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ADDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

ISOLATION AND IDENTIFICATION OF *ESCHERICHIA COLI* K99 AND *SALMONELLA ENTERICA* FROM DIARRHEIC CALVES IN AND AROUND AWASH FENTALE DISTRICT OF AFAR REGION, EASTERN ETHIOPIA

MVSc THESIS

BY

HABTAMU GIRMA TEGENAW

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH
MVSc PROGRAM IN VETERINARY MICROBIOLOGY

SEPTEMBER, 2020
BISHOFTU, ETHIOPIA

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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Microbiology

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As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by: **Habtamu Girma** entitled: **Isolation and Identification of *Escherichia Coli* K99 and *Salmonella enterica* from Diarrheic Calves in and around Awash Fentale District of Afar Region, Eastern Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Veterinary Science in Veterinary Microbiology.

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DEDICATION

I dedicate this thesis to my beloved mother Atsede Assefa who has meant and continue to mean so much to me. Although she is no longer of this world, her memories continue to regulate my life. Thank you so much, I will never forget you.

STATEMENT OF AUTHOR

First, I declare that this thesis is my own work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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BIOGRAPHICAL SKETCH

The author was born on February 28, 1986 in Woyra Amba, North Shewa. He attended his Elementary and secondary school education at Woyra Amba Elementary school and Haile Mariam Mamo Preparatory School respectively. He joined the then University of Gondar, Faculty of Veterinary Medicine and graduate with DVM degree in Veterinary Medicine in July 2009.

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LIST OF ABBREVIATIONS

API	.Analytical profile index
AST	Antimicrobial susceptibility testing
BGA	Brilliant green agar
CD	Cluster of differentiation
CLSI	Clinical and Laboratory Standards Institute
CSA	Central Statistics Agency
CTSMAC	Cefixime and tellurite sorbitol-MacConkey
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic *Escherichia coli*
FAO	Food and Agriculture Organization
HUS	Hemolytic uremic syndrome
LIA	Lysine iron agar
LPS	Lipopolysaccharide
MR	Methyl- Red
MSRV	Modified semi-solid Rappaport Vassiliadis
OIE	Office International des Epizooties
PCR	Polymerase chain reactions
PHE	Public Health England
SMAC	Sorbitol-MacConkey
SPI	Salmonella Pathogenicity Island
SSA	Salmonella Shigella Agar
USAID	United States Agency for International Development
TCBS	Thiosulfate-citrate-bile salts-sucrose agar
TSIA	Triple Sugar Iron Agar
XLD	Xylose lysine deoxycholate

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ABSTRACT

The current cross sectional study was conducted from December 2018 to September 2020 with the aim to isolate and identify E.coli K99 and Salmonella enterica and associated risk factors for occurrence of these two pathogens. Fecal samples were collected from a total of 188 diarrheic calves aged up to 6 months from 150 households in Awash Fentale district Afar region, eastern Ethiopia. These samples were processed during the study period for the isolation and identification of E.coli K99 and Salmonella enterica using culture media, biochemical tests, Pathasure kit and Biolog GEN III microplate. Out of 188 samples, 20.74% and 5.32% were found to be positive for E.coli K99 and Salmonella enterica respectively. Antimicrobial sensitivity patterns of Salmonella enterica were also investigated using 12 commonly used antibiotics. All isolates from calves were susceptible (100%) to amikacin, chloramphenicol, ampicillin, amoxicillin+clavulanic acid, ceftriaxone, cephalothin ciprofloxacin, tetracycline, gentamycin and trimethoprim+sulfamethoxazole. Multivariable logistic regression analysis was done to identify significant risk factors associated with examined bacteria. Thus study showed that the occurrence of E.coli K99 in male calves was higher (25.27%) than in female calves (16.49%). This might be the reason that more care is given to female calves than male calves. Because female calves are needed by the pastoralist where compared with male calves for milk production. On the other hand age and colostrum feeding within 24 hours were significantly associated with Salmonella enterica shedding. Calf age was significantly associated with Salmonella enterica (P=0.014, OR=13.15, 95% CI=1.677-103.127). The highest occurrence of Salmonella enterica was recorded in the 61-120 days of age (13.79%) than 1-60 days of age (2.86%) and 121-180 days of age (10.53%) diarrheic calves. In conclusion, isolation and identification of bacteria and risk factors associated with the spreading of bacteria causing diarrhea may be helpful for construction of suitable methods for prevention and control.

Keywords: Calf diarrhea, *E. coli* K99, *Salmonella enterica,* Culture, Isolation, Risk factors, Awash Fentale

1. INTRODUCTION

Ethiopia has the largest livestock inventories in Africa, estimated at 59.5 million cattle, 30.70 million sheep, 30.20 million goats, 2.16 million horses, 8.44 million donkeys, 0.41 million mules, 1.21 million camels, and 56.53 million poultry. Out of 59.5 million total cattle population, under 6 months ages of calves constitute about 9.6 percent (CSA, 2016). Despite the large number of livestock, productivity in general is low in the country, mainly due to the low genetic quality of local breeds, poor nutrition, and animal health problems (Ayele *et al*., 2003; Negassa *et al*., 2011). Young stock mortalities constitute the larger share of constraints to herd expansion and genetic improvement (Inamdar, 2012).

Neonatal calf mortality is the most important constraint in young stock and accounted for 8.7 to 84% of the total mortality (Inamdar, 2012). In some African countries, for instance, calf mortality rates range from 9% to 45%, 10% to 25%, 4.9% reported in dairy farms in Khartoum, in Tanzania, Mali and Sudan, respectively(Wymann *et al.*, 2006; Chenyambuga and Mseleko, 2009; Changa *et al*., 2010; Abdullatief *et al*., 2014).

In Ethiopia, a 30% pre-weaning calf mortality rate was reported in mixed crop-livestock production systems in Amhara Region (Ferede *et al*., 2014) and an 18% mortality rate was found in market-oriented dairy farms in Central Ethiopia (Wudu *et al*., 2008). According to Asmare and Kiros, 2016; the overall morbidity and mortality recorded are 66.7 % and 20 %, respectively.

Diarrhea is one of the most important causes of morbidity and mortality, leading to economic losses due to the cost of treatment, prophylaxis, increased susceptibility to other infections, reduced growth rates, and death of calves Worldwide (Parkinson *et al*., 2010; Selles *et al*., 2018).The main causes of calf diarrhea are grouped into two categories: non-infectious cause and infectious causes. Infectious diarrhea is the most significant cause of morbidity and mortality in neonatal dairy calves throughout the world (E-Seedy *et al*., 2016). The important viral infectious agents include bovine rotavirus group A, bovine corona virus, and bovine viral diarrhea virus. Among bacterial causes, *Salmonella* Typhyimurium, *Escherichia coli* K99+ as “white scour”, and *Clostridium perfringens* type C and among protozoa, *Cryptosporidium parvum* and coccidiosis either singly or in combination are affected young calves (Acha *et al*., 2004; Manickam and Ponnusamy, 2017; Elitok, 2018; Park *et al.,* 2018).

Enterotoxigenic *Escherichia coli* and *Salmonella* are known to be the most common and economically important causative agents of diarrhea in calves; lipopolysaccharides located on cell walls play an important role in the development of endotoxemia (Acha *et al*, 2004; Gharieb *et al*., 2015; E-Seedy *et al*., 2016). An Enterotoxigenic strain of ETEC, possessing fimbrial adhesins K99, is of particular importance in neonatal diarrhoea. K99 adhesins is encoded by plasmids (Quinn, 2011). In Ethiopia, *S.* Typhimurium appears to be more important than others, because it was one of the dominant isolates in cattle (17.4%) (Alemayehu *et al*., 2003; Alemu and Zewde, 2012).

Several risk factors for calf diseases, particularly diarrhoea, have been identified, including farm size, presence of a calving pen and hygiene, the quality of colostrum and the route of colostrum feeding as well as type of calf housing (Klein-Jobstl *et al*., 2014). The applications of false drugs and feed additives also play a very important role in predisposition of the etiology in adult calf diseases (Elitok, 2018).

Antimicrobial resistance is a global public and animal health concern that is influenced by the usage of antimicrobial agents in humans, animals and elsewhere (OIE, 2015). The historical and growing emergence of drug resistance among *E. coli* and *Salmonella spp*. strains isolates from humans and animals has increased the debate on public health hazard associated with the use of antibiotics in animal production (Hur *et al*., 2012; Rostagno and Callaway, 2012; Tadesse *et al*., 2012; Keelara *et al*., 2013).

Although there are some research works carried out in certain parts of the country, on problem of calf mortality and its economic significance, information on calf morbidity and mortality as result of diarrhea caused by the two important enteric bacteria, *E.coli* K99 and *Salmonella enterica* is limited in study area, in and around Awash Fentale district. Hence isolation and identification of *E.coli* K99 and *Salmonella enterica* in calves is needed for planning a proper control and preventive measure in the area as well as in country.

Therefore the objectives of the present study were intended:-

- To isolate and identify *E. coli* K99 *and Salmonella enterica* from diarrheic calves.
- To identify associated risk factors for transmission or exposure to *E.coli* K99 *and Salmonella enterica*
- To asses antimicrobial susceptibility profile of *Salmonella* isolates

2. LITERATURE REVIEW

2.1. Calf Diarrhea

Diarrhea is defined as a rapid movement of fluids through the intestine resulting in poor absorption of water, nutritive elements and electrolytes. Calf diarrhea is the commonest disease in young calves and is the greatest single cause of death. Diarrhea is one of the most important causes of morbidity and mortality, leading to economic losses due to the cost of treatment, prophylaxis, increased susceptibility to other infections, reduced growth rates, and death of calves Worldwide (Parkinson *et al*. 2010; Selles *et al*., 2018). It has a multifactorial etiology in which viruses, bacteria, and protozoa and also management factors (shelter, nutrition, hygienic conditions) play a role (Elitok, 2018).

Newborn calves are susceptible to neonatal calf diarrhea especially during their first 28 days of life. The diarrheic calf becomes dehydrated and suffers from electrolyte loss and acidosis (Bozukluhan *et al*., 2017). The loss of electrolytes causes a condition called metabolic acidosis, which will kill the calf if it is not corrected promptly (in less than 2 days) (Vermunt, 2002). Scours occurring within the first several days of life are the most important cause of calf sickness and death. Almost no herd goes through a calving season without some scours. In severe outbreaks, the effects of scours in an individual herd can be overwhelming (Ata *et al*., 2013).

2.2. Causative Agents

2.2.1. Historical background of bacteria

Escherichia coli

The German Austrian pediatrician Theodor Escherich discovered this organism in the feces of healthy individuals in 1885. He called it *Bacterium coli commune* because it is found in the colon. Its present name *Escherichia coli* was officially accepted in 1958 in honor of its discoverer. Early classifications of prokaryotes placed these in a handful of genera based on their shape and motility (Kuhnert *et al*., 2000; Croxen *et al*., 2013; Farrar *et al*., 2013).The genus *Escherichia* consists of five species namely, *Escherichia coli, E. fergusonii, E. hermanni, E. vulneris and E. blattae* of which *E.coli* is the most common and clinically most important (Gillespie and Hawkey, 2006; Kumar, 2012). *E. coli* O157:H7 was first recognized in 1982 as a human pathogen and cattle have been identified as a major source of *E. coli* O157:H7 infection of human but it is not pathogenic in cattle and present in the feces of healthy cattle (Zinnah *et al*., 2007).

Salmonella

The genus *Salmonella* was named after Daniel Salmon, an American veterinarian first isolated *Salmonella choleraesuis* from pigs with hog cholera in 1884 hence the name "*Salmonella*". *Salmonella* was first visualized in 1880 by Karl Eberth in the Peyer's patches and spleens of typhoid patients. Four years later in 1884 Georg Theodor Gaffky was able to successfully grow the pathogen in pure culture. A year after that, medical research scientist Theobald Smith discovered what would be later known as *Salmonella enterica* (Eberth, 1880; Hardy, 1999; Umeh and Enwuru, 2014). *Salmonella* is a ubiquitous and hardy bacterium that can survive several weeks in a dry environment and several months in water. It has many pathogenic species and strains. *Salmonella* is an enteric pathogen and is the major cause of bacterial foodborne infections (*Salmonella* Typhimurium and *Salmonella* Enteritidis), enteric or typhoid fever(primarily *Salmonella typhi* and *Salmonella paratyphi*), bacteremia, endovascular infections, focal infection and enterocolitis (typically *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Salmonella* Heidelberg) (Umeh and Enwuru, 2014).

2.2.2. Characteristics

Escherichia coli

The genus *Escherichia* includes motile and non-motile bacteria which belong to Class *Gammaproteobacteria*, the family *Enterobacteriaceae.* They are Gram-negative, oxidase negative, non-spore-forming, rod-shaped bacteria, facultative anaerobes, which are often motile by peritrichously arranged flagella. They are capable of fermenting a wide variety of carbohydrates with production of both acid and gas, although anaerogenic biotypes exist. Rapid fermentation of lactose is a characteristic feature of many strains, particularly those of *E. coli,* whilst other *Escherichia* species, including strains of enteroinvasive *E.coli* (EIEC), as well as some *E. coli* (metabolically inactive) strains ferment it slowly or fail to utilise this substrate at all (Gillespie and Hawkey, 2006; Leboffe and Pierce, 2010; Hakkani *et al*., 2016). *E. coli* is the major facultatively anaerobic bacterium in the intestinal tract of most animal species and is typically present at $10^{7} – 10^{9}$ organisms per gram in feces. *E. coli* is usually the dominant organism recovered on aerobic culture of feces, but pet birds are an exception as *E. coli* are recovered from only a low percentage of healthy pet birds(Gyles *et al*., 2010).

Cells are typically straight, round-ended rods, 0.5–0.8 ×1–4 μm, normally occurring singly or in pair growth occurs on a range of media. The optimum growth temperature is 37°C, and the minimum doubling time is ∼20 minutes. Colonies of *E. coli* on nutrient agar (37°C/24 hours) are 1–3 mm in diameter and may be smooth, entire, low-convex, greyish-translucent, and easily emulsifiable or rough, dry, and not easily emulsifiable. Mucoid and slime forming strains also occur (Singleton and Sainsbury, 2006). It is able to grow both aerobically and anaerobically, preferably at 37°C, and can either be nonmotile or motile, with peritrichous flagella. Fimbriae and capsules are found in some strains (Kumar, 2012; Croxen *et al.*, 2013).

Salmonella species

Salmonella species are gram negative, small rod-shaped nonspore-forming, non-capsulated, aerobic and facultative anaerobic organisms predominantly motile enterobacteria with cell diameters between about 0.7 and 1.5 μm, lengths from 2 to 5 μm, and peritrichous flagella (Fabrega and Vila, 2013; Hakkani *et al*., 2016). They are generally motile, aerogenic, nonlactose fermenting, urease negative, citrate utilizing and acetyl methyl carbinol negative (Gillespie and Hawkey, 2006).

Salmonellae are aerobes and facultatively anaerobes, growing readily over a range of pH 6 to 8 and temperature 15 to 45° C (optimum 37°C)(Kumar, 2012). They are chemotrophs, obtaining their energy from oxidation and reduction reactions using organic sources. They are also facultative anaerobes, capable of surviving with or without oxygen that inhabit the intestinal tract of animals, and may be found in a wide variety of sources, such as poultry, swine, human, food and the environment(Tortora, 2008; Khoo *et al*., 2014).

Table 1: Important distinguishing features of *Escherichia* and *Salmonella*

Test	*Escherichia*	*Salmonella*
Motility	+	+
Gas from glucose	+	+
Acid from lactose	+	-
Acid from sucrose	D	-
Growth in potassium cyanide	-	D
Indole	+	-
Methyl red	+	+
Voges-Proskauer	-	-
Citrate	-	+
Hydrogen sulfide	-	+
Urease	-	-
Phenylalanine deaminase	-	-
Arginine dehydrolase	D	+
Lysine decarboxylase	+	+
Ornithine decarboxylase	D	+

(d = results different in different species or strains). Source (Kumar, 2012. pp.349)

2.2.3. *Biochemical reactions*

E. coli ferments glucose, lactose, mannitol, maltose and many other sugars with the production of acid and gas. Typical strains do not ferment sucrose. Indole and methyl red positive, and Voges-Proskauer and citrate negative (IMViC + + – –). The four biochemical tests widely employed in the classification of enterobacteria are the indole, methyl red (MR), Voges-Proskauer (VP) and citrate utilization tests, generally referred to by the mnemonic 'IMViC'. It is negative for phenylalanine deaminase test, urease test, hydrogen sulfide production, gelatin liquefication, growth in the presence of potassium cyanide, and malonate utilization (Kumar, 2012; Parija, 2012).

Salmonellae ferment glucose, mannitol, arabinose, maltose, dulcitol and sorbitol, forming acid and gas except *S.* Typhi, Gallinarum and rare anaerogenic variants in other serotypes form only acid and no gas (Kumar, 2012). The biochemical properties of *Salmonella spp*. show that almost all *Salmonella* serovars do not produce indole, hydrolyze urea, and deaminate phenylalanine or tryptophan. Most of the serovars readily reduce nitrate to nitrite and most ferment a variety of carbohydrates with the production of acid, and reported to be negative for Voges-Proskauer (VP) reaction (Popoff and Le Minor, 2005).

Table 2: Biochemical reactions of subspecies *Salmonella enterica*

S/NO.	**Test**	***Subspecies***						
		I	**II**	**IIIa**	**IIlb**	**IV**	**V**	**VI**
1	Dulcitol fermentation	+	+	-	-	-	+	+
2	Lactose fermentation	-	-	+	+	-	-	+
3	Sorbitol fermentation	+	+	+	+	+	+	-
4	Malonate utilization	–	+	+	+	-	-	-
5	Salicin fermentation	-	-	-	-	+	-	-
6	Culture in potassium cyanide	-	-	-	-	+	+	-
7	Gelatinase	-	+	+	+	+	-	+
8	ONPG hydrolysis	-		+	+	-	+	+

Subspecies are: I, .*enterica*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae*; V, *bongori*; VI, *indica*. (Source: Kuman, 2012).

2.2.4. Taxonomy

Taxonomy is the science that studies organisms in order to arrange them into groups; those organisms with similar properties are grouped together and separated from those that are different. Taxonomy can be viewed as three separate but interrelated areas:

1. Classification: The process of arranging organisms into similar or related groups, primarily to provide easy identification and study.
2. Identification: The process of characterizing organisms.
3. Nomenclature: The system of assigning of names to organisms (Kumar, 2012).

Escherichia coli

Escherichia species are intestinal bacteria belonging to the *Enterobacteriaceae*, which are taxonomically placed within the gamma subdivision of the *Proteobacteria* phylum (Croxen *et al*., 2013). The determination of six different *E. coli* ‘‘pathotypes’’ that cause enteric diarrheal, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC), greatly enhanced our understanding of pathogenic *E. coli* (Marrs *et al.*, 2005).

Salmonella species

The genus *Salmonella* has two species, *Salmonella bongori* and *Salmonella enterica* (Goldman and Green, 2009; Parija, 2012). *Salmonella enterica* subspecies are found worldwide in all warm-blooded animals, and in the environment. *Salmonella bongori* is restricted to cold-blooded animals, particularly reptile (Tortora, 2008). *Salmonella enterica* is divided into six subspecies, which are distinguishable by certain biochemical characteristics and susceptibility to lysis by bacteriophage (Figure 1). These subspecies are:

Original subgenera Current nomenclature

- Subspecies I = subspecies enterica
- Subspecies II = subspecies salamae
- Subspecies IIIa = subspecies arizonae
- Subspecies IIIb = subspecies diarizonae
- Subspecies IV = subspecies hautenae
- Subspecies VI = subspecies indica(OIE, 2016)

More than 2600 serovars are recognised (Issenhuth-Jeanjean *et al.*, 2014). The most common serovars that cause infections in humans and food animals belong to *subspecies enterica* (OIE, 2016).

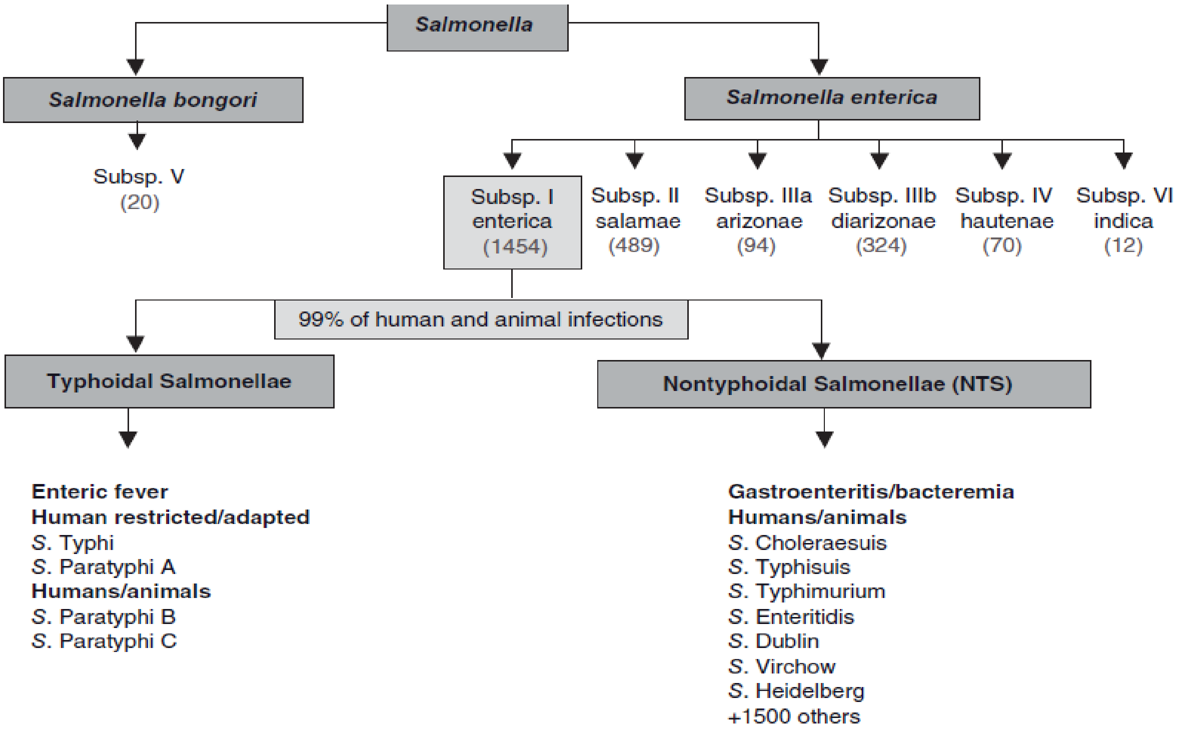

Figure 1: The genus *Salmonella***.** Numbers in brackets indicate the total number of serotypes included in each subspecies. Common serotypes are listed, but other serotypes may cause bacteremia or focal infection (Gyles *et al*., 2010)

2.2.5. Virulence factors

The basic mechanism underlying *Salmonella* virulence includes the ability to invade the intestinal mucosa, multiply in lymphoid tissues, and evade host defense systems, leading to systemic disease. For *Salmonella* pathogenesis, the organism should be capable of invading intestinal epithelial cells, surviving within macrophages, and causing enteropathogenicity. *Salmonella* pathogenicity island 1 (SPI-1) and SPI-5 are known to influence the type III secretion system and are mainly responsible for *Salmonella*-induced diarrhea in calves. SPI-2 is involved in the second type III secretion system and is responsible for intracellular survival of the organism (Cho and Yoon, 2014). *Salmonellae* possess the following antigens based on which they are classified and identified (Figure 2):

1. Flagellar antigen H
2. Somatic antigen O
3. Surface antigen Vi, found in some species.

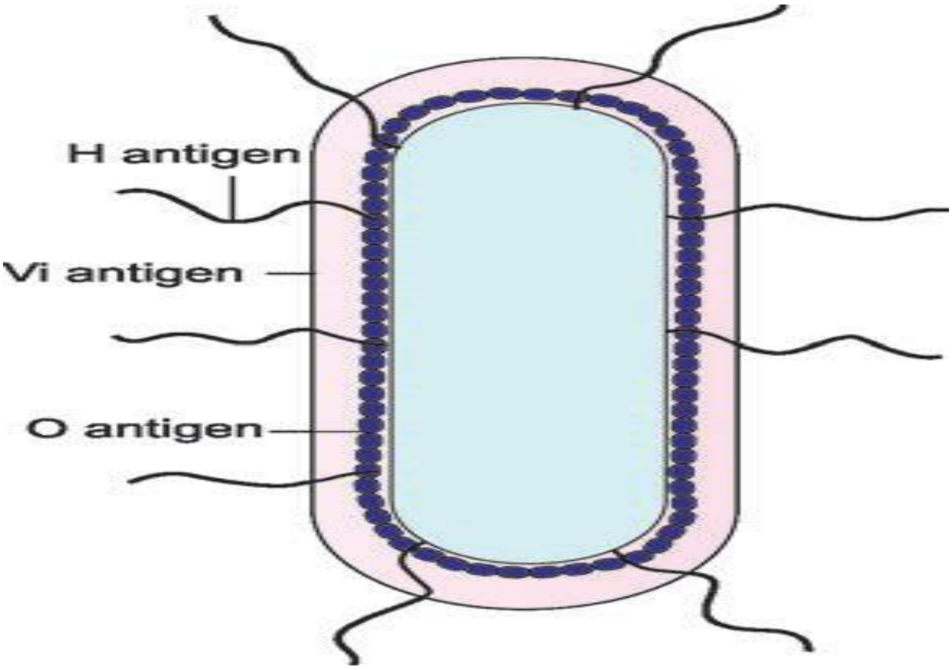

Figure 2: Antigenic structure of *Salmonellae* (Kumar, 2012)

Two types of virulence factors have been recognized in *E. coli* surface antigens and toxins.

