that non-participation in this project will not subject me or my family to any health service denial from the health facility either now or in the times to come. I and /or my parent confirm that all the information provided to me is clear and has been conveyed by the language that I fully understand. Finally, I and/or my child declare that wehave been given enough time to deliberate before we agree in participation of my child in the study, I as parent/legal guardian of my child(under 18 yrs) signed this informed consent.

Name of parent/guardian_____Signature_____Date_____

Name of researcher obtaining consent_____signature_____ Date_____

Name and signature of witnesses

1. _____
2. _____
3. _____

Name and address of contact person
Tadesse Eguale
Tele: +251911435759,
School of Pharmacy, Addis Ababa University, Ethiopia

Annex 7: <u>የጥናቱ ተሳታፊዎች የስምምነት ቅፅ</u>

የጥናቱ ተሳታፊ ስም _____ እድሜ _____ ፆታ _____

የሚስጢር ቁጥር _____ የጥናቱ ቦታ / የጤና ተቋም _____

እኔ ስሜ (ወይም የቤተሰቤ ስም እድሜያቸዉ ከ15 ዓመት በታች ለሆኑት) ከዚህ በላይ የተግለፀዉ ግለሰብ በመጋል ኢትዮጵያ የሰልሞኔላ ባክቴርያ ስርጭትና መድሃኒት የመቋቋም ብቃቱን ለመፈተሽ የሚደረግ ጥናት/ "Study on drug resistance salmonella in central Ethiopia" በጥናቱ ለመሳተፍ ከመወሰኔ በፊት ስለጥናቱ በቂ ዝርዝር መረጃ የተሰጠኝ መሆኑን አረጋግጣለሁ። በዚሁም መሰረት እኔ ወይም ቤተሰቤ (እድሜያቸዉ ከ15 ዓመት በታች ለሆኑት) የጥናቱ ተሳታፊ በመሆን የሰገራ ናሙና ለመስጠት ተጠይቀናል።በጥናቱ በመሳተፍ እና ናሙና በመስጠት ጋር በተያያዘ ሊከሰቱ የሚችሉ ችግሮች እንደሌሉ በተመራማሪዎቹ ተገልፆልኛል። ለቤተሰቤም ተገልፆላቸዋል:: በተጨማሪም የእኔ የላቦራቶሪና የህክምና ዉጤት በሚስጢር እንደሚያዝና ለታቀደዉ ምርምርና ጥናት ብቻ እንደሚዉል ተገልፆልኛል ። በጥናቱ መሳተፍ ሙሉ በሙሉ በፍቃድግንንት ላይ የተመሰረተ እንደሆነና አስፈላጊ ከሆነም ምንም አይነት ምክንያት መስጠት ሳያስፈልግ በማንኛዉም ጊዜ የጥናቱ ተሳታፍነቴን ማቋረጥ እንደምችል ተረድቻለሁ። ይህን በማድረጌም የህክምና አገልግሎት እንደማይቋረጥብኝ ተገልፆልኛል። በመጨረሻም የተገለፀልኝን እንዲሁም ለቤተሰቤ የተገለፀዉን በቂ መረጃ ከተረዳሁ በኃላ በጥናቱ ለመሳተፍ መስማማቴን በፊርማዬ አረጋግጣለሁ።