A. Surface Antigens

1. Somatic Antigen (O Antigen):- The somatic lipopolysaccharide surface O antigen, besides exerting endotoxic activity, also protects the bacillus from phagocytosis and the bactericidal effects of complement.

2. K Antigen: - The envelope or K antigens also afford protection against phagocytosis and antibacterial factors in normal serum, though it is not effective in the presence of antibody to O or K antigen.

3. Fimbriae:- Like many other members of the Enterobacteriaceae, strains of *E. coli* exhibit common fimbriae which are chromosomally determined, present in large numbers and causing mannose sensitive hemagglutination and probably not relevant in pathogenesis.

B. Toxins

Exotoxins: - *E. coli* produce two kinds of exotoxins: hemolysins and enterotoxins. Hemolysins do not appear to be relevant in pathogenesis though they are produced more commonly by virulent strains than by a virulent strain. Enterotoxins are important in the pathogenesis of diarrhea (Kumar, 2012). The virulence attributes of the strain of *E. coli*, particularly its capacity to invade tissues and produce a septicemia, or to produce an enterotoxin that causes varying degrees of severity of diarrhea (Radostits *et al*., 2006).

2.2.6. Mode of infection

Salmonella species infection occurs when a susceptible animal ingest feed or water that has been contaminated with feces from animals shedding the organism. Sources of infection may be rodents, birds, flies, feral cats, dogs. The principal route of infection is fecal-oral. Saliva, nasal secretions, colostrum and milk can also be the source of organisms shed from sick animals and oral transmission from these sources is another way to spread disease on a dairy. Cow to cow transmission is typical but other animals, insects, birds, people, vehicles, medications, equipment and utensils can facilitate transmission of the organisms. It is also possible that aerosol transmission of *Salmonella* may occur under some types of housing conditions (Quinn *et al*., 2011).

Pathogenic *E. coli* can be found in contaminated environment (water and soil) because they are being shed in the faeces of infected animals and humans (Shah *et al*., 2018). Strains of *E.coli* capable of producing diseases reside in the lower gastrointestinal tract and area abundant in environments inhibited by animals. Animal faeces are considered to be the major source of pathogenic *E. coli*. The close contact among animals in the farms may lead to the transmission to other animals (Karch *et al*., 2005). Transmission is through the fecal oral route (Hirsh and Zee, 1999). Animal wastes, sewages from farming operations, manure/slurries which are frequently used as fertilizers for the crops or silage preparation and cattle grazing also contribute to the infection and re-infection of cattle (Jiang *et al*., 2002).

2.2.7. Pathogenesis

Escherichia coli

Escherichia coli is among the most important cause of diarrhoea in both animals and humans. Among diarrheagenic strains of *E. coli,* ETEC is most commonly associated with diarrhoea in calves and lambs and typically produce only enterotoxin heat stable and fimbrial adhesins F5 (K99) and F41 (Gyles *et al*., 2010; Wani *et al*., 2013).

Pathogenic *E. coli* strains have different virulence factors that allow them to colonize the host's small intestine, avoiding the immune response and stimulating the deleterious inflammatory response to produce diarrhea (Croxen and Finlay, 2010). ETEC enter the animal by the oral route, and when present in sufficient numbers, colonize the small intestine following attachment by fimbrial adhesins to receptors on the small intestinal epithelium or in the mucus coating the epithelium (Gyles *et al.*, 2010). The ETEC then proliferate rapidly to attain massive numbers to the order of 10^{9} per gram of intestine in the mid - jejunum to the ileum and produce enterotoxins, which cause an increase in net secretion of fluid and electrolytes from the systemic circu1ation (Figure 3). The adhesion of *E. coli* to the intestinal epithelial cells is mediated by bacterial to microvilli of enterocytes of the small intestine (Radostits *et al.*, 2006; Gyles *et al.*, 2010; Yousif *et al.*, 2013). The enterotoxigenic form of colibacillosis occurs most commonly in calves and piglets and less commonly in foals and lambs. Septicemia, bacteremia, diarrhea, dehydration and metabolic acidosis are the major pathogenetic events in the various forms of colibacillosis (Radostits *et al.*, 2006).

E. coli causes a watery diarrhea and weakness in 1–4 day old newborn calves, progressive dehydration, high temperature, foul-smelling white or yellowish to tarry black coloured feces (Cho *et al.*, 2010). Death usually occurred within 24 hours due to severe dehydration. The fimbrial adhesion F5 (K99) promotes the adhesion of bacterial cells to glycoproteins on the epithelial surface of the jejunum and/or ileum, and bacterial enterotoxin also causes damage to the epithelial cells, resulting in fluid secretion and diarrhea (El-Seedy *et al.*, 2016). Only the most successful combinations of virulence factors have persisted to become specific *E. coli* pathotypes that are capable of causing disease in healthy individuals (Natalia *et al.*, 2015).

Under natural conditions less than 10% of beef cow colostrum contains antibodies to ETEC (K99). As ETEC diarrhea occurs during the first three days of life the neonate does not have time to mount a protective immune response to vaccination. Protection is afforded by vaccinating cows in late gestation to ensure high concentrations of colostral antibodies. The protective efficacy of ETEC bacterins needed to ensure calves receive maternal antibodies (Ashenafi, 2013).

The bacterial fimbriae attach to specific receptor sites on villous epithelial cells, following which the bacteria multiply and form microcolonies that cover the surface of the villi. The capsular polysaccharide of *E. coli* may also be involved in adhesion and colonization. The fimbriae of *E. coli* are strongly immunogenic, a factor that is utilized in the production of vaccines (Radostits *et al*., 2006).

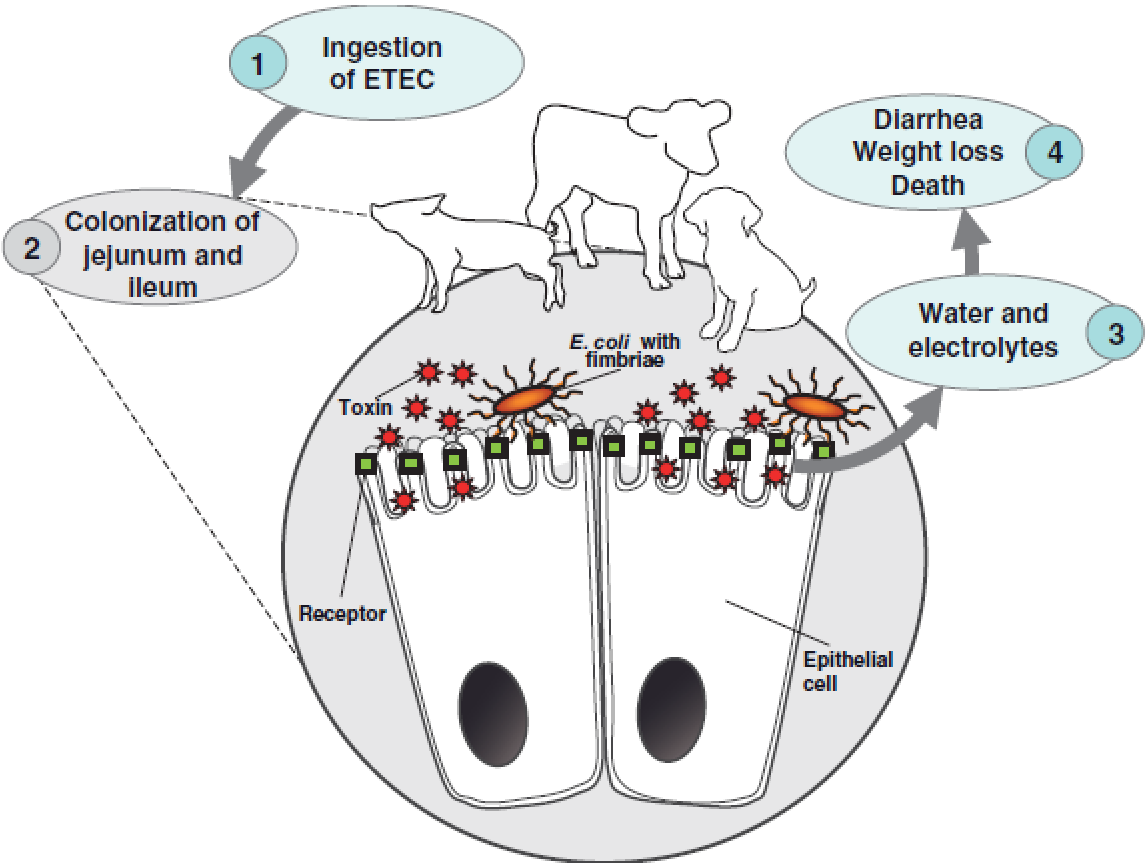

Figure 3: Schematic representation of the steps involved in the pathogenesis of ETEC infection. ETEC in the animal's environment are ingested (1), pass through the stomach, adhering to the small intestinal epithelium where they produce enterotoxins (2) that stimulate the secretion of water and electrolytes into the intestinal lumen (3). Loss of water and electrolytes leads to diarrhea, weight loss, and possibly death (4). (Source: Gyles *et al*., 2010, Pp. 266)

Salmonella

Salmonella causes diarrhoea, dehydration, abortion, pneumonia, septic arthritis, meningitis, gangrene of distal extremities, and sudden death in domestic animals (Gupta and Saxena, 2014). *Salmonella* cells are able to enter macrophages via macro pinocytosis. Much of the success of *Salmonella* in causing infection is attributed to two type III secretion systems which function at different times during infection. One is required for the invasion of nonphagocytic cells, colonization of the intestine, and induction of intestinal inflammatory responses and diarrhea. The other is important for survival in macrophages and establishment of systemic disease. These systems contain many genes which must work co-operatively to achieve infection (Haraga *et al,* 2008).

The source of *salmonella* infection in a herd can be from cattle, birds, cats, rodents, the water supply or a human carrier (Bell and Kyriakides, 2002; Bashahun and Amina, 2017). The disease is more severe in young or debilitated calves. Finding a membrane like coating in the intestine on necropsy is strong presumptive evidence that *Salmonella* might be involved (Bashahun and Amina, 2017). Young, pregnant and lactating animals are more susceptible to *Salmonella* infection and transmit the infection to human through direct and indirect means (Abdullah *et al*., 2010).

Salmonella enterica is a major pathogen in humans as well as in animals. The highly adapted *S. enterica* Typhi causes typhoid fever only in humans, whereas other serotypes, namely nontyphoid *Salmonella* serotypes, can cause a wide spectrum of diseases in humans and animals (Tegegne, 2019). *Salmonella enterica* colonizes the digestive of both adult cattle and calves but the infection is often recorded in the first 3 months of age and often causing severe symptoms. The organism *Salmonella* Dublin or *Salmonella* Typhimurium were the main cause of salmonellosis in calves. *S.* Typhimurium is the most common serotype affecting calves. *Salmonella* Dublin serovar is commonly detected in calves and adult cattle. Most infections are introduced into *Salmonella* free herds by the purchase of infected animals that might have acquired infection on farm premises, in transit or on dealer’s premises (Cho, 2012; Cho and Yoon, 2014; Andino and Hanning, 2015). Diarrhea due to *Salmonella* infection is watery and mucoid with the presence of blood and fibrin (Fossler *et al*., 2005). Calves can shed *Salmonella* for variable periods of time and intermittently depending on the degree of infection (Cho and Yoon, 2014).

Economic losses of salmonellosis in cattle include increased mortality especially in young animals, decreased weight gain in calves with inferior performance as well as costs associated with treatment and control (Rushton, 2009).

2.2.8. Detection and identification of E.coli and Salmonella

Culture

Culture methods remain the norm for detection and identification of bacterial enteric pathogens in clinical laboratories (Liu *et al*., 2012). As proposed by Robert Koch, a pure culture is the foundation of all research in infectious diseases. The first isolation of a bacterium enables the design of experimental models to analyze virulence and to complete Koch's criteria, thereby establishing a link between microorganisms and infectious diseases. The first culture media were developed empirically using environmental components. Overall, the choice of nutrients, atmosphere, temperature, and time of incubation are the four primary elements that determine the growth of bacteria (Lagier *et al*., 2015). Blood agar plates, MacConkey agar plates, MacConkey agar with sorbitol, Hektoen enteric (HE) plates, and xylose lysine deoxycholate (XLD) plates are used for bacterial culture (Cho and Yoon, 2014).

A pure bacterial culture remains essential for the study of its virulence, its antibiotic susceptibility and its genome sequence in order to facilitate the understanding and treatment of caused diseases. The first culture conditions empirically varied incubation time, nutrients, atmosphere, and temperature; culture was then gradually abandoned in favor of molecular methods (Lagier *et al*., 2015). However, an enrichment step is necessary for enhancing the detection and isolation of target pathogens when healthy cattle with possibly low levels of shedding are being evaluated (Pao *et al*., 2005).

Nonselective culture media contain no inhibitors and should permit the growth of most of the microorganisms present in the clinical samples studied. Meat infusions or heart or brain extracts are the initial substrates used empirically. Yeast extracts remain among the major components of several culture media. Vegetable components can also be used (Lagier *et al*., 2015).

Most of the species implicated in clinical microbiology are Mesophilic bacterial species and these species grow at medium temperatures of 25°C to 45°C. Most clinical pathogens grow easily over 24 to 48 hours in plate media, but several bacterial species require a much longer time, whereas most routine laboratories maintain cultures within days(Lagier *et al*., 2015).

Escherichia coli

A special request is required to test *E. coli,* which uses Sorbitol-MacConkey agar, eosin methylene blue agar, Luria agar or Luria broth at 37°C overnight or combination of broth culture and a Shiga toxin enzyme immunoassay (EIA). If the sample is positive, the culture tested confirms the presence of *E. coli*. Another method is using a culture of stool or rectal swab samples on thiosulfate citrate bile salts-sucrose agar (TCBS) agar or other media (Bennett *et al*., 2009). Optimum growth of *E. coli* occurs at 37 °C, but some laboratory strains can multiply at temperatures up to 49 °C (Fotadar *et al*., 2005).

Salmonella

Individual samples for bacteriological tests are collected as aseptically as possible and in the case of clinical disease or routine monitoring, samples should be collected before any antibiotic treatment has commenced. There are numerous methods for isolation and detection of *Salmonella* in use worldwide. The culture techniques and media that may work best in a particular diagnostic situation depend on a variety of factors including the *Salmonella* serovar, source and type of specimens, animal species of origin, experience of the microbiologist and availability of selective enrichment and selective plating media. The core of the standard method is pre-enrichment in buffered peptone water, enrichment on modified semisolid Rappaport Vassiliadis (MSRV) and isolation on xylose lysine deoxycholate (XLD) and an additional plate medium of choice(OIE, 2016).

Most subspecies of *Salmonella* produce hydrogen sulfide which can readily be detected by growing them on media containing ferrous sulfate such as used in the triple sugar iron test. *Salmonella* can also be detected and subtyped using multiplex (Alvarez, 2004) or real-time polymerase chain reactions (PCR) from extracted *Salmonella* deoxyribonucleic acid *(*DNA). *Salmonella* reproduce asexually with a cell division interval of 40 minutes (Wymann, 2006).

After 24± 3 hours and after 48± 3 hours if extended incubation has been applied, examine the selective agar plates for typical and atypical colonies of *Salmonella* (PHE, 2019). These are solid selective agars that permit differential growth to varying degrees. They inhibit growth of bacteria other than *Salmonella* and give information on some of the principal differential biochemical characteristics usually non-lactose fermentation and hydrogen sulphide (H_2S) production. The results are read after 24 and 48 hours of culture at 37°C. *Salmonellae* form characteristic colonies on such media that are usually distinguishable from the colonies of other bacteria on the plate (OIE, 2016). The presumptive *Salmonella* colonies (4-5 colonies/plate) appearing slightly transparent red halo with a black center surrounded by a pink-red zone on xylose lysine deoxycholate (XLD) agar will be screened further for its biochemical characterization (Nair *et al.*,2015). *Salmonella* species do not normally ferment sucrose or lactose and produce red colonies surrounded by a bright red medium on brilliant green agar (PHE, 2019).

Biochemical tests

E. coli produces strong acids and usually gas forms by the fermentation of d-glucose (positive in the methyl red test) and do not produce acetyl-methyl carbinols (acetoin) (negative in the Voges-Proskauer test). Lysine is decarboxylated by the majority of the strains. Indole test is also positive in case of *E. coli.* (Kar *et al.*, 2017).

Escherichia coli STEC is sorbitol fermenter (unless O157) but *Escherichia coli* O157 sorbitol nonfermenter. Sorbitol nonfermenting *E. coli* isolated on Sorbitol-MacConkey agar (SMAC) or Cefixime and tellurite sorbitol-MacConkey (CTSMAC) agar should be tested in O157 specific antiserum or latex reagents in order to confirm O157 STEC. Caution must be used with these reagents as other species of bacteria may cross-react with O157 antiserum requiring biochemical confirmation of *E. coli* prior to reporting (Humphries and Linscott, 2015). The majorities of *Salmonellae* are non-lactose fermenters (Quinn *et al*., 2011).

A straight inoculating needle is used to take isolated colony from culture. The TSI slant is inoculated by stabbing the butt down to the bottom and then streaked over the surface of the slant. Then, the TSI slant is incubated overnight at 37ºC. Test result for *Salmonella* positive is production of H_2S; blackening of the medium (Kar *et al.*, 2017).

Biochemical confirmation using analytical profile index 20 E must be carried out on suspect colonies that auto-agglutinate or give agglutination with any of the polyvalent 'O' or 'H' antisera. If all colonies have the same morphology and exhibit the same serological reactions perform API on one pick only. After incubation at 37 ± 1°C for 21 ± 3 hours it will be necessary to add reagents to the API 20E test strip. Disposable gloves must be worn and reagents must be added in a fume hood. Acceptable profiles are good, very good or excellent identification with a percentage of identification $\geq$ 90% and a T index ≥ 0.25. If a doubtful or unacceptable profile is obtained recheck the purity; if pure and the presence of *Salmonella* remains a possibility, send the strain to the reference laboratory for further identification. If biochemical results exclude the presence of *Salmonella* and the strain is pure no further action is required (PHE, 2019).

2.3. Diagnosis of Calf Diarrhoea

Diarrhea can be fatal to neonatal calves due to dehydration and acidosis that may result in anorexia and ataxia. Clinical and farm history is not always conclusive in determining the cause of diarrhea. Classical approach includes examining fecal sample are examined by microscopy (for *C. parvum* and Coccidia), bacterial culturing (for *Salmonella spp., E. coli,* and *C. perfringens*), and PCR (for BRV and BCoV). In addition, intestinal tissues can be subjected to immunohistochemistry or bacterial culturing. Since various pathogens or factors have been implicated in the development of diarrheic disease, laboratory testing is necessary for accurate assessment of the problem (Cho and Yoon, 2014).

A quick diagnosis is critical for not only quickly confirming the cause but also helping clinicians and cattle producers to implement appropriate interventions in a timely manner (Nossair *et al.*, 2018). Difficulties in the clinical diagnosis of infectious diarrhoea arise from frequent nonspecific clinical signs and lesions, the presence of asymptomatic infections, the involvement of multiple agents and the interplay of intrinsic and extrinsic factors that predispose the host to enteric dysfunction and pathology (Brar *et al.*, 2017). Laboratory test methods for enteric pathogens have typically included pathogen isolation and identification and histopathology as the gold standard for agent and disease confirmation (Cho, 2012).

Fecal bacteria culture is a commonly used laboratory test to isolate and identify bacterial pathogens in fecal swabs and intestinal contents. *Salmonella spp*., *E. coli* K99+ and *C. perfringens* are primary bovine enteric bacterial pathogens. In order to prevent any cross contamination, feces should be collected directly from diarrheic calves through either rectal swabs or rectal stimulation. Once collected, fecal samples should be stored in a transport medium or special stool container in a cooler or on ice for submission to a diagnostic lab in order to minimize loss of viability ((Larson *et al.*, 2004; Ferrarezi *et al.*, 2008; Cho, 2012).

However, many of enteric pathogens are difficult to isolate from gastrointestinal environment conditions. Direct visualization (e.g. microscopy, electron microscopy) of pathogens in feces or intestinal contents or detection of antigens (e.g., antigen-capturing ELISA) or nucleic acids (e.g., PCR) of pathogens in specimens (feces, content and intestine) have been widely accepted alternative methods. Most of veterinary diagnostic laboratories use a variety of tests concurrently when to test the samples for enteric pathogens (Cho, 2012). Molecular diagnostic assays on fecal specimens have recently emerged for enteric pathogens including several multiplex PCR approaches using real-time cyclers, gel electrophoresis or hybridization-based detection (Liu *et al*., 2012).

2.4. Public Health Importance

Over 1.7 billion global cases of diarrheal disease are reported annually and are associated with an estimated 2.2 million deaths. The burden of diarrheal disease is most critical in developing countries, facilitated by unsafe water supplies, poor sanitation and nutritional deficiencies (Humphries and Linscott, 2015). Diarrheal diseases are a major cause of illness and death in low and middle-income countries, where there are over 1.5 billion diarrhea cases that occur annually among children less than 5 years old resulting in nearly 700,000 deaths (Walker *et al.*, 2013). According to the World Health Organization (WHO), diarrheal diseases are the second leading cause of death (~760,000 per year) in children less than 5 years of age (Shaaban *et al*., 2018). Diarrhea may be infectious caused by bacteria, viruses or parasites (Humphries and Linscott, 2015).

Human salmonellosis is one of the most common and economically important zoonotic diseases. The Centers for Disease Control and Prevention (CDC) estimates that salmonellosis causes more than 1.2 million illnesses each year in the United States of America with more than 23,000 hospitalizations and 450 deaths (CDC, 2013). The most common cause of infection with *Salmonella* is eating contaminated foods including raw or under cooked eggs or egg products, meat, poultry, contaminated fresh fruit and vegetables and soft cheeses made from unpasteurized milk (OIE, 2016).

In developing countries including Ethiopia up to 2 million people die per year due to disease of foodborne pathogens. Over the last 20 years, the emergence of major foodborne pathogens such as *Salmonella* and *E. coli* have persisted as a major public health concerns and provide clear examples of the persistence of foodborne pathogens despite considerable efforts aimed at prevention and control (Mengistu *et al*., 2017).

Ethiopia ranks second after Nigeria in the health burden of zoonotic diseases in Africa. This country is located in a sub-region that experiences the second highest foodborne disease burden in the world, where *E. coli* O157 is one of the leading causes of foodborne disease disability adjusted life years. In Ethiopia, years of life lost due to diarrheal diseases was 2.6 million and diarrheal diseases are the second leading cause of premature death after lower respiratory infections. A study conducted on diarrheic children under 5 years in northern part of Ethiopia, *E. co*li O157:H7 was 28.9% (Beyi *et al*, 2017).

Escherichia coli and non-typhoidal *Salmonella enterica* infections have a high occurrence in most countries (Seimenis and Battelli, 2018). *Escherichia coli* is one of the causes of gastrointestinal diseases worldwide causing millions of illness annually (Assefa and Bihon, 2018). *Salmonella* can also be spread to people through contact with infected birds, livestock, reptiles, amphibians, and dogs and cats. These animals may carry the bacteria even when apparently healthy. Many serovars including some that are host-adapted such as *S. Choleraesuis* and *S. Dublin* have been shown to cause serious disease in humans. Abattoir workers, animal attendants and veterinarians may be infected directly during the course of their work when in contact with infected animals. Laboratory personnel may also acquire the infection if safe working practices are not implemented (OIE, 2016).

2.5. Antimicrobial Susceptibility Testing

Antimicrobial agents are essential drugs for human and animal health and welfare. The World Organisation for Animal Health (OIE) recognises the need for access to antimicrobial agents in veterinary medicine. Antimicrobial agents are essential for treating and controlling infectious diseases in animals (OIE, 2015). Antimicrobial agents are considered popular to fight diarrhea in calves. Nevertheless, their wide spectrum of activity, the emergence of microbial tolerance of different antimicrobial agents has become a well-known phenomenon, which represents a major concern (Hajipour *et al*., 2013).

Antimicrobial resistance is a global problem in general, but it might be more severe in Ethiopia where there is lack of antimicrobial resistance assessments of *Salmonella* and lack of rigorous regulations, but there is easy access of antimicrobials for purchase of people without prescription and incomplete treatment courses as the result of patient non-compliance (Beyene *et al*., 2011). The historical and growing emergence of drug resistance among *E. coli* and *Salmonella spp*. strains isolates from humans and animals has increased the debate on public health hazard associated with the use of antibiotics in animal production (Hur *et al*., 2012; Rostagno and Callaway, 2012; Tadesse *et al*., 2012; Keelara *et al*., 2013).

The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognised by the World Organisation for Animal Health (OIE), the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as a serious global human and animal health problem. The development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon. It is, however, an increasingly troublesome situation because of the frequency with which new emerging resistance phenotypes are occurring among many bacterial pathogens and even commensal organisms (OIE, 2015).

Tests to determine the most suitable antibiotic for the effective treatment in a given disease can be conducted on isolates from clinical cases (Quinn *et al.*, 2011). The purpose of performing antimicrobial susceptibility testing (AST) is to provide in vitro data to help ensure that appropriate and adequate antimicrobial therapy is used to optimize treatment outcomes(Goldman and Green, 2009). A number of antimicrobial susceptibility testing (AST) methods are available to determine bacterial susceptibility to antimicrobials. The selection of a method is based on many factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, and individual preference (OIE, 2015). Three methods have been shown to consistently provide reproducible and repeatable results when followed correctly:

1. Disk diffusion:-Disk diffusion refers to the diffusion of an antimicrobial agent of a specified concentration from disks, tablets or strips, into the solid culture medium that has been seeded with the selected inoculum isolated in a pure culture. Disc diffusion tests are the most commonly used methods in a laboratory to determine susceptibility of bacteria isolates to antibiotics (OIE, 2012; Parija, 2012).
2. Broth and agar dilution: - The aim of the broth and agar dilution methods is to determine the lowest concentration of the assayed antimicrobial that inhibits the visible growth of the bacterium being tested (OIE, 2012).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Awash Fentale district, zone 3, Afar Regional State of Ethiopia from December 2018 to September 2020. The zone is located in the part of the middle Awash basin about 225 km north east of Addis Ababa. Geographically, the study area Awash Fentale located between $8^0$52'to $9^0$24'North latitude and $39^0$40'to 40^0 20'East longitudes (Figure 4). The altitude range is from 743 – 1387m above sea level and the majority of the land is rocky with annual rain fall range 150–500mm per year. The average daily ranges of minimum and maximum temperature are 25^0C and 39^0C, respectively. Awash Fentale is bordered on the south by the Oromia Region, on the west by the Amhara Region, on the north by Dulecha, and on the east by Amibara. The district has 5 kebeles with total population 29,076 within 5101 households (Alelign *et al*., 2018; Assefa *et al*., 2018). Towns in Awash Fentale include Awash Sebat Kilo and Sabure. Rivers in this woreda include the Awash and its tributary the Germama. A large portion of this woreda is occupied by the Awash National Park. The soil texture of the area is characterized under sandy loam which accounts 55% sand, 40% silt (mud) and 5% of clay. The water holding capacity of such soil is very poor (Mesfin, 2017). The dry season is from May to June while the main rainy season extends from July to September accounts for above 60% of the annual total rainfall. The livestock populations of the Awash Fentale district are composed of cattle 86085, sheep 35389, goat 83691, horses, asses 45260, mules 4355, camels 41245 and poultry 4355 (CSA, 2012).

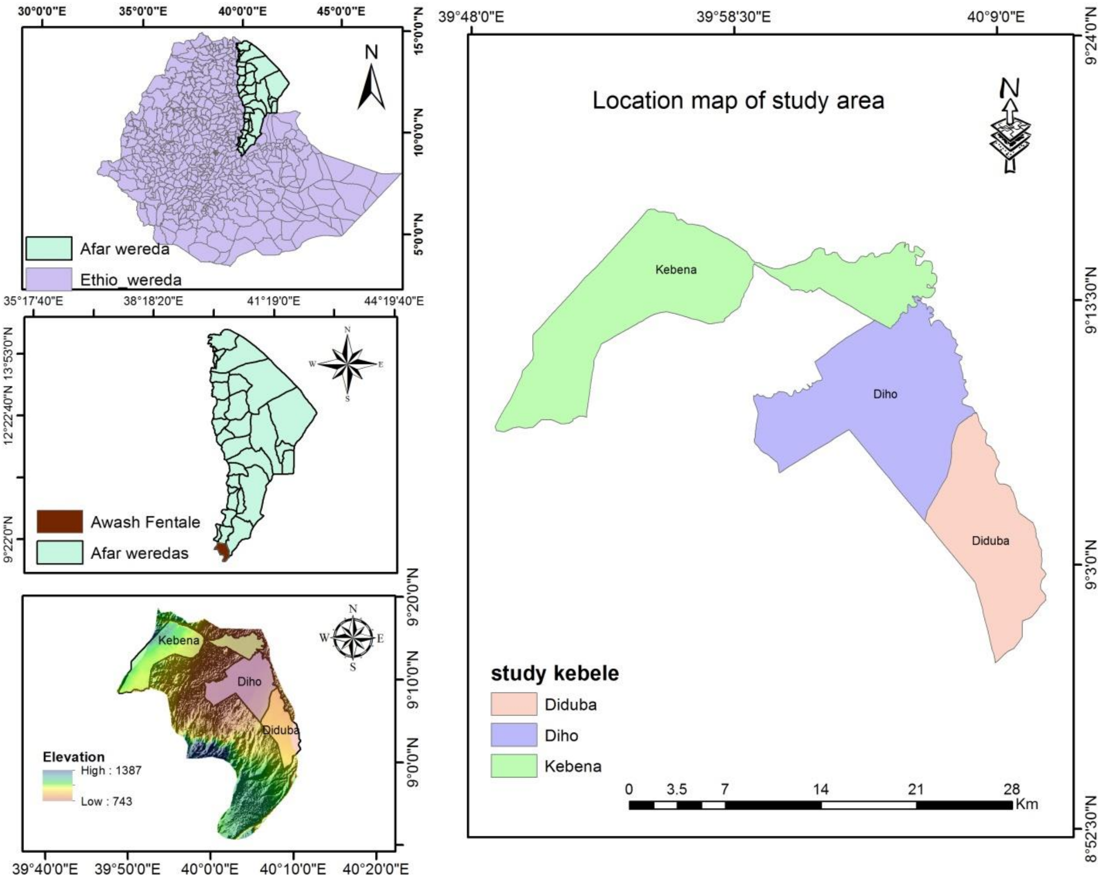

Figure 4: Map of study area, Diduba, Diho and Kebena in Awash Fentale district (Map constructed using Arc Version 10.4 GIS Software, 2015)

3.2. Study Animals

Calves of both sexes up to 6 months old affected with diarrhea and exhibiting clinical signs were included in the study. These calves showed, but had not been treated with antibiotics before. The study calves were local breeds, kept under traditional extensive management system from three selected Kebele namely Diho, Diduba and Kebena.

3.3. Study Design and Sampling Method

A cross-sectional study design was undertaken to isolate and identify *E.coli* K99 and *Salmonella enterica* from calves' diarrhea and assess associated risk factors. Individual animals were carefully identified and sex, age, species were recorded. Purposive type sampling method was conducted to select households and calf sample. The sample size was determined based on the availability of diarrheic calf (case). 50 households from each three Kebele were selected purposively by the “The project Addressing causes of young stock mortality in Ethiopia” based by considering on accessibility to vehicle or proximity to road and distribution of study animal population. Accordingly a total of 188 fecal samples from diarrheic calves were collected from 150 households in 3 Kebeles namely Diho, Diduba and Kebena in Awash Fentale district. Then collected samples were confirmed on the basis of their, culture, pathasure kit, biochemical tests, Biolog and antimicrobial testing using standard bacteriological procedures described by Quinn *et al*. (2011) and OIE (2016).

For the questioner survey, questionnaire was administered and about 50 households were interviewed as calves taken for sample collection to gather information about risk factors to exposure of *E.coli* K99 and *Salmonella enterica*.

3.4. Sample Collection and Transportation

Fecal samples were aseptically collected from the rectum of each the calves with sterile hand glove those showing clinical manifestations of calf diarrhea by rectal stimulation while avoiding environmental contamination. Approximately 10 ml of fecal samples were collected from non-treated diarrheic calves directly from the rectum by using disposable plastic gloves and transferred immediately to sterile plastic container. Gloves were changed between calves to reduce the risk of contamination during sample collection which will affect the result after laboratory diagnosis. The containers were labeled legibly using permanent marker with individual identifier and placed in ice box. The samples were protected from light, extreme temperatures and desiccation. The samples were transported in ice box to Aklilu Lemma Institute of Pathobiology laboratory, microbiology department for isolation and identification of bacteria. Feces were stored at 4 °C until the time of processing (OIE, 2008).

3.5. Questionnaire Survey

A structured questionnaire was administered to calves' owners to assess the general calf husbandry practices of the households. Management as well as herd and calf-level risk factors associated with diarrhea causing bacteria, *E.coli* K99 and *Salmonella enterica* were observed and assessed during sample collection. Questionnaire formats and checklists were developed. The checklist was contained household ID, young stock ID (code number of the calf), sex, age and diarrhea type. Thus the information that was included in the questionnaires were colostrum feeding, age of dam at parturition, dam gestation number (parity), area where the animal lies down look clean and dry, animal housed on bedding, general health care, herd size, occurrence of calf diarrhea as well as disease control measures practiced in the herd. The format was filled directly by face to face interviewing (Annex I).

3.6. Laboratory Investigation

3.6.1. Isolation and identification of E.coli K99

Antigen test kit (ELISA) for the detection of *Escherichia coli* K99

Pathasure Enteritis 4 is a sensitive and specific diagnostic kit based on the ELISA technology. The presence of infectious agents in tested samples is detected using highly specific antibodies and is revealed by means of a colour reaction easily detected by eye. Homogenizing the concentrated wash solution, it was diluted at 1/10 with purified water. Fecal samples were diluted in 1X wash solution at 1/10 and each dilution was properly mixed before being distributed into the wells. Then diluted fecal samples were incubated in wells coated with a mixture of 4 antibodies (Abs) specific to rotavirus, coronavirus, *E. coli* K99 and *Cryptosporidium parvum* at 23 ± 2°C for 30 minutes. After washing each well 5 times with wash solution to eliminate unbound substances, a conjugate (an antibody coupled to an enzyme) targeted at rotavirus, coronavirus, *E. coli* K99 and *Cryptosporidium parvum* was added. After incubation at 23 ± 2°C for 30 minutes, excess of this conjugate was eliminated by a second wash and its attachment to the specific pathogen was revealed with a chromogenous substrate. Following incubation period at 23 ± 2°C for 10 minutes, the enzyme reacted with the substrate and a color was developed. The intensity of the color allowed the determination of the type of sample tested (Annex II). A negative sample was displayed a weak reaction (colorless, pale blue) whereas a positive sample showed a reaction stronger than the one for the negative control (red, darker blue, green and violet than negative control) (Biovet, 2009).

Formerly positive fecal samples in ELISA test were plated directly on to buffered peptone water. Then incubated feces to minimise overgrowth by other organisms were 6 hours at 37°C. Enrichment broths were pre-warmed to prevent cold-shocking the organisms and slowing their initial growth. Selective culture was done by inoculating on MacConkey agar containing 1% D-sorbitol. MacConkey agar with sorbitol was used because inexpensive medium on which nonsorbitol fermenting *E. coli* grow as small, round greyish-white colonies(OIE, 2016). Medium size and bright ink to red with flat or elevated surface and complete weight edge colonies was seen on MacConkey agar (Quinn *et al.*, 2011). Media were inoculated in a logical order from least selective to most selective to avoid the inhibition of organisms to the selective agent. First indicator media MacConkey and second selective media Eosin methylene blue agar were used and bright pink, green metallic sheen colonies were observed respectively (Annex X, Figure 10).

3.6.2. Isolation and identification of Salmonella enterica

The conventional bacteriological methods were used to isolate *Salmonella enterica* from faecal samples. All media were prepared according to manufacturer's direction. **Non-selective pre-enrichment**: The fecal samples at ratio of 1:10 were inoculated in 0.1% buffered peptone solution and incubated at 37^0C for 18-20 hours for pre-enrichment. **First selective enrichment**: 0.1 ml and 1 ml of the culture obtained from incubated buffered peptone water broth were transferred to a tube containing 10 ml of the RVS broth and 10 ml MKTTn broth respectively. Then inoculated RVS broth and MKTTn broth were incubated at 41.5 °C, 37 °C for 24 and 48 hours respectively.

Second selective enrichment and plating out and identification: A loop full from the inoculated and incubated RVS and Muller-Kauffmann tetrathionate broth was streaked on XLD and on SSA agar plates and incubated at 37^oC for 24 hours. After development of characteristic black centered colony the positives were selected and inoculated onto nutrient agar plates to allow well-isolated colonies at 37 °C for 24 hours (Annex X, Figure 11). Presumptive *Salmonella* colonies from nutrient agar were phenotypically confirmed by biochemical properties in differential agars, such as TSI, LIA and Simmons citrate after incubation at 37 ºC for 16-24 hours (Quinn *et al.*, 2011; OIE, 2008).

Biochemical confirmation of *Salmonella enterica*

Urease test: A portion of well isolated colony was streaked on the surface of a urea agar slant to detect the ability of an organism to produce an enzyme urease which splits urea to carbon dioxide and ammonia. Then it was left the cap on loosely and incubated the test tube at 37^0c in ambient air for up to 24 hours.

Triple sugar iron agar: A straight inoculating needle was used to pick up isolates from culture of isolated colony. The TSI slant was inoculated by stabbing the butt down to the bottom, and then streaked over the surface of the slant back and forth. The TSI slant was then incubated for 16 hours and 24 hours at temperature of 37^0C. The positive result for *Salmonella* was detected based on the properties blackening of the medium provided by hydrogen sulphide gas production. The typical reaction for *Salmonella* in TSI agar, a red (alkaline) slant, yellow(acid) butt and superimposed (black) H_2S production (R/Y/H_2S+) was observed(Table 3).

Lysine iron agar (LIA) test: Lysine iron agar was inoculated with a straight needle by stabbing to the base of the butt and streaking the slant. The caps of the tubes were replaced loosely so that aerobic conditions prevail on the slant. Then incubated at 37°C in ambient air for 24 hours. After 24 hours incubation period purple slant/black butt, color was observed (Figure 5).

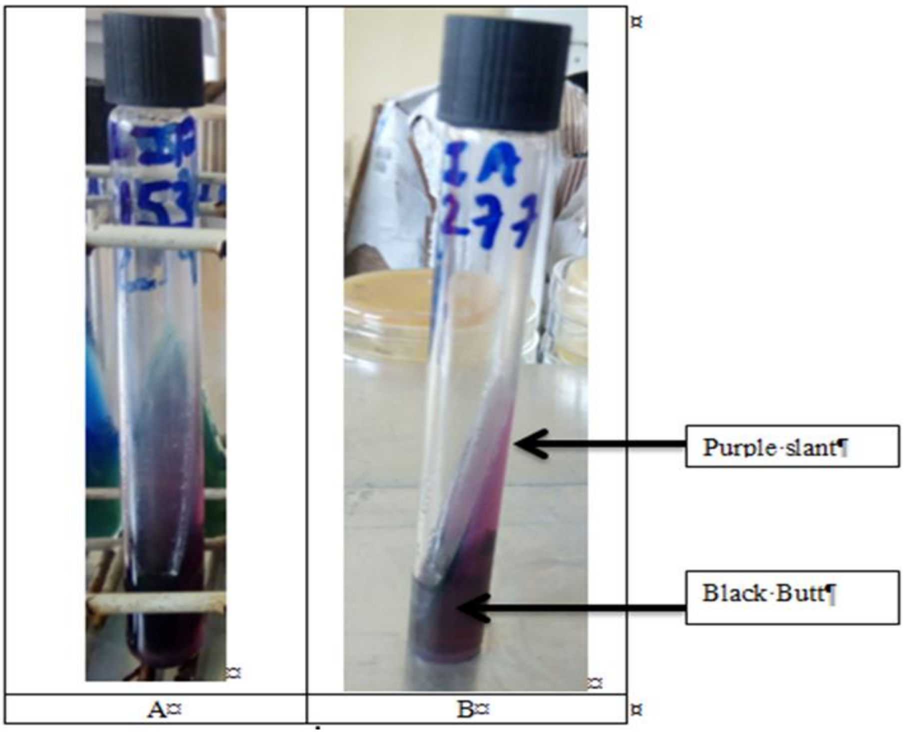

Figure 5: Lysine iron agar:-Both A and B are positive for *Salmonella species*

Citrate utilization test: Simmons citrate solid slant agar medium was inoculated with *Salmonella* isolated colony to test for ability to use citrate as carbon source. Then, the Simmons citrate slant was incubated at 35^0C to 37^0C for 18 to 24 hours. After 24 hours incubation blue color was observed (Figure 6).

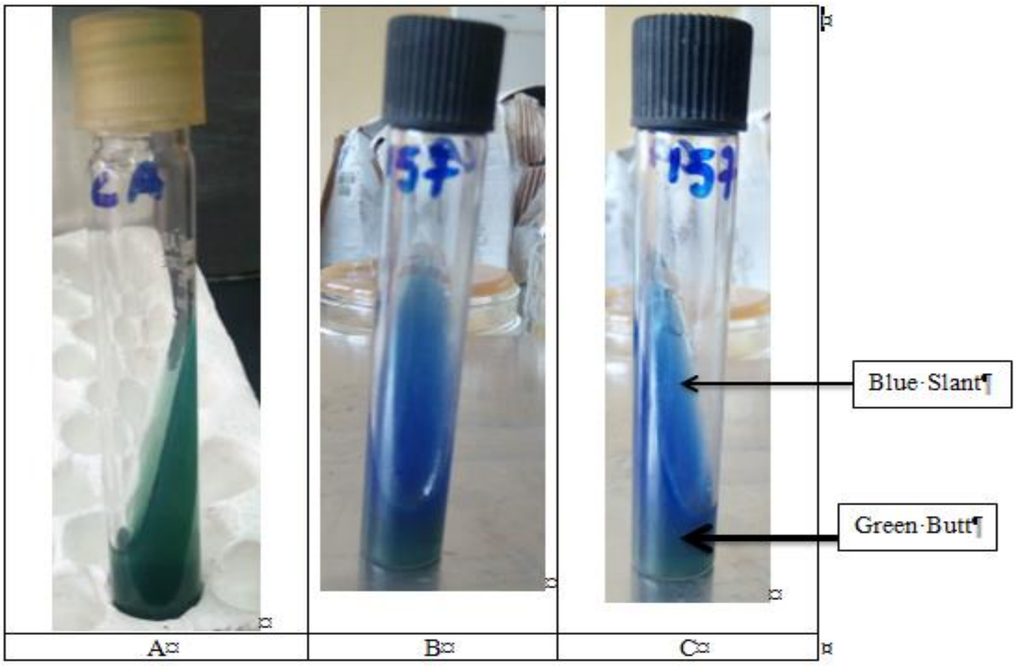

Figure 6: Citrate utilization test:-A: control, B and C are *salmonella species*

Table 3: Summary of biochemical tests results used to identify *Salmonella enterica*

S/No.	**Biochemical test**	**Positive reaction**		
		Color change		**H_2S production**
		Slant	**Butt**	
1.	TSI	Red	Yellow	Yes, Black color
2.	LIA	Purple	Black	Yes, Black color
3.	Citrate utilization test	Blue	Green	Yes, Black color
4.	Ureases test	Pale yellow color	Pale yellow color	No change in black color

Identification of *Salmonella enterica* by using Biolog GEN III microplate

Biolog is powerful carbon source utilization technology accurately identifies environmental and pathogenic microorganisms by producing a characteristic pattern or “metabolic fingerprint” from discrete test reactions performed within a 96 well microplate. The scope of the 96 assay reactions, coupled with sophisticated interpretation software, delivers a high level of accuracy that is comparable to molecular methods. The one minute per sample set up is much simpler and faster than DNA sequencing and the automated pattern matching eliminates the need for training and expertise in gene sequence interpretation (Biolog, 2011).

13 *Salmonella* isolates were confirmed using biochemical tests from a total of 188 fecal samples. Then these 13 isolates were sent to Ethiopian Biodiversity institute for identification *Salmonella enterica* by using Biolog GEN III microplate. Agar plates was streaked using correct techniques on Biolog Universal growth (BUG) agar medium to generate well isolated colonies. A single colony grown on BUG agar medium was selected and emulsified into inoculating fluid A. According to the manufacturer’s instructions, cell density of the bacterial inoculum was measured for a specified transmittance (90 to 98%) using a turbidimeter, as specified in the user guide. For each isolate, 100 µl of the cell suspension was inoculated in to each of the 96 well coated micro plates, using automatic multichannel pipette and incubated aerobically at 33°C for 22 hours (Biolog, 2011). The OmniLog identification system automatically read each micro plate and provided identification called species/sub-species ID. The

result was read in the BIOLOG Micro Station reader after 22 hours incubation outside GEN III incubation then printed (Annex III).

3.6.3. Antimicrobial susceptibility testing for isolated Salmonella enterica

Antimicrobial susceptibility testing of *Salmonella enterica* isolates was performed using disc diffusion method on Muller Hinton agar medium according to Office of International Epizootics Terrestrial Manual (OIE, 2012). Results, sizes of the zones of inhibition were interpreted in accordance with Clinical and Laboratory Standards Institute guidelines (CLSI, 2016). The following 12 commonly antibiotic discs were used: ampicillin (10µg), amoxicillin+clavulanicacid (20/10µg), amikacin (30µg), chloramphenicol (30µg), cephalothin (30µg), ceftriaxone (30µg), ciprofloxacin (5µg), tetracycline(30µg),sulfamethoxazole+trimethoprim(23.75/1.25µg),sulfisozazole(0.25mg), streptomycin (10µg) and gentamycin (10µg). The antimicrobial susceptibility was determined according to the standard diameter of inhibition for each antibiotic used according to guidelines set by the Clinical and Laboratory Standards Institute (CLSI, 2016).

Table 4: Antimicrobial discs, concentration and interpretation standards of their action on *Salmonella* isolates

Antimicrobial agent	**Disk content**	**Zone diameter interpretive criteria (nearest whole mm)**		
		S	**I**	**R**
Ampicillin(AM)	10µg	≥ 17	14–16	≤ 13
Amoxicillin clavulanic acid(AMC)	20/10 µg	≥ 18	14–17	≤ 13
Amikacin(AN)	30 µg	≥ 17	15–16	≤ 14
Chloramphenicol(C)	30µg	≥ 18	13–17	≤ 12
Cephalothin(CF)	30µg	≥ 18	15–17	≤ 14
Ceftriaxone(CRO)	30µg	≥ 23	20–22	≤ 19
Ciprofloxacin (CIP)	5 µg	≥ 21	16–20	≤ 15
Tetracycline(TE)	30µg	≥ 15	12–14	≤ 11
Trimethoprim(1.25µg)- sulfamethoxazole (SXT)(23.75µg)	25µg	≥ 16	11–15	≤ 10
Sulfisoxazole (G)	0.25mg	≥ 17	13–16	≤ 12
Streptomycin(S)	10µg	≥ 15	12–14	≤ 11
Gentamycin(GM)	10µg	≥ 15	13–14	≤ 12

Legend: µg = Microgram, S= Susceptible, I= Intermediate, R= Resistant strains, source (CLSI, 2016)

3.6.4. Salmonella enterica serotyping

Salmonella enterica isolates identified by Biolog GEN III microplate were sent to Public Health Agency of Canada, National Microbiology Laboratory at Guelph Reference Service and Laboratory OIE *Salmonella* Reference Laboratory 110 Stone Rd. West, Guelph, Ontario for *Salmonella* typing. Serotyping of *Salmonella enterica* was done from National Microbiology Laboratory at Guelph Reference Service and Laboratory OIE Salmonella Reference Laboratory 110 Stone Rd. West, Guelph, Ontario, and NIG3W4 (519)822-3300 on the basis of their antigen.

3.7. Data Management and Analysis

Data which describing the diarrheagenic conditions suggestive of *E.coli* K99 and *Salmonella enterica* infection observed on calves along with age, sex, herd size, time of birth, type of delivery, colostrum fed within 24 hours and parity were classified, filtered and coded. Data after laboratory test result was recorded in Microsoft excel® 2010 from prepared result recording format paper. The data was then imported to the software STATA windows version 14 (StataCorp LP, College Station, Texas, USA) for appropriate statistical analysis. Pearson Chi-square (X^2) test followed by multivariable logistic regression analysis was utilised to assess the degree of association between each risk factor and calf diarrhea causing *E.coli* K99 and *Salmonella enterica*. An association was regarded as significant if the p-value is <0.05.

3.8. Ethical Clearance

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This study was conducted after obtaining approval from Addis University Aklilu Lemma Institute of Pathobiology “Animal Experiments Ethics Committee”. In the study area there was a proper oral consent with the animal owner or household to collect fecal sample from their animal with the cooperation of veterinarians who give service to the pastoral community in the study site.

3.9. Limitations of the Study

- Failure to conduct antimicrobial susceptibility of *E.coli* K99 isolates in order to know drug resistance of the pathogen in calves due to lack of testing antibiotic disc. Because the objective of the project is addressing causes of young stock mortality likes infectious disease, such as *E. coli* K99, *Salmonella* and management problem.
- Failure to molecular characterization of the isolated bacteria.
- Lack of some chemicals and reagent which are important for isolation and identification of *Salmonella* using biochemical tests supplied by the project.
- Failure to present the result at the given schedule due to the project time management, shortage of transport vehicle and availability of laboratory resources.