የተሳታፊ ስም _____ፊርማ _____ቀን _____

የመላጅ/ያሳዳጊ ስም _____ፊርማ _____ቀን _____

የተሳታፊ ስምምነት የተቀበሉ ባለሞያ ስም _____ፊርማ _____ቀን _____

በስምምነት ወቅት የነበሩ እማኞች ስምና ፊርማ

1. _____
2. _____
3. _____

የምርምሩ ተጠሪ ስምና አድራሻ :-

ታደሰ እጓለ

የፋርማሲ ትምህርት ክፍል፣ አዲስ አበባ ዩኒቨርስቲ፣ አዲስ አበባ፣ ኢትዮጵያ

የመልክት ሳጥን ቁጥር- 1176

ስልክ 0911435759

Annex 8. የ<u>ጥናቱ ተሳታፊዎች የስምምነት ቅፅ</u>

የጥናቱ ተሳታፊ ስም _____ እድሜ _____ ፆታ _____

የመላጅ/ያሳዳጊ ስም _____ እድሜ _____ ፆታ _____

የሚስጢር ቁጥር _____ የጥናቱ ቦታ / የጤና ተቋም _____

እኔ እና እድሜዉ/ዋ ከ 15 ዓመት በታች የሆነ ልጄ ሥሚ/ሙ ከዚህ በላይ የተግለፀዉ ግለሰብ በመጎል ኢትዮጵያ የሰልሞኔላ ባክቴርያ ስርጭትና መድሃኒት የመቋቋም ብቃቱን ለመፈተሽ የሚደረግ ጥናት "Study on drug resistance salmonella in central Ethiopia" በጥናቱ ለመሳተፍ ከመወሰናችን በፊት ስለጥናቱ በቂ ዝርዝር መረጃ የተሰጠን መሆኑን አረጋግጣለሁ። በዚሁም መሰረት እድሜዉ/ዋ ከ15 ዓመት በታች የሆነ ልጄ የጥናቱ ተሳታፊ በመሆን የሰገራ ናሙና ለመስጠት ተጠይቃለች።በጥናቱ በመሳተፍ እና ናሙና በመስጠት ጋር በተያያዘ ሊከሰቱ የሚችሉ ችግሮች እንደሌሉ በተመራማሪዎቹ ተገልፆልናል። በተጨማሪም የላቦራቶሪና የህክምና ዉጤት በሚስጢር እንደሚያዝና ለታቀደዉ ምርምርና ጥናት ብቻ እንደሚዉል ተገልፆልናል ። በጥናቱ መሳተፍ ሙሉ በሙሉ በፍቃድግኝነት ላይ የተመሰረተ እንደሆነና አስፈላጊ ከሆነም ምንም አይነት ምክንያት መስጠት ሳያስፈልግ በማንኛዉም ጊዜ የጥናቱ ተሳታፍነቷን ማቋረጥ እንደምትችል ተረድተናል። ይህን በማድረጋችንም የህክምና አገልግሎት እንደማይቋረጥብን ተገልፆልናል። በመጨረሻም የተገለፀልንን በቂ መረጃ ከተረዳሁ በኃላ በጥናቱ ለማሳተፍ መስማማቴን በፊርማዬ አረጋግጣለሁ።

የተሳታፊ ስም _____ፊርማ _____ቀን _____

የመላጅ/ያሳዳጊ ስም _____ፊርማ _____ቀን _____

የተሳታፊ ስምምነት የተቀበሉ ባለሞያ ስም _____ፊርማ _____ቀን _____

በስምምነት ወቅት የነበሩ እማኞች ሰምና ፊርማ

1. _____
2. _____
3. _____

የምርምሩ ተጠሪ ስምና አድራሻ :-

ታደሰ እጓለ

የፋርማሲ ትምህርት ክፍል፣ አዲስ አበባ ዩኒቨርስቲ፣ አዲስ አበባ፣ ኢትዮጵያ

የመልክት ሳጥን ቁጥር- 1176

ስልክ 0911435759

Deceleration

I, Tadesse Eguale, hereby declare that the submitted dissertation entitled “Phenotypic and Molecular Characterization of Non-typhoidal *Salmonella* Species in Humans and Animals in Central Ethiopia and Inhibition of Biofilm Formation Using Small Molecule Adenosine Mimetics” is my own work. This dissertation or parts thereof have not been submitted to any other university for the award of academic degree. All materials and publications used for the dissertation have been fully acknowledged.

Name: Tadesse Eguale

Signature: _____

Date: June 20, 2016