4. RESULTS

4.1. Isolation and Identification of *Escherichia coli* K99

A total of 188 fecal samples were examined in laboratory for the isolation and identification of bacterium, of which 39 (20.74%) samples were positive for *E. coli* K99 by antigen test kit (ELISA). Highest percentage of *E. coli* K99 was found in Diho (29.6%) followed by Diduba (17.9%) and Kebena (13.6%) (Table 6). There was no significant difference (P>0.05) (P= 0.147) among three location for the prevalence of *E. coli* K99. Among 188 samples 3 of them were infected with both *E.coli* K99 and *Salmonella enterica* 1.6 %(3/188) (Table 5).

Table 5: Presence of mixed infection with both *E.coli* K99 and *Salmonella enterica* in diarrheic calves

Number of samples examined	Both *E.coli* K99 and *Salmonella enterica* positive	
	No. of positive	**% positive**
188	3	1.6

Table 6: Frequency distribution of *E.coli* K99 isolated from fecal sample of diarrheic calves within three Kebeles in Awash Fentale district

S/No.	Kebele	*E.coli* K99		Percentage of positive *E.coli* K99
		Negative	**Positive**	
1.	Diho	38	16	29.6
2.	Diduba	92	20	17.9
3.	Kebena	19	3	13.6
Total		149	39	20.7%

Pearson chi2 (2) = 3.8369, P value= 0.147

4.2. Isolation and Identification of *Salmonella enterica*

Salmonella species were identified using Biolog GEN III microplates, 10 (5.32%) were *Salmonella enterica.* Among these, *Salmonella enterica subspecies enterica, Salmonella enterica subspecies salamae* and *Salmonella enterica subspecies diarizonae* 5(38.46%), 4(30.78%) and 1(7.69%) were isolated respectively (Table 7).

Highest prevalence of *Salmonella enterica* was found in Diduba (7.1%) followed by Diho (3.7%) (Table 8). No samples were found positive in Kebena. There was no statistically significant difference (P>0.05) (P=0.324) in prevalence of *Salmonella enterica* subspecies for different locations within three Kebeles in Awash Fentale district. After culturing on media, *Salmonella species* revealed red colony with black center on MacConkey agar and pale, colorless, transparent, with a black center H_2S produced on Salmonella Shigella (SS) Agar. A typical *Salmonella* colony on XLD agar has a slightly transparent zone of reddish colour and a black centre; a pink-red zone was seen in the medium surrounding the colonies. On the basis of culture and morphological properties all suspected colonies of *Salmonella* were subjected to selected biochemical tests such as ureases test, citrate utilization test, lysine iron agar and triple sugar iron agar test.

4.2.1. *Identification of Salmonella enterica by using Biolog GEN III microplate*

After incubation of 13 *Salmonella* isolates aerobically at 33°C for 24 hours, then OmniLog identification system was automatically read each microplate and provided identification species ID. The result was read in the BIOLOG Micro Station reader then it was printed. Out of 13 *Salmonella* isolates 10 were positive for *Salmonella enterica* (Table 7).

Table 7: Result of Biolog isolated *Salmonella enterica*

Isolated *Salmonella enterica*	**No. of positive samples**	**Percentage**
Salmonella enterica ss enterica	5	38.46%
Salmonella enterica ss salamae	4	30.78%
Salmonella enterica ss diarizonae	1	7.69%
Citrobactor koseri/youngae	3	23.8%
Total	13	100%

Legend: *Salmonella enterica ss enterica =Salmonella enterica subspecies enterica, Salmonella enterica ss salamae=Salmonella enterica subspecies salamae* and *Salmonella enterica ss diarizonae =Salmonella enterica subspecies diarizonae*

Table 8: Frequency distribution of *Salmonella enterica* isolated from fecal samples of diarrheic calves within three Kebeles in Awash Fentale district

S/No.	**Kebele**	***Salmonella enterica***		**Percentage of positive**
		Negative	**Positive**	***Salmonella enterica***
1.	Diho	52	2	3.7%
2.	Diduba	104	8	7.1%
3.	Kebena	22	0	0
	Total	178	10	5.32%

Pearson chi2 (2) =2.2554; P= 0.324

4.2.2. *Salmonella enterica serotyping*

Serotyping of *Salmonella enterica* was done from National Microbiology Laboratory at Guelph Reference Service and Laboratory OIE Salmonella Reference Laboratory 110 Stone Rd. West, Guelph, Ontario, and NIG3W4 (519)822-3300 on the basis of their “K” antigen. The 10 *Salmonella enterica* isolates were serotyped as 8 S. ZANZIBAR (80%) and 2 (20%) unclassified (Table 9).

Table 9: Serotyping frequency distribution of *Salmonella enterica*

S/No.	**Serotype**	**Antigens**	**No.**	**Percentage**
1	II:58:k:1,6(not classified)	58:K:1,6	2	20
2	ZANZIBAR	10:K:1,5	8	80
	Total		10	100

4.3. Antimicrobial Susceptibility Testing of *Salmonella enterica* Isolates

Out of total 10 isolates screened for antimicrobial susceptibility test against twelve antibiotics discs, all isolates from calves were susceptible(100%) to ampicillin, amoxicillin+clavulanic acid, amikacin, chloramphenicol, cephalothin, ceftriaxone, ciprofloxacin, tetracycline, gentamycin and trimethoprim+sulfamethoxazole. Three isolates (30%) and one isolate (10%) were found to be susceptible to streptomycin and sulfisoxazole respectively. On the other hand, six isolates (60%) were found to be resistant for sulfisoxazole, whereas 7 isolates (70%) were intermediate against streptomycin (Table 10).

Table 10: Antimicrobial susceptibility testing results of *Salmonella enterica* isolates from diarrheic calves **(n= 10)**

Antibiotics	Susceptible (n (%))	Intermediate (n (%))	Resistant (n (%))
Ampicillin(AM)	10(100)	-	-
Amoxicillin clavulanic acid(AMC)	10(100)	-	-
Amikacin(AN)	10(100)	-	-
Chloramphenicol(C)	10(100)	-	-
Cephalothin(CF)	10(100)	-	-
Ceftriaxone(CRO)	10(100)	-	-
Ciprofloxacin (CIP)	10(100)	-	-
Tetracycline(TE)	10(100)	-	-
Trimethoprim sulfamethoxazole (SXT)	10(100)	-	-
Sulfisoxazole (G)	1(10)	3(30)	6(60)
Streptomycin(S)	3(30)	7(70)	0
Gentamycin(GM)	10(100)	-	-

4.4. Risk Factors Associated with the Presence of *E.coli* K99 and *Salmonella enterica*

Due to similar management of households in pastoral production system, no statistical comparisons were done for most of the household factors and no relevant association was found to various variables. However, some risk factors associated to the exposure of *E.coli* K99 and *Salmonella enterica* are presented in Table 11 and Table 12 below. The positive samples of *E. coli* K99 and *Salmonella enterica* in 1-60 days, 61-120 days and 121 -180 days age groups were 31, 5, 3 for *E. coli* K99 and 4, 4, 2 for *Salmonella enterica* respectively. Thus the prevalence of *E. coli* K99 and *Salmonella enterica* in 1-60 days, 61-120 days and 121-180 were 22.14%, 17.24% and 15.79 for *E. coli* K99 and 2.86%, 13.79, 10.53% for *Salmonella enterica* respectively within the age groups. There was statically significant difference within age groups in the presence of *Salmonella enterica* infection (P=0.033).

The total number of positive samples in male calves were 23,7 and the prevalence were 25.27%,7.69% and in the case of female calves, the total number of positive samples were 16,3 and the prevalence were 16.49%,3.09% for *E. coli* K99 and *Salmonella enterica* respectively. No significant difference between two sexes was found for both bacteria (P>0.05). The prevalence of *E. coli* K99 was 18.81% (n=19/101) in day time of birth and

20.99% (n=17/81) in night time of birth. The prevalence of *E. coli* K99 was similar in both time of birth. The p-value was calculated as 0.187 (P>0.05) that mean the results were statistically not significant. In the case of type of delivery, the occurrence of *E. coli* K99 was 20.93 %(36/172) in normal delivery and 16.67% (1/6) in assisted delivery. There was no association between type of delivery and occurrence of *E.coli* K99 in calves (P=0.967).

Colostrum fed within 24 hours was found to affect significantly the occurrence of *E.coli* K99 (P< 0.05; P=0.000). Thus, 73.33 %(11/15) of the cases were found to be not fed colostrum within 24 hours whereas 16.18 %(28/173) were fed colostrum within 24 hours. Continuous access of calf to dam during the first 24 hours significantly reduce the infection rate with *E.coli* K99 (P < 0.05; P=0.000). Thus, 14 out of 21(66.67%) infected calves did not get continuous access of colostrum to dam during the first 24 hours, whereas 25 of 167(14.97%) calves were have got continuous access of colostrum to dam during the first 24 hours.

Infection with *Salmonella enterica* was found to be significantly affected by colostrum fed within 24 hours (P< 0.05; P=0.008) (Table 11). Thus, 3 cases (20.00%) were found not fed colostrum within 24 hours, whereas 7 cases (4.05%) were fed colostrum within 24 hours. Significant association was not found between continuous access of calf to dam during the first 24 hours and infection with *Salmonella enterica* (P>0.05; P=0.362); two calves (9.52%) infected with *Salmonella enterica* were found not to be accessed the calf to the dam during the first 24 hours, while eight (4.79%) were found to be accessed the calf to the dam during the first 24 hours.

Host associated risk factors with the presence of *E.coli* K99 and *Salmonella enterica* in diarrheic calves such as sex, time of birth and type delivery did not affect significantly *E.coli* K99 and *Salmonella enterica* infection in the present investigation. On the other hand, dam and environmental associated risk factors in diarrheic calves including age of dam at parturition, parity, herd size and birth location did not affect *E.coli* K99 and *Salmonella enterica* infection significantly in the current study.

Table 11: Host associated risk factors with the presence of *E.coli* K99 and *Salmonella enterica*

Risk factors	Identification for	Category	No. examined	Positive	Percentage positive (%)	P-value
Age	*E.coli* K99	1-60 days	140	31	22.14	0.17
		61-120 days	29	5	17.24	
		121-180 days	19	3	15.79	
		Total	188	39	20.74	
	Salmonella enterica	1-60 days	140	4	2.86	0.033*
		61-120 days	29	4	13.79	
		121-180 days	19	2	10.53	
		Total	188	10	5.32	
Sex	*E.coli* K99	Male	91	23	25.27	0.138
		Female	97	16	16.49	
		Total	188	39	20.74	
	Salmonella enterica	Male	91	7	7.69	0.160
		Female	97	3	3.09	
		Total	188	10	5.32	
Time of Birth	*E.coli* K99	Day	101	19	18.81	0.187
		Night	81	17	20.99	
		Don't know	6	3	50.00	
		Total	188	39	20.74	
	Salmonella enterica	Day	101	6	5.94	0.362
		Night	81	3	3.70	
		Don't know	6	1	16.67	
		Total	188	10	5.32	
Type of delivery	*E.coli* K99	Normal	172	36	20.93	0.967
		Assisted	6	1	16.67	
		Don't know	10	2	20.00	
		Total	188	39	20.74	
	Salmonella enterica	Normal	172	8	4.65	0.346
		Assisted	6	1	16.67	
		Don't know	10	1	10.00	
		Total	188	10	5.32	
Colostrum fed within 24 hours	*E.coli* K99	Yes	173	28	16.18	0.000*
		No	15	11	73.33	
		Total	188	39	20.74	
	Salmonella enterica	Yes	173	7	4.05	0.008*
		No	15	3	20.00	
		Total	188	10	5.32	
Continuous access to dam during the first 24 hours	*E.coli* K99	Yes	167	25	14.97	0.000*
		No	21	14	66.67	
		Total	188	39	20.74	
	Salmonella enterica	Yes	167	8	4.79	0.362
		No	21	2	9.52	
		Total	188	10	5.32	

*P-value <0.05

Table 12: Distribution of *E.coli* K99 and *Salmonella enterica* with dam and environmental associated risk factors

Risk factor	Identification for	Category	No. examined	Positive	Percentage positive (%)	P-value
Age of dam at parturition	*E.coli* K99	3-7 years	125	27	21.60	
		8-12 years	58	1	18.97	0.19
		13-16 years	5	1	20.00	
		Total	188	39	20.74	
	Salmonella enterica	3-7 years	125	8	6.40	
		8-12 years	58	2	3.45	0.614
		13-16 years	5	0	0.00	
		Total	188	10	5.32	
Parity	*E.coli* K99	< 3	96	21	21.88	
		3	37	6	16.22	0.750
		>3	55	12	21.82	
		Total	188	39	20.74	
	Salmonella enterica	< 3	96	8	8.33	
		3	37	0	0.00	0.127
		>3	55	2	3.64	
		Total	188	10	5.32	
Herd size	*E.coli* K99	4-26 head	104	20	19.23	
		27-49 head	57	15	26.32	0.407
		50-70 head	27	4	14.81	
		Total	188	39	20.74	
	Salmonella enterica	4-26 head	104	7	6.73	
		27-49 head	57	1	1.75	0.353
		50-70 head	27	2	7.41	
		Total	188	10	5.32	
Birth location	*E.coli* K99	In barn	115	27	23.48	
		Outside	73	12	16.44	0.246
		Total	188	39	20.74	
	Salmonella enterica	In barn	115	6	5.22	
		Outside	73	4	5.48	0.938
		Total	188	10	5.32	

4.5. Multivariable Logistic Regression Analysis for the Association of Different Risk Factors with Presence of *E. coli* K99 and *Salmonella enterica*

The results described in Table 13 and 14 showed multivariable logistic regression analysis that was performed to know the association of different risk factors with the occurrence of *E. coli* K99 and *Salmonella enterica*. In multivariable logistic regression analysis, sex was not significantly (P=0.289) associated with occurrence of *E. coli* K99 in which it was not significantly associated with occurrence of *E.coli* K99 in chi square test. The occurrence of *E.coli* K99 was 1.58(95% CI=0.683-3.598) times more likely to occur in male caves than female calves. Here was statistically significant difference observed between the occurrence *E.coli* K99 and different risk factors like colostrum fed within 24 hours (P=0.033) and continuous access of calf to dam during the first 24 hours (P=0.002). With regard to colostrum feeding within 24 hours, the prevalence of *E.coli* K99 was 0.15(95% CI=0.028-0.861) times more likely to occur in calves fed colostrum within 24 hours than calves did not fed colostrum within 24 hours. There was no statistically significant difference observed (P >0.05) between the occurrence of the *E. coli* K99 and age, time of birth, type of delivery, birth location, herd size, age of dam at parturition and dam gestation number similarly with chi-square test.

Similarly colostrum fed within 24 hours (P=0.044) and age (P= 0.014) were significantly (P<0.05) associated with occurrence of *Salmonella enterica*. With regard to colostrum fed within 24 hours, the presence of *Salmonella enterica* was 0.15(95% CI=0.028-0.861) times more likely to occur in calves fed colostrum within 24 hours than calves not fed colostrum within 24 hours. Also, the occurrence of *Salmonella enterica* was 13.15(95% CI=1.677-103.127) times more likely to occur in 61-120 days age group than 1-60 days age group of the calves. Other hand, there was no statistically significant difference observed (P>0.05) between the occurrence of the *Salmonella enterica* with continuous access of calf to dam during the first 24 hours, age, time of birth, type of delivery, birth location, herd size, age of dam at parturition and dam gestation number.

Table 13: Multivariable logistic regression analysis showing the association of different risk factors with the presence of *E. coli* K99

Risk factors	Category	OR	95% CI	P-value
	1-60 days	Ref.		
Age	61-120 days	0.43	0.100-1.872	0.260
	121-180 days	0.48	0.105-2.155	0.335
Sex	Male	1.58	0.683-3.598	0.289
	Female	Ref.		
Time of Birth	Day	0.163	0.010-2.711	0.206
	Night	0.20	0.0123-3.120	0.251
Type of delivery	Normal	3.96	.438-35.808	0.221
	Assisted	0.87	0.016-48.152	0.946
Birth location	In barn	Ref.		
	Outside	0.67	0.261-1.718	0.404
Colostrum fed within 24 hours	Yes	0.15	0.028-0.861	0.033*
	No	Ref.		
Continuous access of calf to dam during the first 24 hours	Yes	0.10	0.022-0.426	0.002*
	No	Ref.		
Herd size	4-26 head	Ref.		
	27-49 head	0.73	0.263-2.050	0.555
	50-70 head	0.32	0.067-1.480	0.144
Age of dam at parturition	3-7 years	Ref.		
	8-12 years	0.33	0.040- 2.674	0.297
	13-16 years	0.11	0.002-4.728	0.246
Dam gestation number	< 3	Ref.		
	3	1.52	0.438-5.254	0.511
	>3	3.85	0.432-34.392	0.227

Legend: *statistically significant; OR-odds ratio; CI- confidence interval; Ref-reference category

Table 14: Multivariable logistic regression analysis showing the association of different risk factors with the presence of *Salmonella enterica*

Risk factors	Category	OR	95% CI	P-value
Age	1-60 days	Ref.		
	61-120 days	13.15	1.677-103.127	0.014*
	121-180 days	4.36	0.494-38.476	0.185
Sex	Male	3.49	0.5293-23.057	0.194
	Female	Ref.		
Time of Birth	Day	0.33	0.009-11.873	0.546
	Night	0.19	0.005-7.606	0.374
Type of delivery	Normal	0.27	0.013-5.712	0.401
	Assisted	Ref.		
Birth location	In barn	Ref.		
	outside	0.86	0.160-4.650	0.864
Colostrum fed within 24 hours	Yes	0.02	0.001-0.913	0.044*
	No	Ref.		
Continuous access of calf to dam during the first 24 hours.	Yes	28.94	0.423- 1979.635	0.119
	No	Ref.		
Herd size	4-26 head	Ref.		
	27-49 head	0.48	0.0433-5.398	0.555
	50-70 head	5.70	0.512-63.377	0.157
Age of dam at parturition	3-7 years	Ref.		
	8-12 years	.15	.0001-248.459	0.619
	13-16 years	1		
Dam gestation number	< 3	Ref.		
	3	1		
	>3	1.80	.001- 2908.273	0.876

Legend: *statistically significant; OR-odds ratio; CI- confidence interval; Ref-reference category

5. DISCUSSION

E.coli K99 and *Salmonella enterica* are the main pathogen and most commonly associated with diarrhoea in calves (Gyles *et al.*, 2010; Wani *et al.*, 2013; Tegegn, 2019). The involvement of *E.coli* K99 and *Salmonella enterica* in calf diarrhea have not been well studied in Ethiopia. This current cross sectional study was aimed to isolate and identify *E.coli* K99 and *Salmonella enterica* from diarrheic calves at the age of one day to six months old.

In the present study, the prevalence of *E. coli* K99 in diarrheic calf was 20.74%. This finding had nearly coincide with the findings of Kumar *et al*. (2018) in India (18.75 %), Rakib, (2016) in Bangladesh(19%), El Ayis *et al*. (2015) in Sudan(17.3%%) , Shahrani *et al*. (2014) (28.4%) and Selles *et al*. (2018) in Algeria(16 %) in diarrheic calves. A higher prevalence of *E. coli* K99 was reported by Acha *et al.* (2004) in Mozambique (40%) and Langoni *et al*. (2004) in Brazil (35 %). On the contrary, many previous studies mentioned a lower prevalence 5.3%, 5.80%, 11.3%, 11.42%, 12.9 %. of *E.coli* K99 were reported by Patel *et al*. (2019), Yadegari *et al*. (2019), Pourtaghi *et al*. (2016), Lacroute *et al*. (2016) and Picco *et al.* (2015) respectively. The differences in prevalence and frequency rates among the studies may be attributed to regional variations, management and hygienic conditions where *E.coli* K99 infection occurs mainly through ingestion of contaminated food and water, the age of the animals, vaccination of dam, colostrum intake, or different diagnostic methods (Picco *et al*., 2015; Cho and Yoon, 2014 and Içen *et al*., 2013).

Frequency distribution of *E.coli* K99 isolated from fecal sample of diarrheic calves within three Kebeles was 29.6%, 17.9% and 13.6% in Diho, Diduba and Kebena respectively. There is no significant difference within the three Kebeles in the frequency distribution of *E.coli* K99 *(*P>0.05) that was P=0.147. This may be due to the similarity of management; location and the production system within the study area. There was no previous report in this study area for comparation to current result.

Salmonella enterica is an important animal and human pathogen that can cause enteritis and septicaemia in calves (Casaux *et al*., 2019). Furthermore, *Salmonella* *enterica* serotypes are one of the most important foodborne pathogens, resulting in enteric disease, hospitalization, and deaths worldwide (Hur *et al*., 2012).

The occurrence of *Salmonella enterica* infection in diarrheic calves was 5.32% in present study. This finding is agreement with the result reported by Nethra *et al*. (2019) in India (5.0%) and Casaux *et al*. (2019) in Uruguay (6.1%). Thus obtained finding is higher than that previously reported results by Acha *et al*. (2004) in Mozambique (2%) and Aziz *et al*. (2018) in Egypt (1.8%). The detection of *Salmonella enterica* in calves is lower than those findings reported by Rakib (2016) in Bangladesh (10%) and El-Rahman *et al*. (2016) (13%) and Gharieb *et al*. (2015) (16.25%) in Egypt. The differences of the occurrence rates of *Salmonella* in calves than the previous reports may be attributed to the small age of calves which is highly susceptible to infection with *Salmonella* (Akam *et al*., 2004), the difference in breed, geographical locations, poor management practices and standards of hygienic measures as well as used laboratory diagnostic tests. There is no previous report in Ethiopia showing distribution of *Salmonella enterica* in calves up six months of age. In the current study, serotyping of *Salmonella enterica* isolates revealed that 8 *S. ZANZIBAR* (80%) and 2 unclassified (20%).

The intent of this study was also aimed to assess risk factors associated with *E.coli* K99 and *Salmonella enterica* infections in newborn diarrheic calves. Multivariable logistic regression analysis was done to identify the significant risk factors associated with examined bacteria. Colostrum feeding within 24 hours and continuous access of calf to dam during the first 24 hours were found to affect significantly the occurrence of *E.coli* K99 in diarrheic calves. Thus study showed that the occurrence of *E.coli* K99 in male calves was higher (25.27%) than in female calves (16.49%). This might be the reason that more care is given to female calves than male calf. Because female calf is needed by the pastoralist where compared with male calf. Further investigation should be conducted to reach conclusion to this assumption.

Calves in age group 1 to 60 days were more susceptible while that age group 61-120 days and 121-180 days were less susceptible. The high prevalence of *E.coli* K99 shown by calves in age group 1-60 days (22.14%) is in agreement to finding that was reported by Olaogun *et al*. (2016) in Nigeria (18.42%). The probable explanation for this finding may be related to either a poor and / or no absorption of colostrum and weakening of passive immunity. There was no statistically significant difference between age group in the occurrence of this bacterium in this finding.

It was evident that calves in household, which received colostrum within 24 hours from their dames were less frequent to be infected with *E.coli* K99 (P=0.033; OR=0.15; 95% CI=0.028-0.861) than those did not fed colostrum within 24 hours calves. Similarly, calves had continuous access of calf to dam during the first 24 hours were less frequent to be infected with the pathogen (P=0.002, OR=0.10, 95% CI=0.022-0.426) those did not have continuous access of calf to dam during the first 24 hours. There was high occurrence of *E.coli* K99 in calves that did not fed colostrum within 24 hours (73.33%) as compared to calves fed colostrum within 24 hours (16.18%). This result coincided with that previously recorded by Younis *et al*. (2009) who reported that *E.coli* K99 was significantly affected by colostrum feeding. This result agreement with that immunity to *E.coli* K99 infections was mainly promoted by passive transfer of antibodies to neonatal calves. The enterotoxigenic *E. coli*, bearing the K99 attachment factor, can be found in calves younger than three days, especially in animals that did not receive any colostrum or received colostrum without any specific antibodies against this pathogen. The antibodies produced by cows in response to natural infection or vaccination are transmitted to the calf at birth via the colostrum (Constable *et al*., 2016).

Final multivariable logistic regression analysis showed that age and colostrum fed within 24 hours were significantly associated with *Salmonella enterica* shedding. Calf age was significantly associated with *Salmonella enterica* (P=0.014, OR=13.15, 95% CI=1.677-103.127). This result agreed with that reported by Younis *et al*. (2009) who recorded age was significantly associated with *Salmonella enterica* in diarrheic calves in Egypt. The highest occurrence of this pathogen was recorded in the 61-120 days of age (13.79%) than 1-60 days of age (2.86%) and 121-180 days of age (10.53%) diarrheic calves. Similarly with previous reports by Fossler *et al*. (2005) that *Salmonella enteric* infection was usually recorded in the first 3 months of age causing severe symptoms. Colostrum fed within 24 hours was significantly associated with the infection of *Salmonella enterica* in diarrheic calves (P=0.044, OR=0.02, 95% CI=0.001-0.913). The higher prevalence *Salmonella enterica* in the present study might be attributed to the variations in environmental and management conditions of the households such as insufficient and/or poor-quality colostrum intake by the calves as stated by Charles *et al.* (2003). In addition to that poor hygiene often allows buildup of pathogenic strains in the young animal's environment.

Antimicrobial agents are indispensable for decreasing mortality and morbidity associated with infectious diseases in animals and humans (Tadesse *et al*., 2012). In veterinary medicine, they have been used for therapy, metaphylaxis, prophylaxis, and growth promotion (Schwarz *et al*., 2001), being the enteric diseases one of the main animal infections treated with antibiotics (Teuber, 2001). In the present study, *Salmonella enterica* isolated from diarrheic feces of calves were examined for antimicrobial susceptibility using 12 antibiotic discs. All 10(100%) isolates showed susceptible to ampicillin, amoxicillin+clavulanic acid, amikacin, chloramphenicol, cephalothin, ceftriaxone, ciprofloxacin, gentamycin, tetracycline and trimethoprim+ sulfamethoxazole. Six isolates (60%) were found to be resistant for sulfisoxazole. The susceptibility of these isolates to antimicrobials may be linked with infrequent use for in the study area. Likewise, similar result, a study conducted in Brazil, also showed that the most common susceptibility profile of *Salmonella enterica* isolated from calves was to amikacin, amoxicillin, ampicillin, ciprofloxacin, gentamicin, tetracycline, and trimethoprim+sulfamethoxazole (Souto, 2017). The susceptibility of *Salmonella* isolates to ciprofloxacin (100%) agreed with the reports of Guesh (2017). On the other hand, all the isolates were resistant to tetracycline (100%), streptomycin (91.4%),trimethoprim+sulfamethoxazole(74.3%),chloramphenicol(85.7%),amoxicillin +clavulanic (62.9%) and gentamycin (60%) reported by Baker *et al*. (2018). Similarly Casaux *et al*. (2019) in Uruguay reported that the most widespread resistance was against tetracycline (87.8%), streptomycin (85.4%) and ampicillin (22%). The wide variation in the observed results for *Salmonella enterica* isolated from calves could be due to differences on the tested antimicrobials, geographical regions, number of isolates and age of animals (Souto, 2017).

6. CONCLUSION AND RECOMMENDATIONS

This study was planned to isolate and identify *E.coli* K99 and *Salmonella enterica* from diarrheic calves with their associated risk factors. Multivariable logistic regression analysis and chi-square test were framed to find the risk factors behind the occurrence of *E.coli* K99 and *Salmonella enterica*. The prevalence of *E.coli* K99 and *Salmonella enterica* was 20.74%, 5.32% that obtained from 188 diarrheic calves, respectively. Three cases were mixed infection by *Salmonella enterica* and *E.coli* K99. In the present study, pathasure enteritis 4 (antigen test kit) and Biolog GEN III microplate enabled identification of *E.coli* K99 and *Salmonella enterica* in diarrheic calves from 1 day to 6 months of age respectively. Biolog GEN III microplate was found delivering a high level of accuracy that is comparable to molecular methods among available diagnostic tests. Pathasure enteritis 4 tests (ELISA) was found to be quick identification method of *E.coli* K99 which is compared with convectional laboratory methods like bacterial culture. Culture characteristics of *E. coli* K99, bright pink to red, yellow and distinct green metallic sheen colonies were observed on MacConkey, XLD and EMB agar media respectively. On the other hand, *Salmonella species* revealed red, pale and colorless, a pink-red zone colony with black center on MacConkey, Salmonella Shigella (SS) and XLD agar respectively. *Salmonella* showed positive reaction to selected biochemical tests such as ureases test, citrate utilization test, lysine iron agar and triple sugar iron agar test. This study revealed that 8 were *S. ZANZIBAR* (80%) from 10 serotyped *Salmonella enterica.*

The results of antimicrobial susceptibility testing revealed that all *Salmonella enterica* isolated from diarrheic feces of calves were susceptible (100%) to ampicillin, amoxicillin+clavulanic acid, amikacin, chloramphenicol, cephalothin, ceftriaxone, ciprofloxacin, gentamycin, tetracycline and trimethoprim+sulfamethoxazole.

Colostrum feeding within 24 hours and continuous access of calf to dam during the first 24 hours were found significantly associated with occurrence of *E.coli* K99 and *Salmonella enterica*. On the other hand there was no statistically significant difference observed between the occurrence of the *E. coli* K99 and *Salmonella enterica* with time of birth, type of delivery, birth location, herd size, age of dam at parturition and dam gestation number. The virulence factors of the organisms were not identified in present study. In general identification of the risk factors associated with the spreading of bacteria causing diarrhea may be helpful for construction of suitable methods for prevention and control.

Based on the above conclusions, the current study led to the following recommendations:-

- Molecular characterization of *E.coli* K99 and *Salmonella enterica* should be conducted.
- Awareness of the pastoral community should be done about the impotence of colostrum feeding to their calves and other management practices.
- Further study also should be made in this area to get more information about *E.coli* K99, *Salmonella enterica* and associated risk factors.
- Antimicrobial susceptibility testing should be conducted regularly on commonly used drugs.

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8. ANNEXES

Annex IV: Consent to Participate in Research

Addressing Young Stock Mortality in Smallholder Farms and Pastoral Herds of Ethiopia
Investigator: Dr. Woutrina Smith, University of California, Davis
Sponsor: USAID - Feed the Future Innovation Lab for Livestock Systems

Introduction

My name is ____________________. I am a graduate student at Addis Ababa University or the University of Gondar, working with Drs. Nigatu Kebede and Tsegaw Fentie. We are planning to conduct a research study, which I invite you to take part in.

You are being invited to participate in this study because your household raises livestock in the region and you have eligible young stock (calves, camel calves, kids and lambs less than six months of age). Participation in this study is completely voluntary. You have the right to decline to participate or to withdraw at any point in this study without penalty or loss of benefits to which you are otherwise entitled.

Signature Block for Capable Subject

Do not sign this document until you have read the consent document, including the appendixes (or the document and appendixes been read to you) and your questions have been answered. Your signature documents your permission to take part in this research.

I VOLUNTARILY AGREE TO PARTICIPATE in the study referenced above.

I understand that I have the right to withdraw my consent at any time. I do understand that I can be withdrawn, or that the study could be terminated by the principal investigator, for my benefit and/or the benefit of the study. I understand I will be given a copy of the information sheet and consent form to keep for my records.

Signature of subject ______ Date ______

Printed name of subject ______

Signature of person obtaining consent ______ Date ______

Printed name of person obtaining consent ______

Signature Block for Consent Documentation or Illiterate subjects

My signature below documents that the information in the consent document and any other written information was accurately explained to, and apparently understood by, the subject, and that consent was freely given by the subject.

Signature of witness to consent process ______ Date ______

Printed name of person witnessing consent process ______

Annex V: Test Procedure for Isolation of *Salmonella Species* from Fecal Sample

1. Add a 10 g sample to ×10 volume of buffered peptone water at ambient temperature.
2. Incubate buffered peptone water for 20 hours at 37°C.
3. Inoculate 10 ml MSRV in a Petri dish with 0.1 ml incubated buffered peptone water.
4. Inoculate 10 ml tetrathionate broth with 1 ml incubated buffered peptone water broth.
5. Incubate MSRV at 41.5°C and tetrathionate broths at 37°C.

6. After 24 and 48 hours of selective enrichment, plate out MSRV by taking a loopful of material from the edge of the turbid growth zone and streaking over one plate of chromogenic agar and one plate of XLD agar.
7. Plate out 10 µl of tetrathionate broth on one plate of chromogenic agar.
8. Incubate plates at 37°C for 24 hours.
9. Check up to five suspect colonies biochemically, using TSI.
10. Subculture strongly suspect colonies that do not agglutinate with poly H antisera on to non-selective media then repeat testing (OIE, 2016).

Annex VI: Antigen Test Kit (ELISA) Pathasure Enteritis 4 Test Procedures for the Detection of *E.coli* K99

Bring all reagents to room temperature and mixed well manually before use. Two controls were supplied with this kit.

1. Make a schematic representation of the plate and the distribution of controls and samples.
2. Select the necessary number of strips by breaking the plastic between the strips. Four wells are needed for each sample and control (1 well for each pathogen).
3. Dispense 2 drops ready-to-use positive control into wells A1, A2, A3 and A4. For a better distribution, hold the dropper bottle vertically above the wells. The drops have to fall freely into the well without touching the sides of the well.
4. Dispense 2 drops ready-to-use negative control into wells B1, B2, B3 and B4.
5. Dispense 2 drops diluted samples (see section B) into wells C1/C2/C3/C4, D1/D2/D3/D4,
6. Incubate at 23 ± 2°C for 30 minutes.
7. Empty the plate contents in a sink and gently wash each well 5 times with 1X wash solution. Throw away all liquid contained in the plate after each wash. After the last wash, dry the plate by tapping it on absorbent paper.
8. Dispense 2 drops ready-to-use red conjugate (anti-rotavirus) into each well of column 1.
9. Dispense 2 drops ready-to-use blue conjugate (anti-coronavirus) into each well of column 2
10. Dispense 2 drops ready-to-use green conjugate (anti-*E. coli* K99) into each well of column 3.

11. Dispense 2 drops ready-to-use violet conjugate (anti-*Cryptosporidium*) into each well of column 4.
12. Incubate at 23 ± 2°C for 30 minutes.
13. Repeat step 7.
14. Dispense 3 drops of substrate into each well.
15. Incubate, away from light, at 23 ± 2°C for 10 minutes.
16. Stir the plate and read the results.

Annex VII: Procedures for Isolation of *E. coli* K99 from Faecal Sample

1. Mix faeces at a dilution of 1:10 in warmed buffered peptone water (BPW) in a labelled container.
2. Incubate at 37°C±2°C for 6 hours.
3. Using a sterile swab, spread the drop over one-third to one-half of the plate to break up the complexes.
4. Using a sterile loop, dilute the bead–bacteria complexes further over one quadrant by streaking out at right angles from the previously streaked area.
5. Using a second sterile loop, streak out at right angles from this quadrant into the final unstreaked area of the plate to obtain single colonies.
6. Incubate at 37°C±2°C for 18 hours (OIE, 2016).

Annex VIII: Laboratory Result Record Sheet Format for *E.coli K99* and *Salmonella enterica*

S/No.	Sample ID	Result of Pathasure kit test			
		Rotavirus	*Coronavirus*	*Escherichia coli K99*	*Cryptosporidium parvum*
1.					
2.					
3.					
4.					
5.					
6.					
7.					
8.					
.					
.					

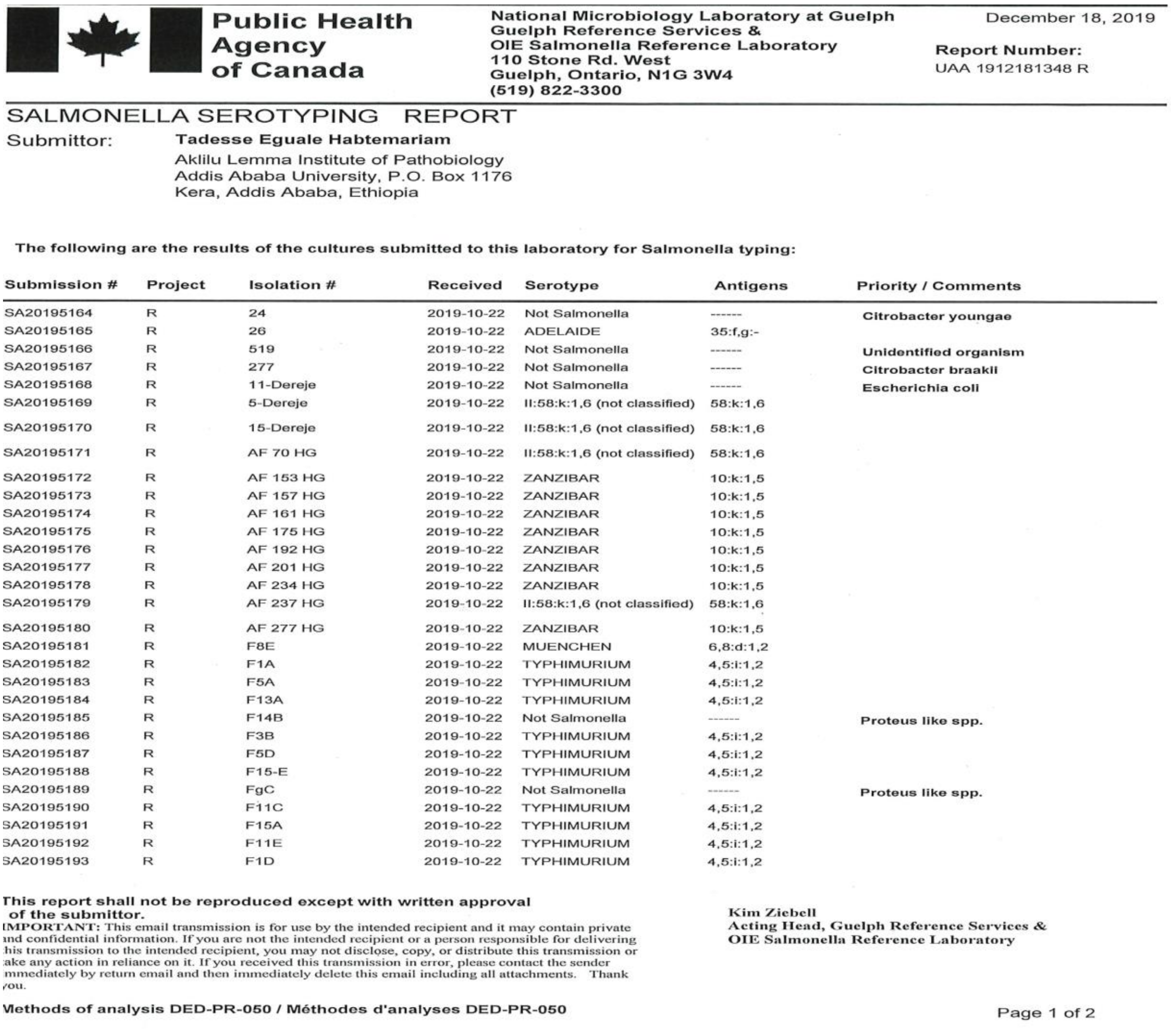
S/No.	Sample ID	Biochemical test				Positive reaction		
		TSI	LIA	Citrate utilization test	Ureases test	Color change		H_2S production
						Slant	Butt	
1.								
2.								
3.								
4.								
5.								
6.								
7.								
8.								
9.								
10.								

Annex IX: Antimicrobial Susceptibility Testing Result Recording Sheet for *Salmonella* Isolates

Sample ID	Antimicrobial Agent with their Zone Diameter in mm											
	AM	AN	AMC	C	CF	CRO	CIP	SXT	G	S	GM	TE

Legend: AM=Ampicillin, AMC=Amoxicillin clavulanic acid, AN=Amikacin, C= Chloramphenicol, CF=Cephalothin, CRO=Ceftriaxone, CIP =Ciprofloxacin, TE=Tetracycline, SXT= Trimethoprim-sulfamethoxazole, G=Sulfisoxazole, S=Streptomycin, GM= Gentamycin

Annex X: *Salmonella* Serotyping Result Report



Public Health Agency of Canada

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December 18, 2019

Report Number:
UAA 1912181348 R

SALMONELLA SEROTYPING REPORT

Submittor: **Tadesse Eguale Habtemariam**
Aklilu Lemma Institute of Pathobiology
Addis Ababa University, P.O. Box 1176
Kera, Addis Ababa, Ethiopia

The following are the results of the cultures submitted to this laboratory for Salmonella typing:

Submission #	Project	Isolation #	Received	Serotype	Antigens	Priority / Comments
SA20195164	R	24	2019-10-22	Not Salmonella	------	**Citrobacter youngae**
SA20195165	R	26	2019-10-22	ADELAIDE	35:f,g:-	
SA20195166	R	519	2019-10-22	Not Salmonella	------	**Unidentified organism**
SA20195167	R	277	2019-10-22	Not Salmonella	------	**Citrobacter braakii**
SA20195168	R	11-Dereje	2019-10-22	Not Salmonella	------	**Escherichia coli**
SA20195169	R	5-Dereje	2019-10-22	II:58:k:1,6 (not classified)	58:k:1,6	
SA20195170	R	15-Dereje	2019-10-22	II:58:k:1,6 (not classified)	58:k:1,6	
SA20195171	R	AF 70 HG	2019-10-22	II:58:k:1,6 (not classified)	58:k:1,6	
SA20195172	R	AF 153 HG	2019-10-22	ZANZIBAR	10:k:1,5	
SA20195173	R	AF 157 HG	2019-10-22	ZANZIBAR	10:k:1,5	
SA20195174	R	AF 161 HG	2019-10-22	ZANZIBAR	10:k:1,5	
SA20195175	R	AF 175 HG	2019-10-22	ZANZIBAR	10:k:1,5	
SA20195176	R	AF 192 HG	2019-10-22	ZANZIBAR	10:k:1,5	
SA20195177	R	AF 201 HG	2019-10-22	ZANZIBAR	10:k:1,5	
SA20195178	R	AF 234 HG	2019-10-22	ZANZIBAR	10:k:1,5	
SA20195179	R	AF 237 HG	2019-10-22	II:58:k:1,6 (not classified)	58:k:1,6	
SA20195180	R	AF 277 HG	2019-10-22	ZANZIBAR	10:k:1,5	
SA20195181	R	F8E	2019-10-22	MUENCHEN	6,8:d:1,2	
SA20195182	R	F1A	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195183	R	F5A	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195184	R	F13A	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195185	R	F14B	2019-10-22	Not Salmonella	------	**Proteus like spp.**
SA20195186	R	F3B	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195187	R	F5D	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195188	R	F15-E	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195189	R	FgC	2019-10-22	Not Salmonella	------	**Proteus like spp.**
SA20195190	R	F11C	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195191	R	F15A	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195192	R	F11E	2019-10-22	TYPHIMURIUM	4,5:i:1,2	
SA20195193	R	F1D	2019-10-22	TYPHIMURIUM	4,5:i:1,2	

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Kim Ziebell
Acting Head, Guelph Reference Services &
OIE Salmonella Reference Laboratory

Methods of analysis DED-PR-050 / Méthodes d'analyses DED-PR-050

Page 1 of 2

Annex XI: Questionnaire Format for Collection of Information about Risk Factors

Household ID: __________________ Date :(dd) ________ (mm) ____ (yyyy) ______

Household Risk factor Identification

Fill out the form once per household, at the very first time the household is enrolled in the study. Write NA if the question is not applicable, NR when the respondent declines to do:

1) Household ID Assigned (per ID protocol, i.e.,G-F001	
2) Interview performed by (full name)	
3) Date(western calendar)	DD:_____MM:___YYY: ___

Owner information

4) Livestock owner name(optional)		
5) Primary telephone contact number		
6) Gender	Male	Female
7) District/Woreda		
8) Kebele		

9) GPS coordinates of farm		Latitude	Longitude
10) Predominant production system	Peri-urban	Mixed crop Livestock	Pastoral
11) Owner educational status			
0=non or preschool 1= primary, 2= secondary 3=higher, 8= don't know 9=no response			

Farm information and facilities

12) How many animals on average have you have during the last year of following types:

	Cattle
Adult females(older than 2 years old)	
Young females(6months- 2years)	
Female calves(less than 6 months old)	
Adult males (older than 6 months)	
Male calves(less than 6 months old)	

13) Out of all the dams that were pregnant in the last 12 months, how many…………?

	Cattle
Dams aborted	
Offspring born dead(stillbirth)	
Offspring born alive	
NR	

14) Where do birth usually happen?

	Cattle
Separate birth area	
Same area as the herd	
Don't know	
NR	

Young Stock Enrollment Form

Household ID: ____________________ Date: (dd) _____ (mm) _____ (yyyy) _________

Young stock ID: _______________ Examiner Name: _____________________

Use this form for each animal less than 6 months old that you enroll in the household.

1) Young Stock Id Assigned (Per ID protocol)	

2) Age	(days)	(Weeks)	(months)	Don't know

3) Sex (circle one)	Male	Female	
4) Predominant Breed (circle one)	Local	Improved/Crossbred	Don't know
5) Name of Breed			Don't know

Animal History

For the following questions, circle one option.

6) Sire	Artificial Insemination	Live Cover		Don't know
7) Time of Birth	Day	Night		Don't know
8) Birth witnessed	Yes	No		Don't know
9) Birth location	In barn	outside	Other	Don't know
10) Type of delivery	Normal	assisted		Don't know
11) Colostrum fed within 24 hours?	Yes	No		Don't know
12) If answer to question 11 is no, was this because				

Calf alert but unable to suck	Calf depressed, unable to suck	Other	Don't know
13) Navel dipped	Yes	No	Don't know
14) Did animal have continuous access to dam for the first 24 hours of life?	Yes	No	Don't know
15) Separate housing from herd?	Yes	No	Don't know
16) Animal housed with dam?	Yes	No	Don't know
17) Animal housed with other young stock?	Yes	No	Don't know
18) Any vaccinations given to animal since birth?	Yes	No	Don't know
19) If yes, what type of vaccines?			
20) Any medications given to animal since birth?	Yes	No	Don't know
21) If yes to question 20, what type of medications? Mark from the following below			
Dewormers	Yes	No	Don't know
Vitamins	Yes	No	Don't know
Oral electrolytes	Yes	No	Don't know
Other, specify	Yes	No	Don't know
22) Has this animal shown any signs of illness since birth?	Yes	No	Don't know
23) If yes to question 22, mark what symptoms of illness has been observed			
Not suckling	Yes	No	Don't know
Diarrhea (not bloody)	Yes	No	Don't know
Diarrhea (bloody)	Yes	No	Don't know
Other, describe			

Animal Environment

Inspect animal housing place. Look at where this animal is housed and answer the following questions.

24) Is animal housed on bedding?	Yes	No	Don't know
25) Does the area where the animal lies down look clean and dry?	Yes	No	Don't know
26) If all the animals housed together	Yes	No	Don't know

were going to stand up at the same time, would they have enough room to do so and turn around without touching each other?			

Dam History

For the following questions, circle one option

27) Age of dam at parturition	(years)	Don't know
28) Dam gestation number		Don't know
29) Dam Body Condition Score (cows/sheep/goats use scale 1 -5; camels use scale 0-5)		Don't know
30) Dam milk production (include all milk, i.e., milk given to calf and milk sold /human consumption)	(Liters/day)	Don't know

Annex XII: Composition and Preparation of Culture Media used for Isolation and Identification of *Salmonella enterica* and *E.coli* K99

1. Buffered peptone water(M1049-500mg, Oxoid, England)

Compostion (g/l): Peptone 10.0 g Sodium chloride 5.0 g, Disodium hydrogen phosphate doxdecahydrate 9.0 g, potassium dihydrogen phosphate 1.5g, water 1000 ml.

Preparation: Dissolve 20 gram the peptone in 1000ml water, adjust pH to 7.0 after sterilization. Dispense into suitable flasks and autoclave at 121^{o}C for 15 min.

2. Rappaport-Vassiliadis medium with soya(M0866,500gm, Oxoid, England)

Composition(g/l): Enzymatic digest of soya, 4.5 g; sodium chloride, 7.2 g; potassium dihydrogen phosphate, 1.44 g; anhydrous magnesium chloride (MgCl2), 13.4 g or magnesium chloride hexahydrate, 28.6 g; malachite green oxalate, 0.036 g.

Preparation: Suspend 26.75 grams of dehydrated medium in 1000 ml purified/ distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired into tubes and sterilize by autoclaving at 115°C as per validated cycle.

3. Muller-Kauffmann tetrathionate novobiocin broth(M14961,500g, Himedia)

Composition(g/l): Meat extract 4.3 g, enzymatic digest of casein 8.6 g, sodium chloride 2.6 g, calcium carbonate 38.7 g, sodium thiosulfate pentahydrate 47.8 g, ox bile for bacteriological use 4.78 g, brilliant green 9.6 mg, water 1000ml.

Preparation: Dissolve 89,42grams the tetrathionate in 1000ml distilled water. Aseptically add 9ml iodine solution. Adjust the pH, if necessary, so that it is 8.2 ± 0, 2 at 25 °C.

4. MacConkey agar(CM0115, 500gm, Oxoid, England)

Preparation: Suspend 51.59 grams in 1000ml of distilled water. Bring to boil to dissolve completely. Sterile by autoclaving at 121^{0}c for 15 minutes.

5. Xylose lysine deoxycholate agar (M0866, 500gm , Oxoid, England)

Composition(g/l): Yeast extract powder 3.0 g, sodium chloride 5.0 g, xylose 3.75 g, lactose 7.5 g, sucrose 7.5 g, l-lysine hydrochloride 5.0 g, sodium thiosulfate 6.8 g, iron (iii) ammonium citrate 0.8 g, phenol red 0.08 g ,sodium deoxycholate 1.0 g, agar 9 g to 18 g and water 1 000 ml.

Preparation: Suspend 53grams in 1000ml of distilled water. Heat with frequent agitation until the medium boils. Do not over heat. Transfer immediately to a water bath at 50 ^{0}c. Pour into plates as soon as the medium has cooled. It is important to avoid preparing large volumes which will cause prolonged heating.

6. Eosine Methylene Blue agar(M0069, 500gm, Oxoid, England)

Preparation: Suspend 37.5 grams in 1000ml of distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121^{0}c for 15 minutes. Cool to 60 ^{0}c and shack the medium in order to oxidize the methylene blue and suspend the precipitate which is essential part of this medium.

7. Salmonella Shigella agar(C6841, 500gm, HARDY)

Preparation: Combine 60 grams of medium with 1000ml of deionized water. Stir using stirrer to mix thoroughly. Boil to dissolve completely. Do not over heat. Do not autoclave. Aseptically dispense into sterile containers after media has cooled to 45-50^{0}c.

8. Nutrient agar(M0003,500gm, Oxoid, England)

Composition(g/l): Meat extract 3.0 g, peptone 5.0 g, agar 9 g to 18 g and water 1 000 ml.

Preparation: Suspend 28grams in 1000ml distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121^{0}c for 15 minutes.

9. Urea agar base (M0053,500gm, Oxoid, England)

Composition(g/l): Peptone1.0 g, glucose 1.0 g, sodium chloride 5.0 g , potassium dihydrogen phosphate 2.0 g, phenol red 0.012 g, agar 9 g to 18 g and water 1 000 ml.

Preparation: Suspend 2.4grams in 95ml of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 115^{0}c for 20 minutes. Cool to 50^{0}c and aseptically add one ampoule of sterile urea solution (SR20). Mix well, distribute 10ml amounts into sterile test tube and allow to set in the slope position.

10. Triple sugar iron agar(500g, Remel product)

Preparation: Suspend 59.5 grams of medium in 1000ml of demineralized water. Heat to boiling with agitation to completely dissolve. Dispense into tubes and sterilize by autoclaving at 121^0c for 15 minutes. Cool in slanted position so that deep butts are formed.

11. Lysine Iron agar(500g, DifcoTM product)

Composition(g/l): Bacteriological peptone 5.0 g, yeast extract 3.0g , glucose 1.0g, l-lysine 10.0g, ferric ammonium citrate 0.5g, sodium thiosulphate 0.04g, bromocresol purple 0.02g and agar 14.5g.

Preparation: Suspend 34 grams of powder in 1000 ml of distilled water. Bring to the boil for 1 minute to dissolve completely. Dispense into tubes and sterilise by autoclaving at 121° C for 15 minutes. Cool the tubes in an inclined position to form slants with deep butts.

12. Simmons Citrate agar(500gm, Oxoid product, England)

Preparation: Suspend 23 grams in 1000ml distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 1210c for 15 minutes.

13. Mueller-Hinton agar(CM0337,500g, Oxoid product, England)

Composition (g/l): Beef, dehydrated infusion from 300.0 g, casein hydrolysate 17.5 g, starch 1.5 g and agar 17.0 g.

Preparation: Add 38 grams to 1 litre of distilled water. Bring to the boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes.

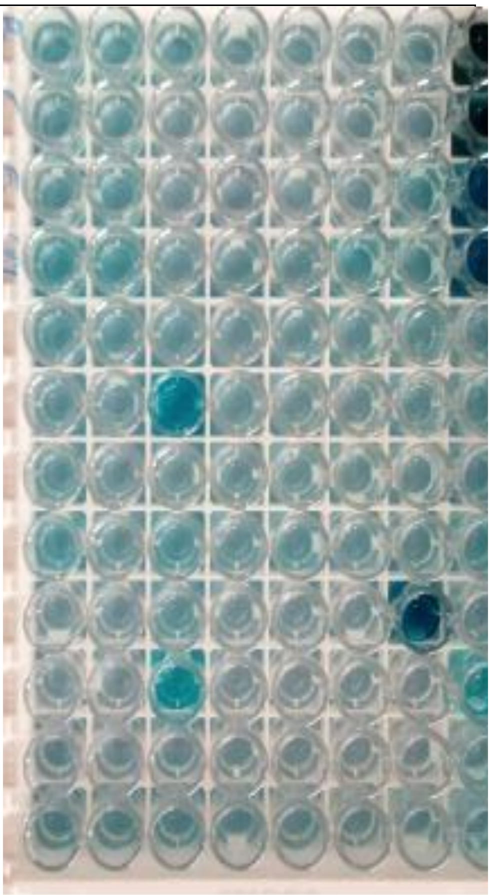
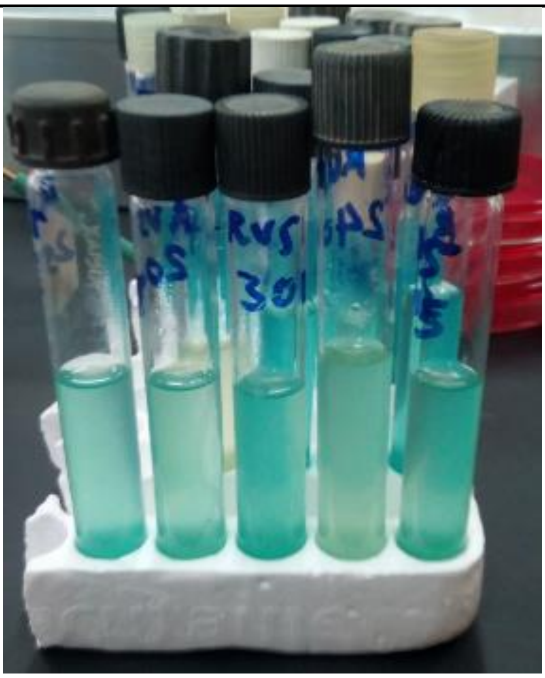
Annex XIII: Some Pictures that have been taken during Field and Laboratory Works

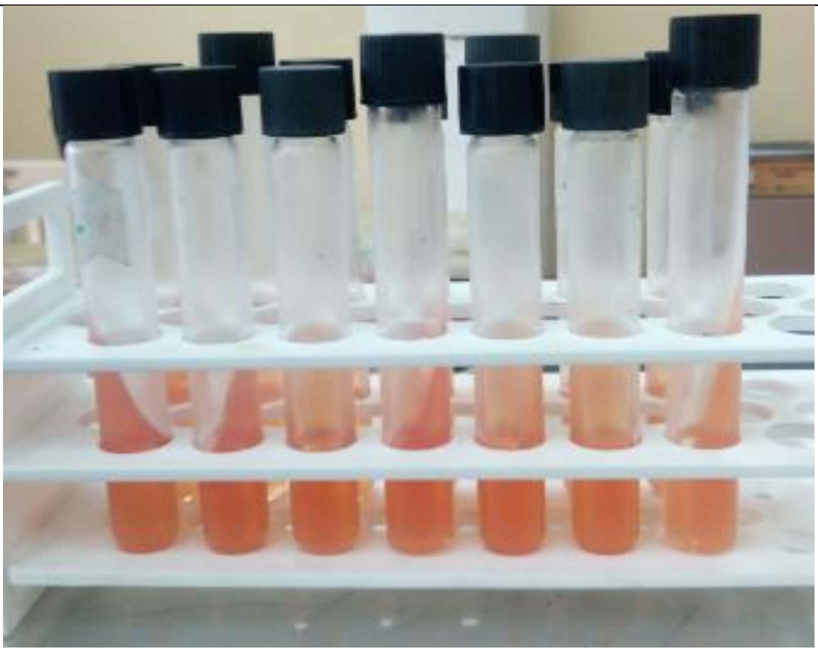
Picture 1: Calves up to 6 months of age in their barn.

Picture 2: Feces with blood that was collected during sample collection.

Picture 3: During antimicrobial susceptibility testing

Picture 4: During peptone water media preparation

	
Picture 5: During Pathasure test for the detection of *E.coli K99*	Picture 6: Stirred plate ready to read the results
	
Picture 7 :- RVS broth after incubation	Picture 8: TSI slant media before inoculation



Picture 9: Urease slant media before inoculation

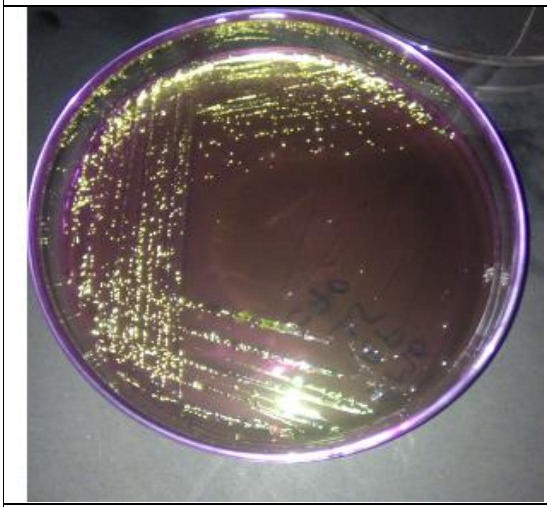


Figure 10: *E.coli* K99 cultured on EMB agar shows characteristic green metallic sheen colonies

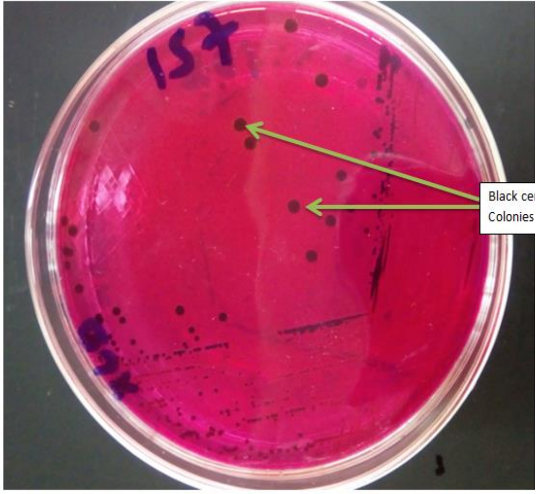


Figure 11: *Salmonella species* colonies after 24 hours incubation at 37ºC growth on XLD agar showing the H_2S production

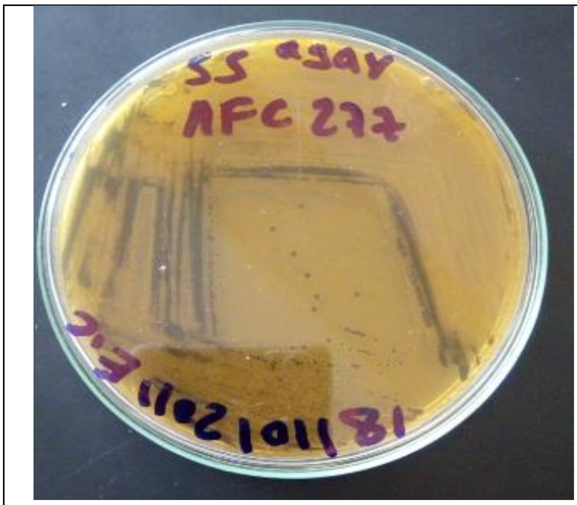


Figure 12: *Salmonella species* colonies after 24 hours incubation at 37ºC growth on SS agar

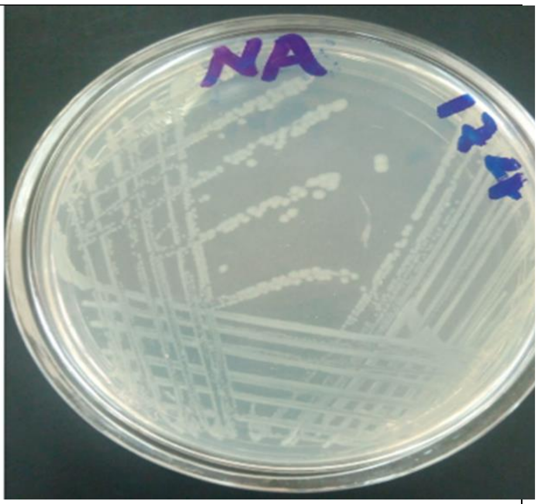


Figure 13: Colonies of *Salmonella* on nutrient agar after 24 hours incubation at 37^0 C

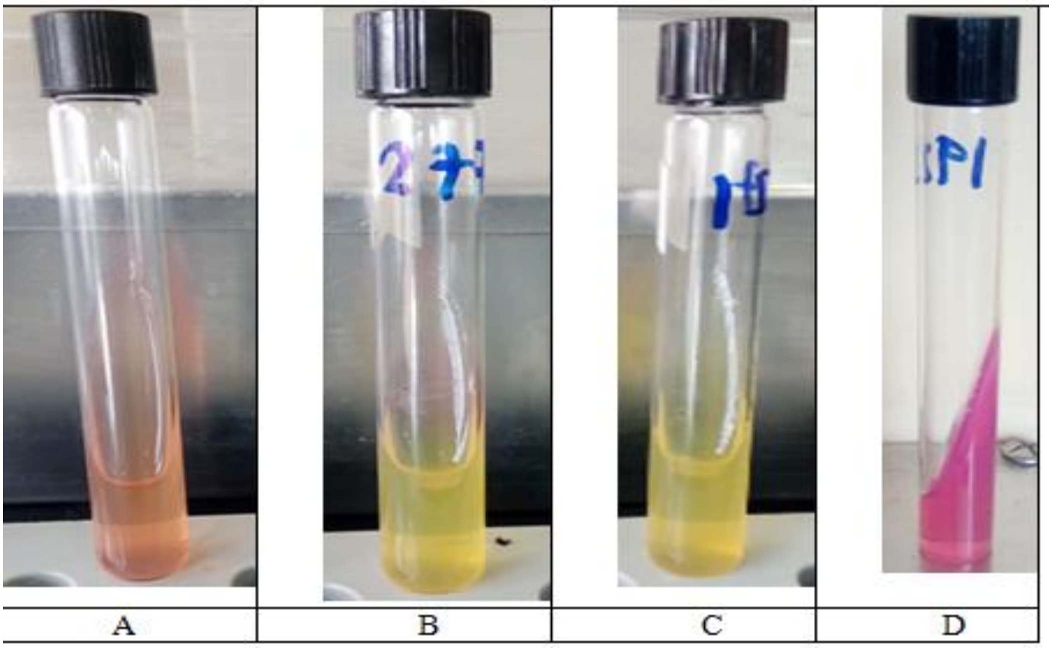
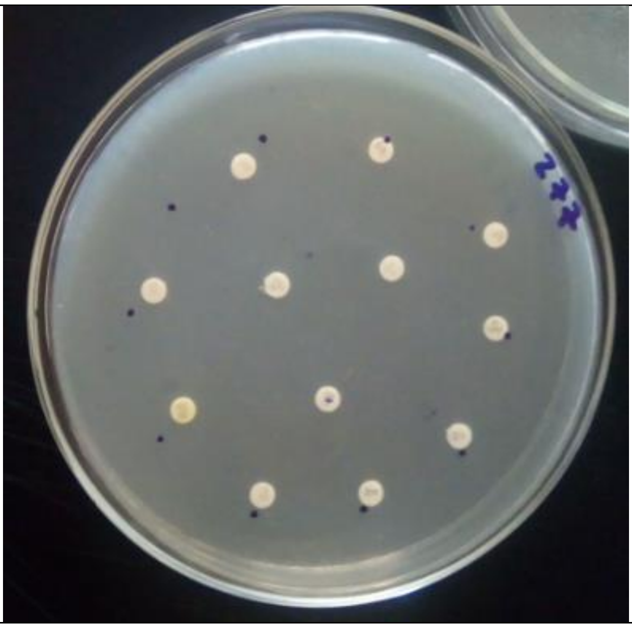
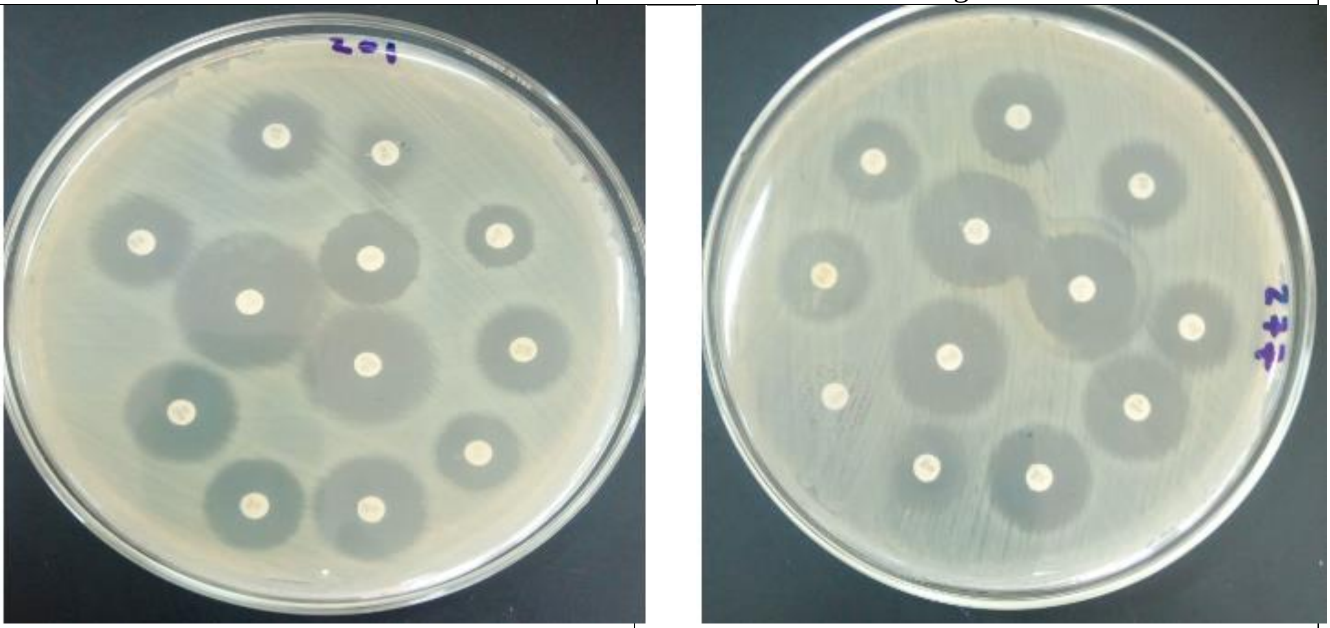


Figure 14: Urease test. A: Uninoculated urea agar slant medium, B and C are Urease negative results, D: urease positive

A. Uninoculated Muller Hinton media



B. Inoculated Muller Hinton agar with Antimicrobial drug before incubation

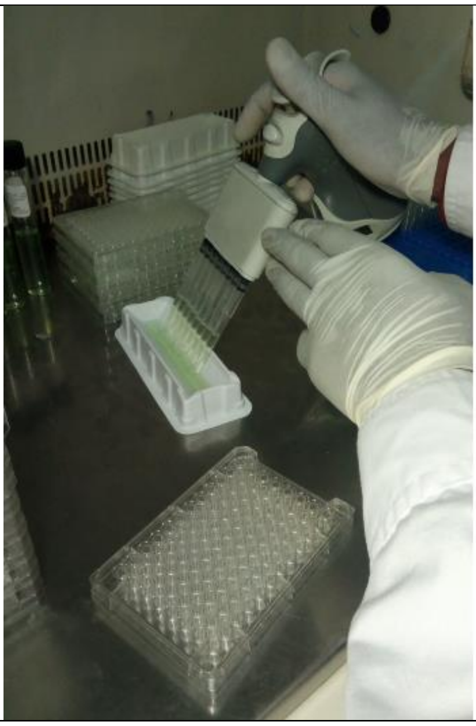
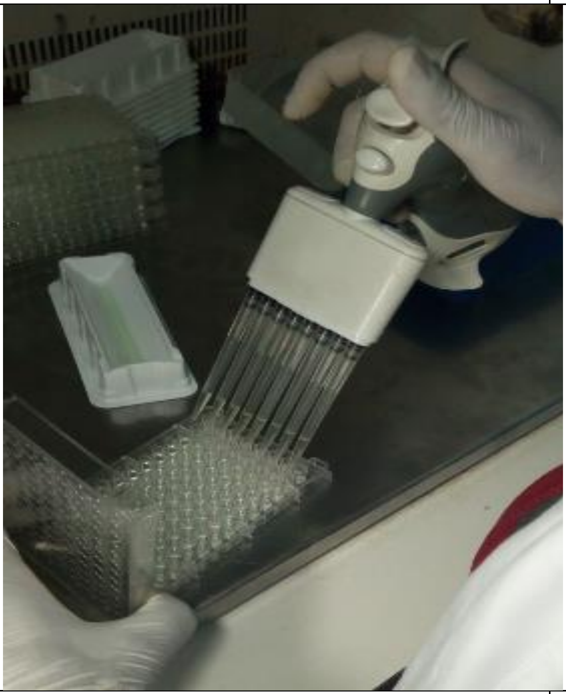


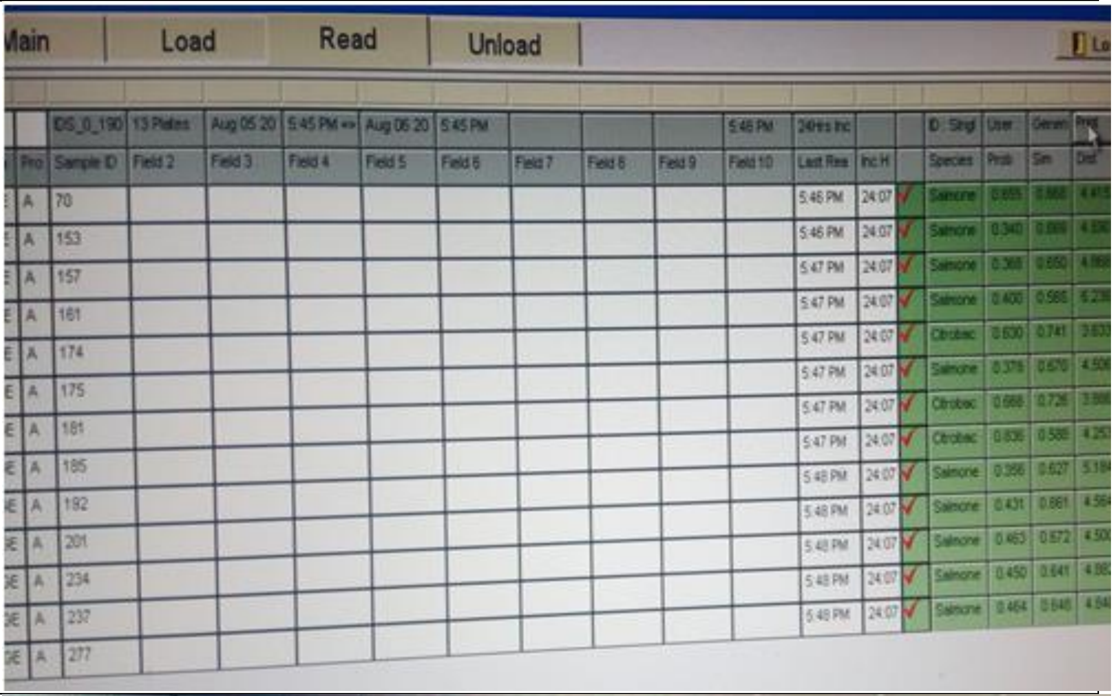
C.

D.

Picture 15: C and D Inoculated Muller Hinton agar with Antimicrobial drug after 24 hours incubation at 37^0C

Annex XIV: Biolog GEN III Microplate Test Procedures and Result Report of *Salmonella enterica*

	
Picture 16: Prepared inoculum for Biolog isolation	Picture 17: Pour the cell suspension into the multichannel pipet reservoir.
	
Picture 18: Filling wells with 100µl of cell suspention during inoculating MicroPlate	Picture 19: Biolog machine used for isolation in Ethiopian biodiversity laboratory



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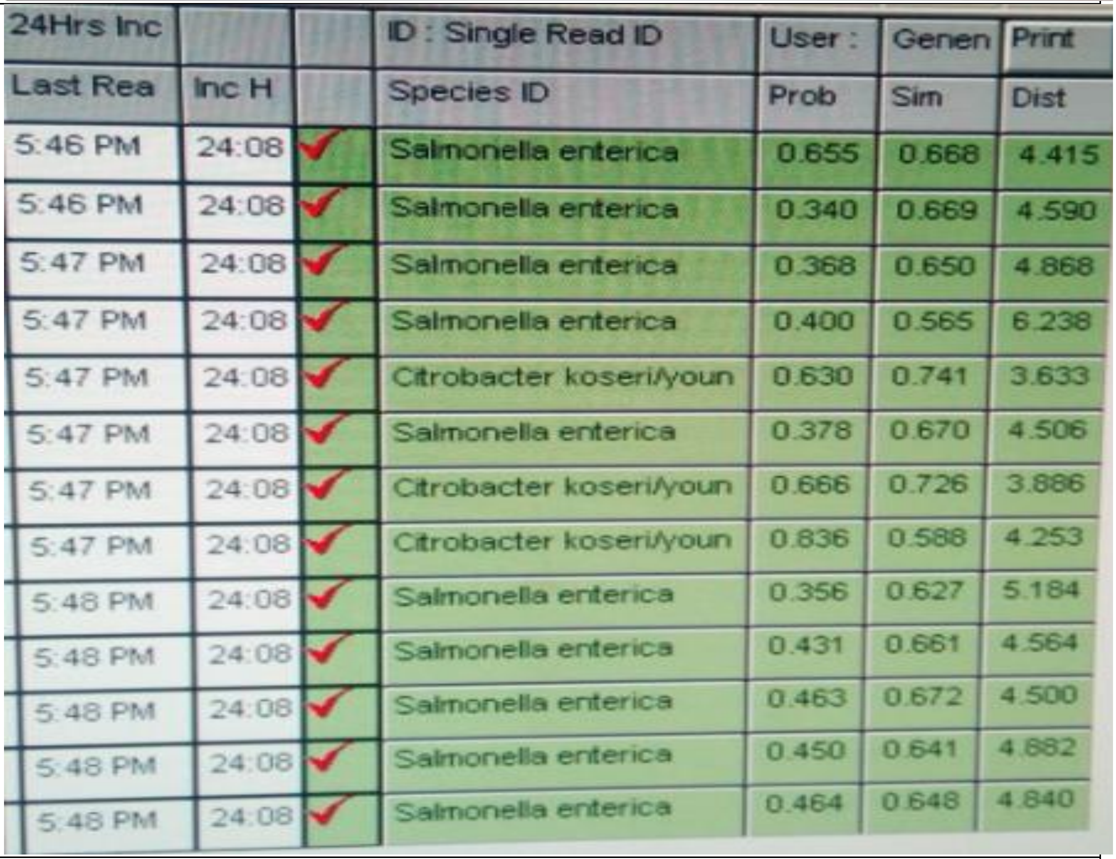
A	153										5:46 PM	24:07	✓	Salmone	0.340	0.669	4.59
A	157										5:47 PM	24:07	✓	Salmone	0.368	0.650	4.86
A	161										5:47 PM	24:07	✓	Salmone	0.400	0.565	6.23
A	174										5:47 PM	24:07	✓	Citrobac	0.630	0.741	3.63
A	175										5:47 PM	24:07	✓	Salmone	0.378	0.670	4.50
A	181										5:47 PM	24:07	✓	Citrobac	0.666	0.726	3.88
A	185										5:47 PM	24:07	✓	Citrobac	0.836	0.588	4.25
A	192										5:48 PM	24:07	✓	Salmone	0.356	0.627	5.18
A	201										5:48 PM	24:07	✓	Salmone	0.431	0.661	4.56
A	234										5:48 PM	24:07	✓	Salmone	0.463	0.672	4.50
A	237										5:48 PM	24:07	✓	Salmone	0.450	0.641	4.88
A	277										5:48 PM	24:07	✓	Salmone	0.464	0.648	4.84



24Hrs Inc			ID : Single Read ID	User :	Genen	Print
Last Rea	Inc H		Species ID	Prob	Sim	Dist
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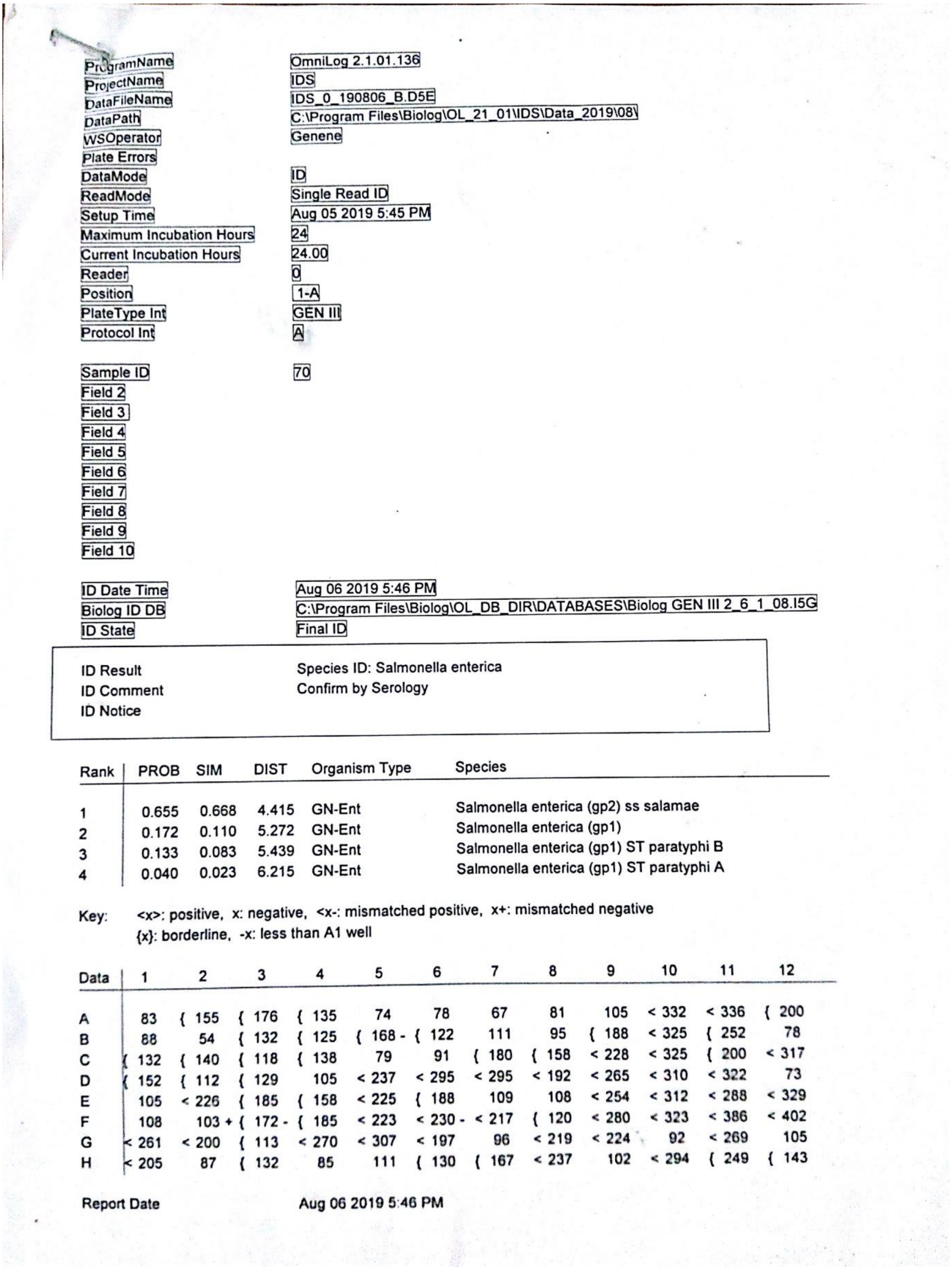
5:46 PM	24:08	✓	Salmonella enterica	0.340	0.669	4.590
5:47 PM	24:08	✓	Salmonella enterica	0.368	0.650	4.868
5:47 PM	24:08	✓	Salmonella enterica	0.400	0.565	6.238
5:47 PM	24:08	✓	Citrobacter koseri/youn	0.630	0.741	3.633
5:47 PM	24:08	✓	Salmonella enterica	0.378	0.670	4.506
5:47 PM	24:08	✓	Citrobacter koseri/youn	0.666	0.726	3.886
5:47 PM	24:08	✓	Citrobacter koseri/youn	0.836	0.588	4.253
5:48 PM	24:08	✓	Salmonella enterica	0.356	0.627	5.184
5:48 PM	24:08	✓	Salmonella enterica	0.431	0.661	4.564
5:48 PM	24:08	✓	Salmonella enterica	0.463	0.672	4.500
5:48 PM	24:08	✓	Salmonella enterica	0.450	0.641	4.882
5:48 PM	24:08	✓	Salmonella enterica	0.464	0.648	4.840

Picture 20: Results of *Salmonella enterica* displayed on BIOLOG Micro Station reader after 24 hours incubation period



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ID State	Final ID

ID Result	Species ID: Salmonella enterica
ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.655	0.668	4.415	GN-Ent	Salmonella enterica (gp2) ss salamae
2	0.172	0.110	5.272	GN-Ent	Salmonella enterica (gp1)
3	0.133	0.083	5.439	GN-Ent	Salmonella enterica (gp1) ST paratyphi B
4	0.040	0.023	6.215	GN-Ent	Salmonella enterica (gp1) ST paratyphi A

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

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B	88	54	{ 132	{ 125	{ 168 -	{ 122	111	95	{ 188	< 325	{ 252	78
C	{ 132	{ 140	{ 118	{ 138	79	91	{ 180	{ 158	< 228	< 325	{ 200	< 317
D	{ 152	{ 112	{ 129	105	< 237	< 295	< 295	< 192	< 265	< 310	< 322	73
E	105	< 226	{ 185	{ 158	< 225	{ 188	109	108	< 254	< 312	< 288	< 329
F	108	103 +	{ 172 -	{ 185	< 223	< 230 -	< 217	{ 120	< 280	< 323	< 386	< 402
G	< 261	< 200	{ 113	< 270	< 307	< 197	96	< 219	< 224	92	< 269	105
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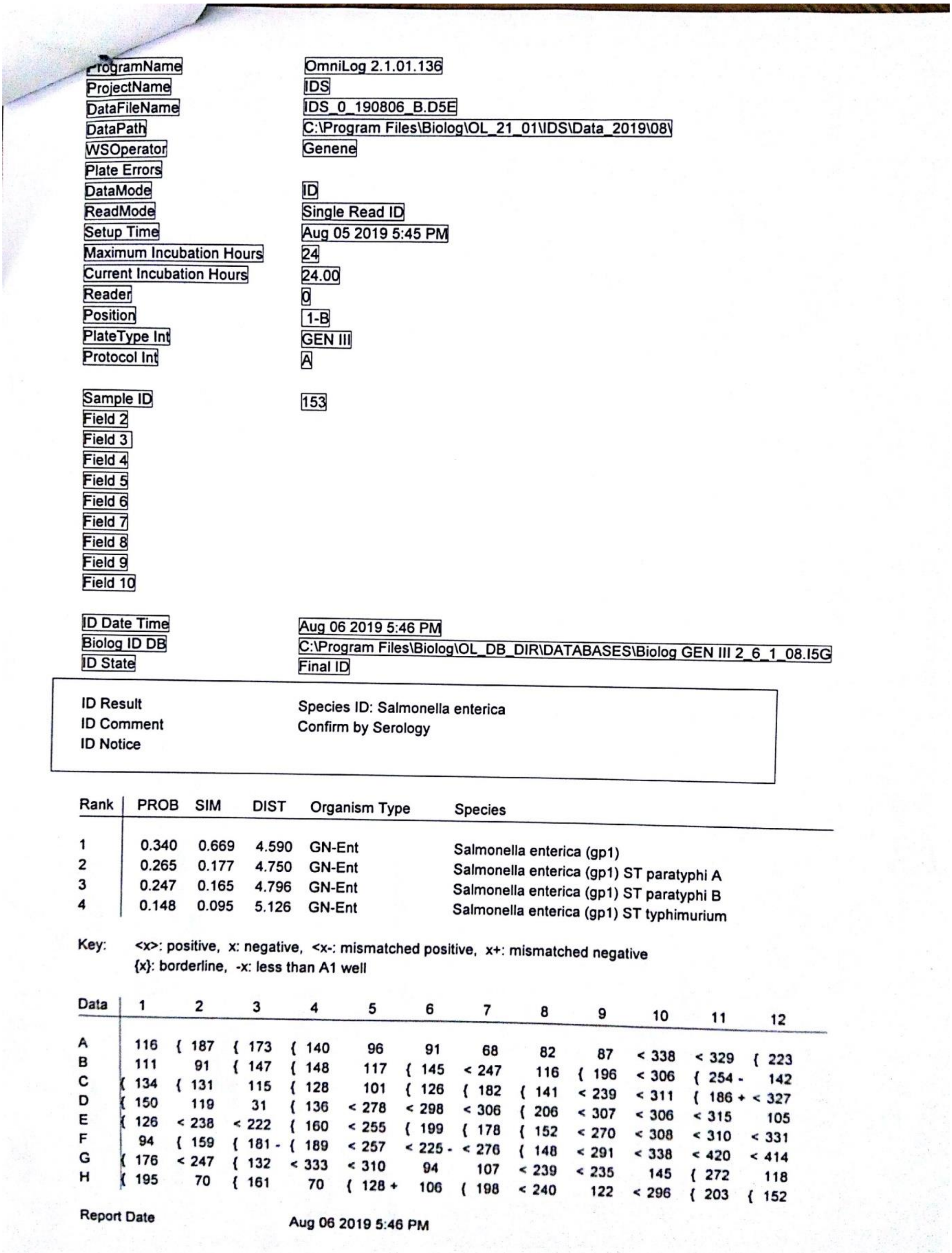

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ID State	Final ID

ID Result	Species ID: Salmonella enterica
ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.340	0.669	4.590	GN-Ent	Salmonella enterica (gp1)
2	0.265	0.177	4.750	GN-Ent	Salmonella enterica (gp1) ST paratyphi A
3	0.247	0.165	4.796	GN-Ent	Salmonella enterica (gp1) ST paratyphi B
4	0.148	0.095	5.126	GN-Ent	Salmonella enterica (gp1) ST typhimurium

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

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C	{ 134	{ 131	115	{ 128	101	{ 126	{ 182	{ 141	< 239	< 311	{ 186 +	< 327
D	{ 150	119	31	{ 136	< 278	< 298	< 306	{ 206	< 307	< 306	< 315	105
E	{ 126	< 238	< 222	{ 160	< 255	{ 199	{ 178	{ 152	< 270	< 308	< 310	< 331
F	94	{ 159	{ 181 -	{ 189	< 257	< 225 -	< 276	{ 148	< 291	< 338	< 420	< 414
G	{ 176	< 247	{ 132	< 333	< 310	94	107	< 239	< 235	145	{ 272	118
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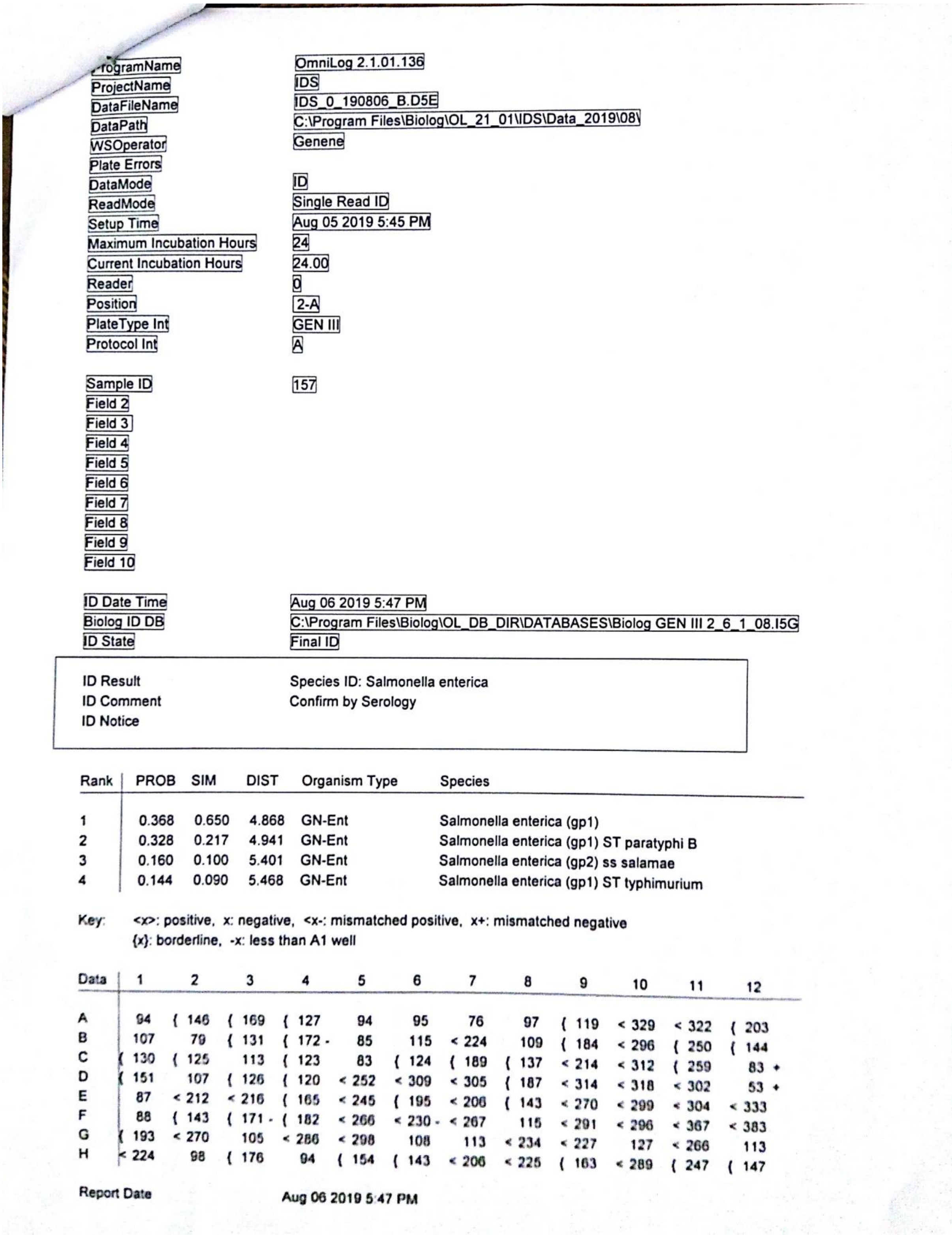

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ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
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2	0.328	0.217	4.941	GN-Ent	Salmonella enterica (gp1) ST paratyphi B
3	0.160	0.100	5.401	GN-Ent	Salmonella enterica (gp2) ss salamae
4	0.144	0.090	5.468	GN-Ent	Salmonella enterica (gp1) ST typhimurium

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

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C	{ 130	{ 125	113	{ 123	83	{ 124	{ 189	{ 137	< 214	< 312	{ 259	83 +
D	{ 151	107	{ 126	{ 120	< 252	< 309	< 305	{ 187	< 314	< 318	< 302	53 +
E	87	< 212	< 216	{ 165	< 245	{ 195	< 206	{ 143	< 270	< 299	< 304	< 333
F	88	{ 143	{ 171 -	{ 182	< 266	< 230 -	< 267	115	< 291	< 296	< 367	< 383
G	{ 193	< 270	105	< 286	< 298	108	113	< 234	< 227	127	< 266	113
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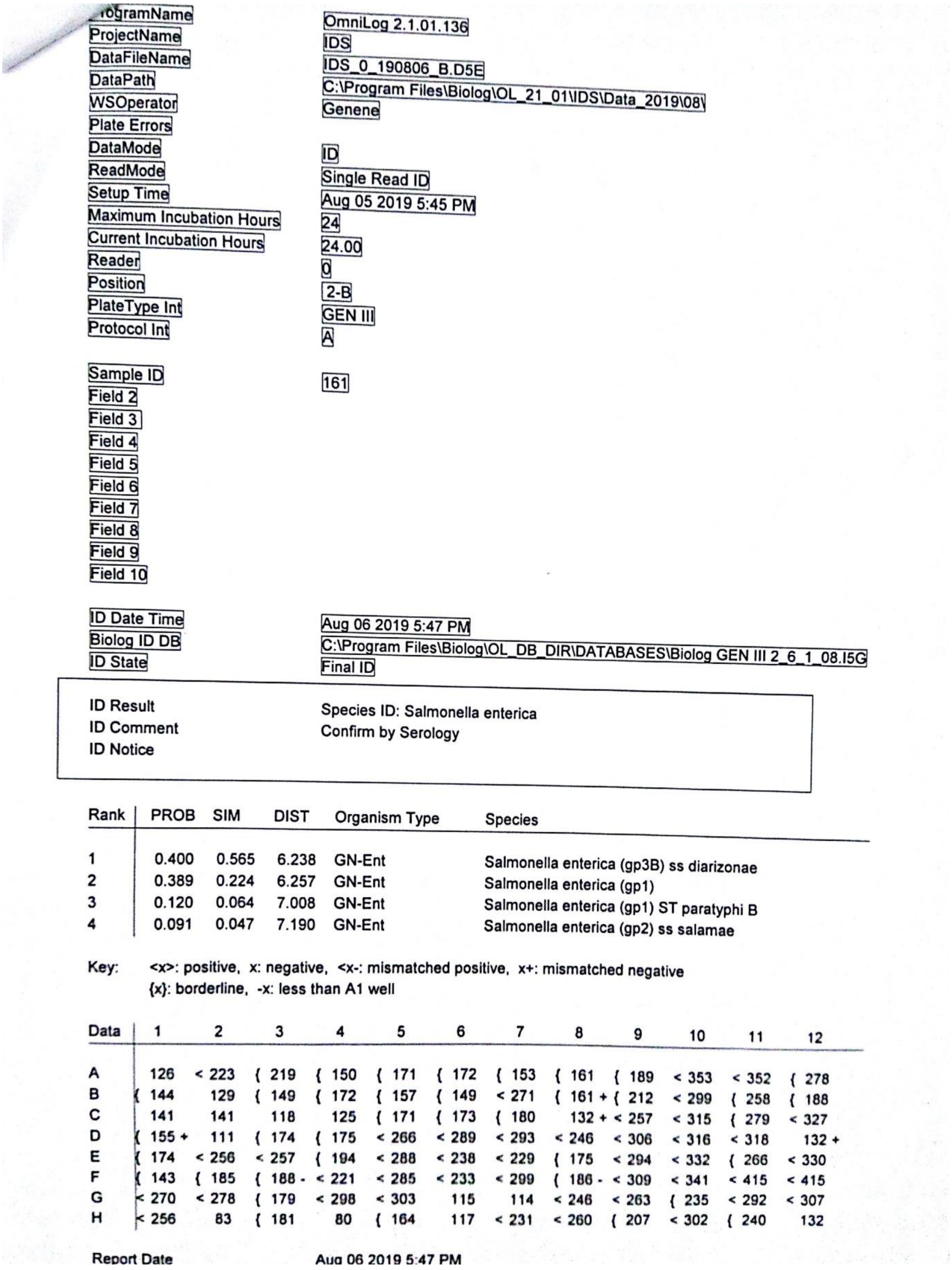

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Protocol Int                 A

Sample ID                    161
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Field 3
Field 4
Field 5
Field 6
Field 7
Field 8
Field 9
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ID State                     Final ID

ID Result                    Species ID: Salmonella enterica
ID Comment                   Confirm by Serology
ID Notice

Rank | PROB   SIM    DIST   Organism Type   Species

1    | 0.400  0.565  6.238  GN-Ent          Salmonella enterica (gp3B) ss diarizonae
2    | 0.389  0.224  6.257  GN-Ent          Salmonella enterica (gp1)
3    | 0.120  0.064  7.008  GN-Ent          Salmonella enterica (gp1) ST paratyphi B
4    | 0.091  0.047  7.190  GN-Ent          Salmonella enterica (gp2) ss salamae

Key:   <x>: positive,  x: negative,  <x-: mismatched positive,  x+: mismatched negative
       {x}: borderline,  -x: less than A1 well

Data |  1       2       3       4       5       6       7       8       9       10      11      12

A    |  126   < 223   { 219   { 150   { 171   { 172   { 153   { 161   { 189   < 353   < 352   { 278
B    |{ 144     129   { 149   { 172   { 157   { 149   < 271   { 161 + { 212   < 299   { 258   { 188
C    |  141     141     118     125   { 171   { 173   { 180     132 + < 257   < 315   { 279   < 327
D    |{ 155 +   111   { 174   { 175   < 266   < 289   < 293   < 246   < 306   < 316   < 318     132 +
E    |{ 174   < 256   < 257   { 194   < 288   < 238   < 229   { 175   < 294   < 332   { 266   < 330
F    |{ 143   { 185   { 188 - < 221   < 285   < 233   < 299   { 186 - < 309   < 341   < 415   < 415
G    |< 270   < 278   { 179   < 298   < 303     115     114   < 246   < 263   { 235   < 292   < 307
H    |< 256      83   { 181      80   { 164     117   < 231   < 260   { 207   < 302   { 240     132

Report Date                  Aug 06 2019 5:47 PM
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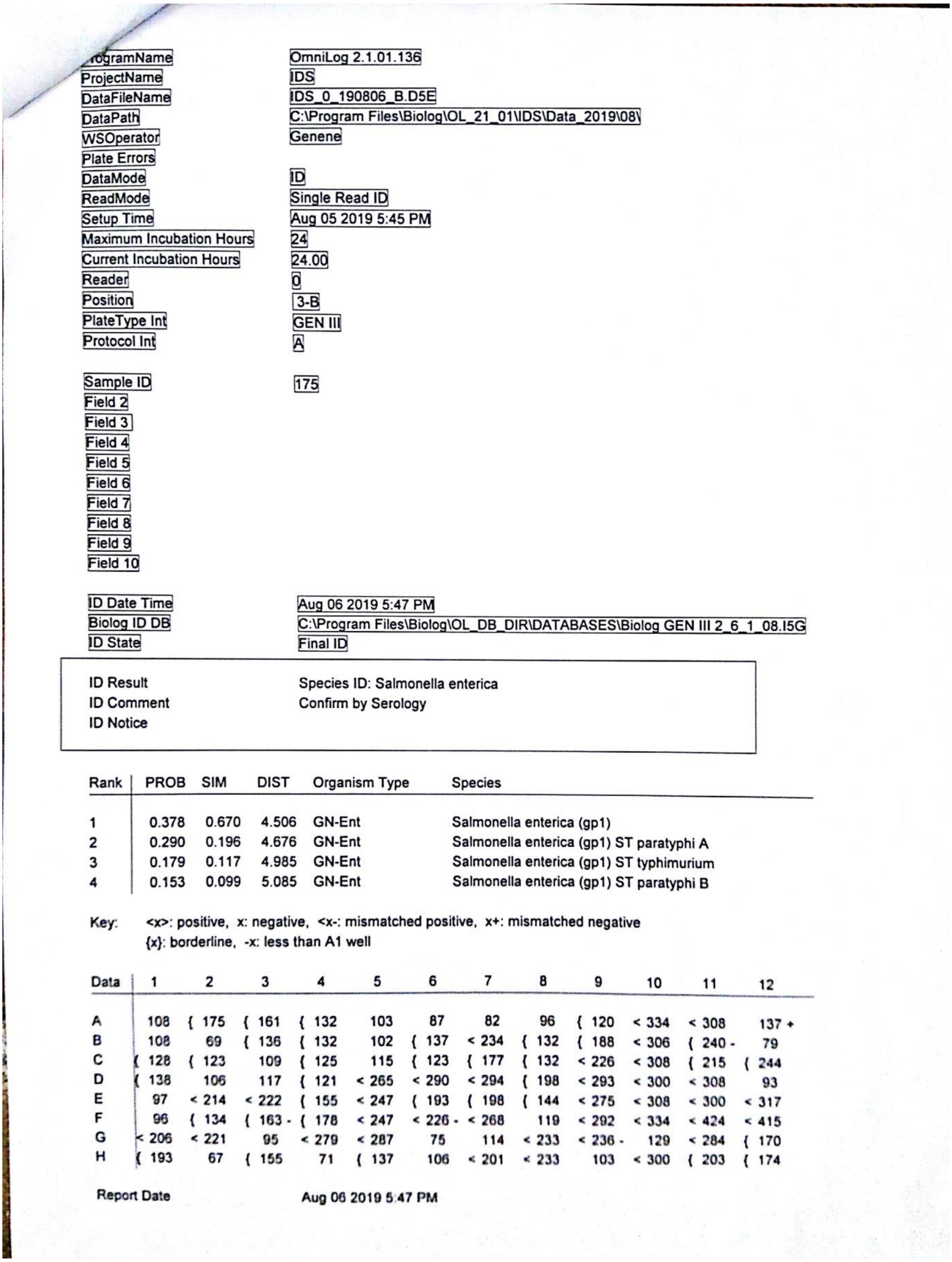


rogramName	OmniLog 2.1.01.136
ProjectName	IDS
DataFileName	IDS_0_190806_B.D5E
DataPath	C:\Program Files\Biolog\OL_21_01\IDS\Data_2019\08\
WSOperator	Genene
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Aug 05 2019 5:45 PM
Maximum Incubation Hours	24
Current Incubation Hours	24.00
Reader	0
Position	3-B
PlateType Int	GEN III
Protocol Int	A

Sample ID	175
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	

ID Date Time	Aug 06 2019 5:47 PM
Biolog ID DB	C:\Program Files\Biolog\OL_DB_DIR\DATABASES\Biolog GEN III 2_6_1_08.I5G
ID State	Final ID

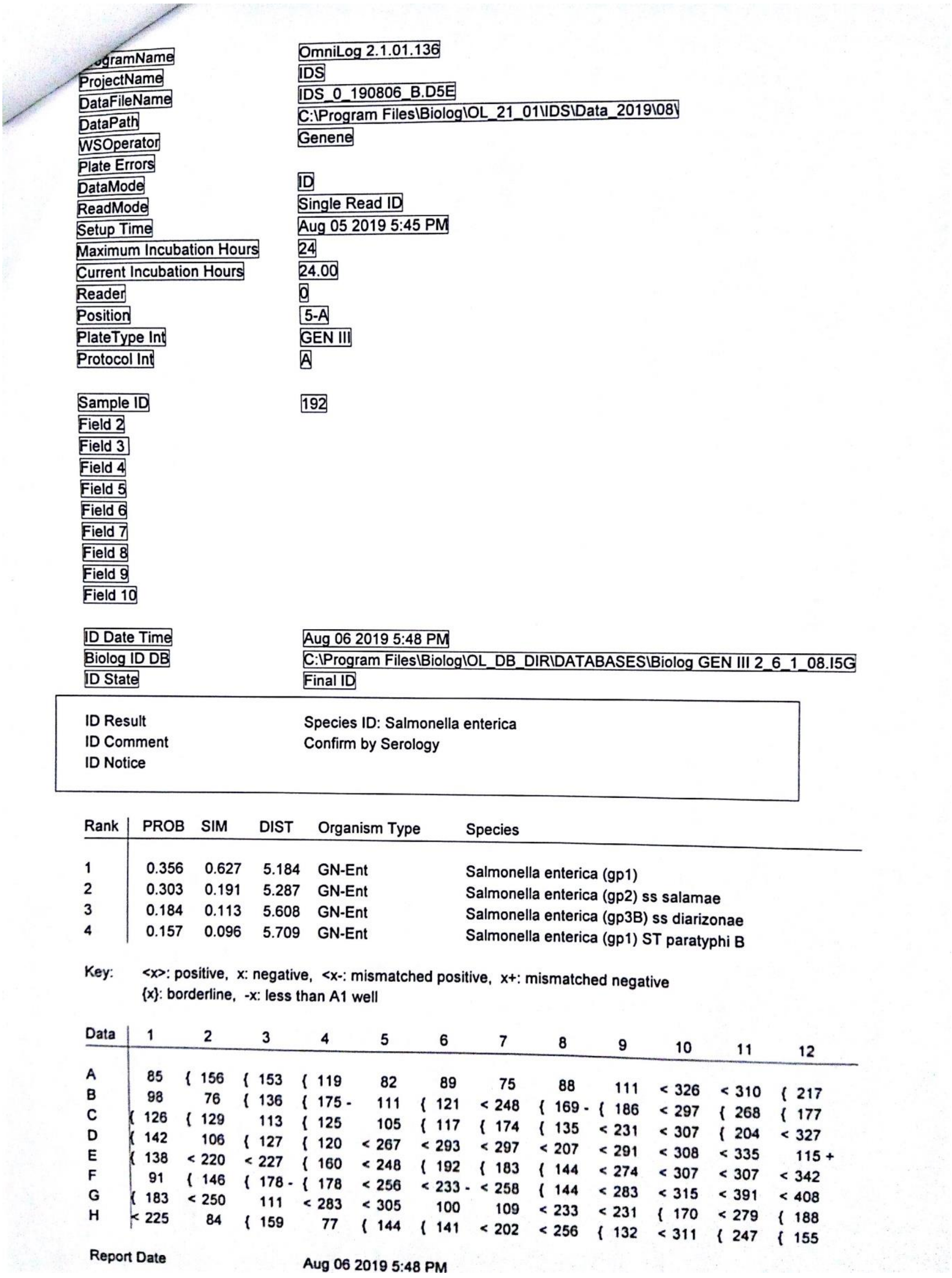
ID Result	Species ID: Salmonella enterica
ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.378	0.670	4.506	GN-Ent	Salmonella enterica (gp1)
2	0.290	0.196	4.676	GN-Ent	Salmonella enterica (gp1) ST paratyphi A
3	0.179	0.117	4.985	GN-Ent	Salmonella enterica (gp1) ST typhimurium
4	0.153	0.099	5.085	GN-Ent	Salmonella enterica (gp1) ST paratyphi B

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	108	{ 175	{ 161	{ 132	103	87	82	96	{ 120	< 334	< 308	137 +
B	108	69	{ 136	{ 132	102	{ 137	< 234	{ 132	{ 188	< 306	{ 240 -	79
C	{ 128	{ 123	109	{ 125	115	{ 123	{ 177	{ 132	< 226	< 308	{ 215	{ 244
D	{ 138	106	117	{ 121	< 265	< 290	< 294	{ 198	< 293	< 300	< 308	93
E	97	< 214	< 222	{ 155	< 247	{ 193	{ 198	{ 144	< 275	< 308	< 300	< 317
F	96	{ 134	{ 163 -	{ 178	< 247	< 226 -	< 268	119	< 292	< 334	< 424	< 415
G	< 206	< 221	95	< 279	< 287	75	114	< 233	< 236 -	129	< 284	{ 170
H	{ 193	67	{ 155	71	{ 137	106	< 201	< 233	103	< 300	{ 203	{ 174

Report Date Aug 06 2019 5:47 PM



gramName	OmniLog 2.1.01.136
ProjectName	IDS
DataFileName	IDS_0_190806_B.D5E
DataPath	C:\Program Files\Biolog\OL_21_01\IDS\Data_2019\08\
WSOperator	Genene
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Aug 05 2019 5:45 PM
Maximum Incubation Hours	24
Current Incubation Hours	24.00
Reader	0
Position	5-A
PlateType Int	GEN III
Protocol Int	A

Sample ID	192
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	

ID Date Time	Aug 06 2019 5:48 PM
Biolog ID DB	C:\Program Files\Biolog\OL_DB_DIR\DATABASES\Biolog GEN III 2_6_1_08.I5G
ID State	Final ID

ID Result	Species ID: Salmonella enterica
ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.356	0.627	5.184	GN-Ent	Salmonella enterica (gp1)
2	0.303	0.191	5.287	GN-Ent	Salmonella enterica (gp2) ss salamae
3	0.184	0.113	5.608	GN-Ent	Salmonella enterica (gp3B) ss diarizonae
4	0.157	0.096	5.709	GN-Ent	Salmonella enterica (gp1) ST paratyphi B

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	85	{ 156	{ 153	{ 119	82	89	75	88	111	< 326	< 310	{ 217
B	98	76	{ 136	{ 175 -	111	{ 121	< 248	{ 169 -	{ 186	< 297	{ 268	{ 177
C	{ 126	{ 129	113	{ 125	105	{ 117	{ 174	{ 135	< 231	< 307	{ 204	< 327
D	{ 142	106	{ 127	{ 120	< 267	< 293	< 297	< 207	< 291	< 308	< 335	115 +
E	{ 138	< 220	< 227	{ 160	< 248	{ 192	{ 183	{ 144	< 274	< 307	< 307	< 342
F	91	{ 146	{ 178 -	{ 178	< 256	< 233 -	< 258	{ 144	< 283	< 315	< 391	< 408
G	{ 183	< 250	111	< 283	< 305	100	109	< 233	< 231	{ 170	< 279	{ 188
H	< 225	84	{ 159	77	{ 144	{ 141	< 202	< 256	{ 132	< 311	{ 247	{ 155

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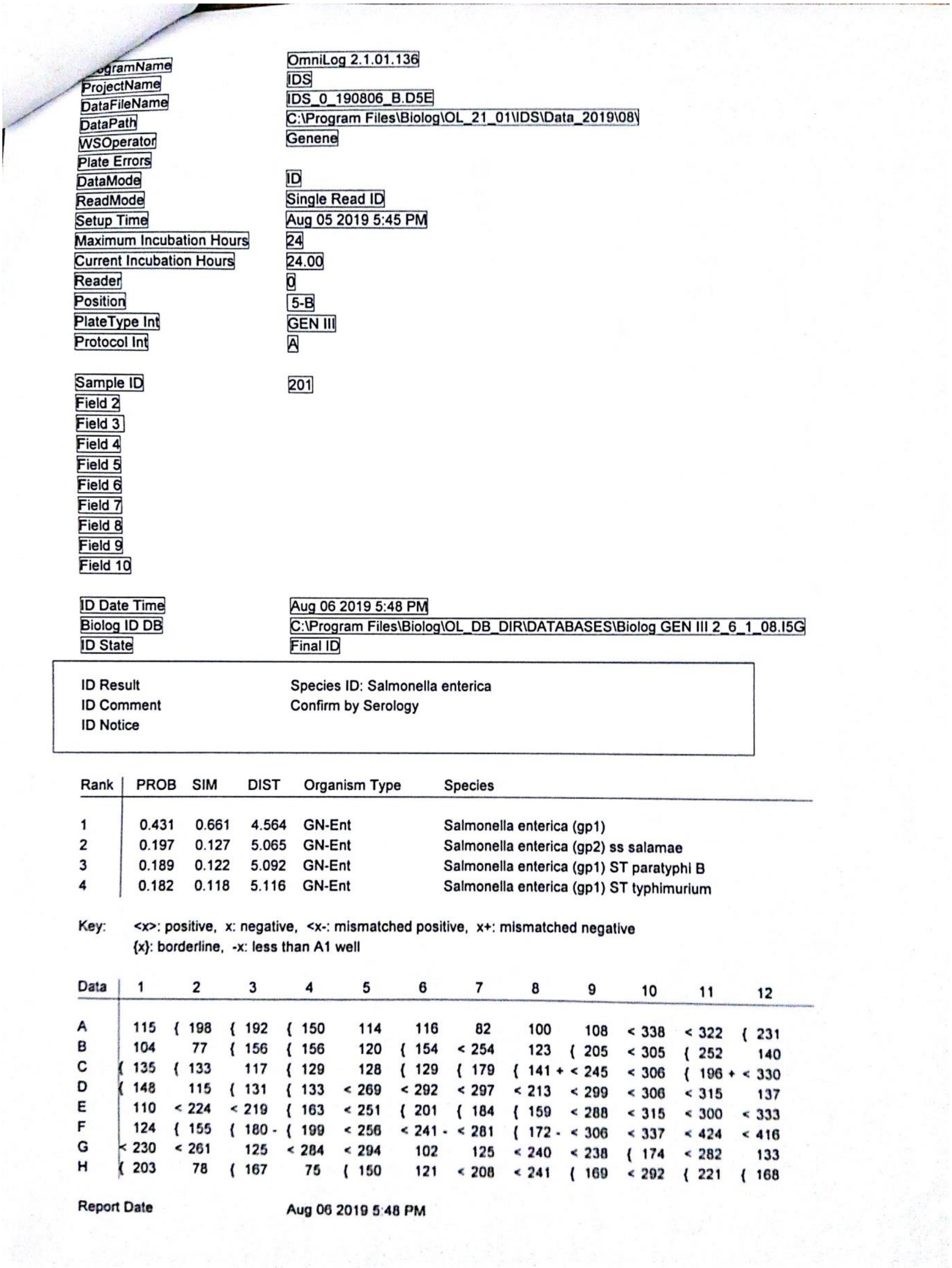

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ProjectName	IDS
DataFileName	IDS_0_190806_B.D5E
DataPath	C:\Program Files\Biolog\OL_21_01\IDS\Data_2019\08\
WSOperator	Genene
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Aug 05 2019 5:45 PM
Maximum Incubation Hours	24
Current Incubation Hours	24.00
Reader	0
Position	5-B
PlateType Int	GEN III
Protocol Int	A
Sample ID	201
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	Aug 06 2019 5:48 PM
Biolog ID DB	C:\Program Files\Biolog\OL_DB_DIR\DATABASES\Biolog GEN III 2_6_1_08.I5G
ID State	Final ID

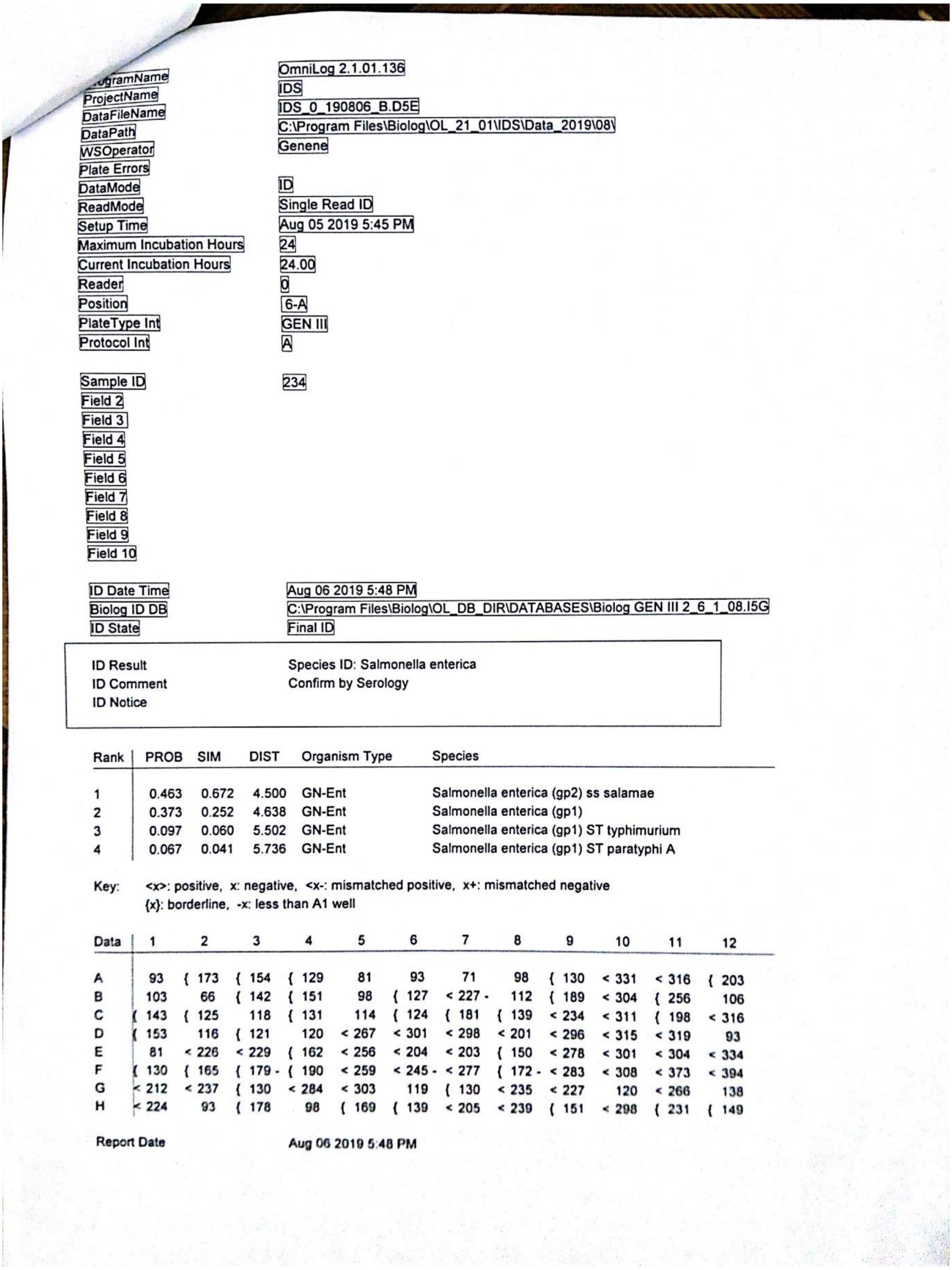
ID Result	Species ID: Salmonella enterica
ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.431	0.661	4.564	GN-Ent	Salmonella enterica (gp1)
2	0.197	0.127	5.065	GN-Ent	Salmonella enterica (gp2) ss salamae
3	0.189	0.122	5.092	GN-Ent	Salmonella enterica (gp1) ST paratyphi B
4	0.182	0.118	5.116	GN-Ent	Salmonella enterica (gp1) ST typhimurium

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	115	{ 198	{ 192	{ 150	114	116	82	100	108	< 338	< 322	{ 231
B	104	77	{ 156	{ 156	120	{ 154	< 254	123	{ 205	< 305	{ 252	140
C	{ 135	{ 133	117	{ 129	128	{ 129	{ 179	{ 141 +	< 245	< 306	{ 196 +	< 330
D	{ 148	115	{ 131	{ 133	< 269	< 292	< 297	< 213	< 299	< 306	< 315	137
E	110	< 224	< 219	{ 163	< 251	{ 201	{ 184	{ 159	< 288	< 315	< 300	< 333
F	124	{ 155	{ 180 -	{ 199	< 256	< 241 -	< 281	{ 172 -	< 306	< 337	< 424	< 416
G	< 230	< 261	125	< 284	< 294	102	125	< 240	< 238	{ 174	< 282	133
H	{ 203	78	{ 167	75	{ 150	121	< 208	< 241	{ 169	< 292	{ 221	{ 168

Report Date Aug 06 2019 5:48 PM



ogramName	OmniLog 2.1.01.136
ProjectName	IDS
DataFileName	IDS_0_190806_B.D5E
DataPath	C:\Program Files\Biolog\OL_21_01\IDS\Data_2019\08\
WSOperator	Genene
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Aug 05 2019 5:45 PM
Maximum Incubation Hours	24
Current Incubation Hours	24.00
Reader	0
Position	6-A
PlateType Int	GEN III
Protocol Int	A

Sample ID	234
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	

ID Date Time	Aug 06 2019 5:48 PM
Biolog ID DB	C:\Program Files\Biolog\OL_DB_DIR\DATABASES\Biolog GEN III 2_6_1_08.I5G
ID State	Final ID

ID Result	Species ID: Salmonella enterica
ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.463	0.672	4.500	GN-Ent	Salmonella enterica (gp2) ss salamae
2	0.373	0.252	4.638	GN-Ent	Salmonella enterica (gp1)
3	0.097	0.060	5.502	GN-Ent	Salmonella enterica (gp1) ST typhimurium
4	0.067	0.041	5.736	GN-Ent	Salmonella enterica (gp1) ST paratyphi A

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	93	{ 173	{ 154	{ 129	81	93	71	98	{ 130	< 331	< 316	{ 203
B	103	66	{ 142	{ 151	98	{ 127	< 227 -	112	{ 189	< 304	{ 256	106
C	{ 143	{ 125	118	{ 131	114	{ 124	{ 181	{ 139	< 234	< 311	{ 198	< 316
D	{ 153	116	{ 121	120	< 267	< 301	< 298	< 201	< 296	< 315	< 319	93
E	81	< 226	< 229	{ 162	< 256	< 204	< 203	{ 150	< 278	< 301	< 304	< 334
F	{ 130	{ 165	{ 179 -	{ 190	< 259	< 245 -	< 277	{ 172 -	< 283	< 308	< 373	< 394
G	< 212	< 237	{ 130	< 284	< 303	119	{ 130	< 235	< 227	120	< 266	138
H	< 224	93	{ 178	98	{ 169	{ 139	< 205	< 239	{ 151	< 298	{ 231	{ 149

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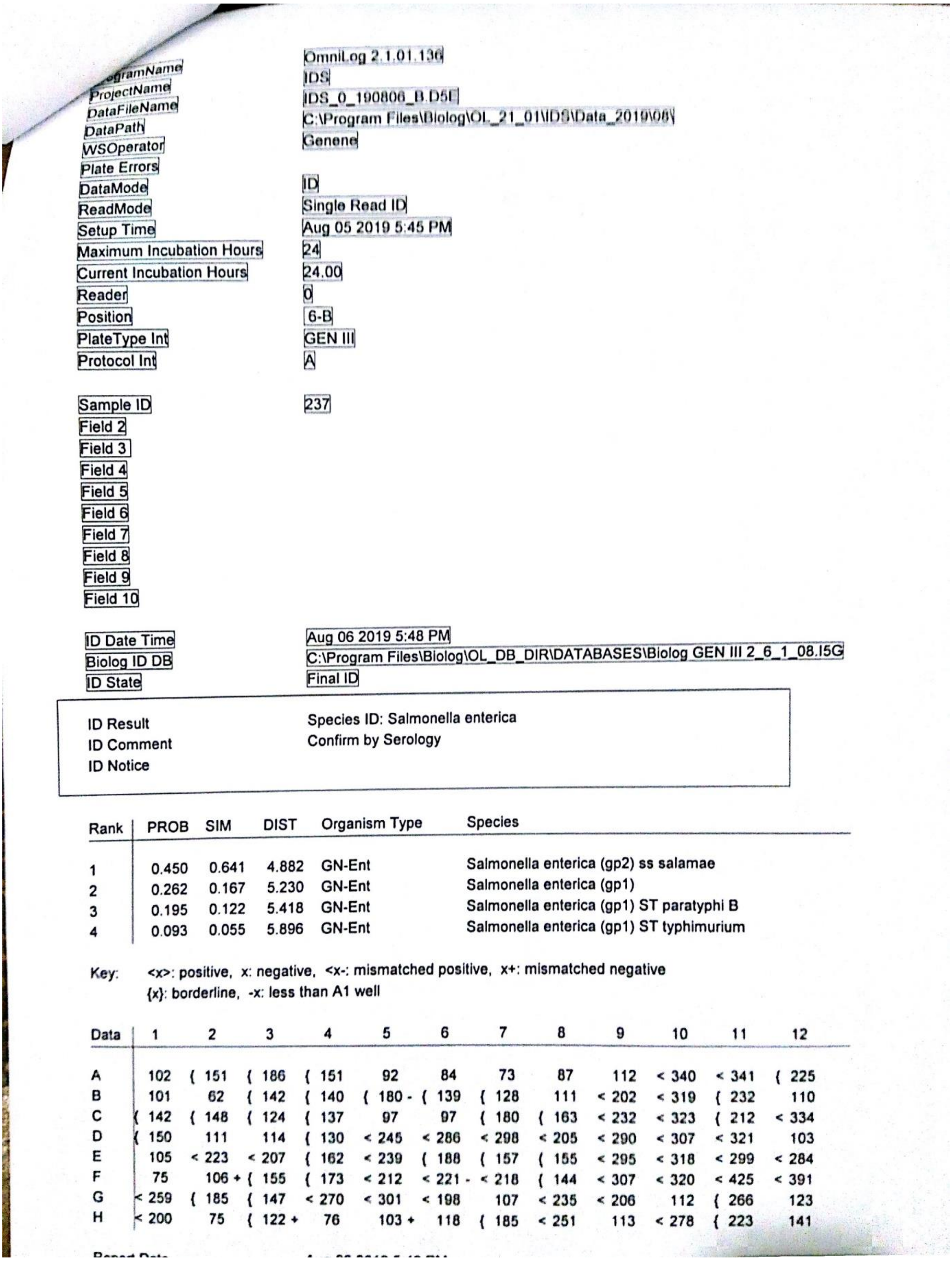


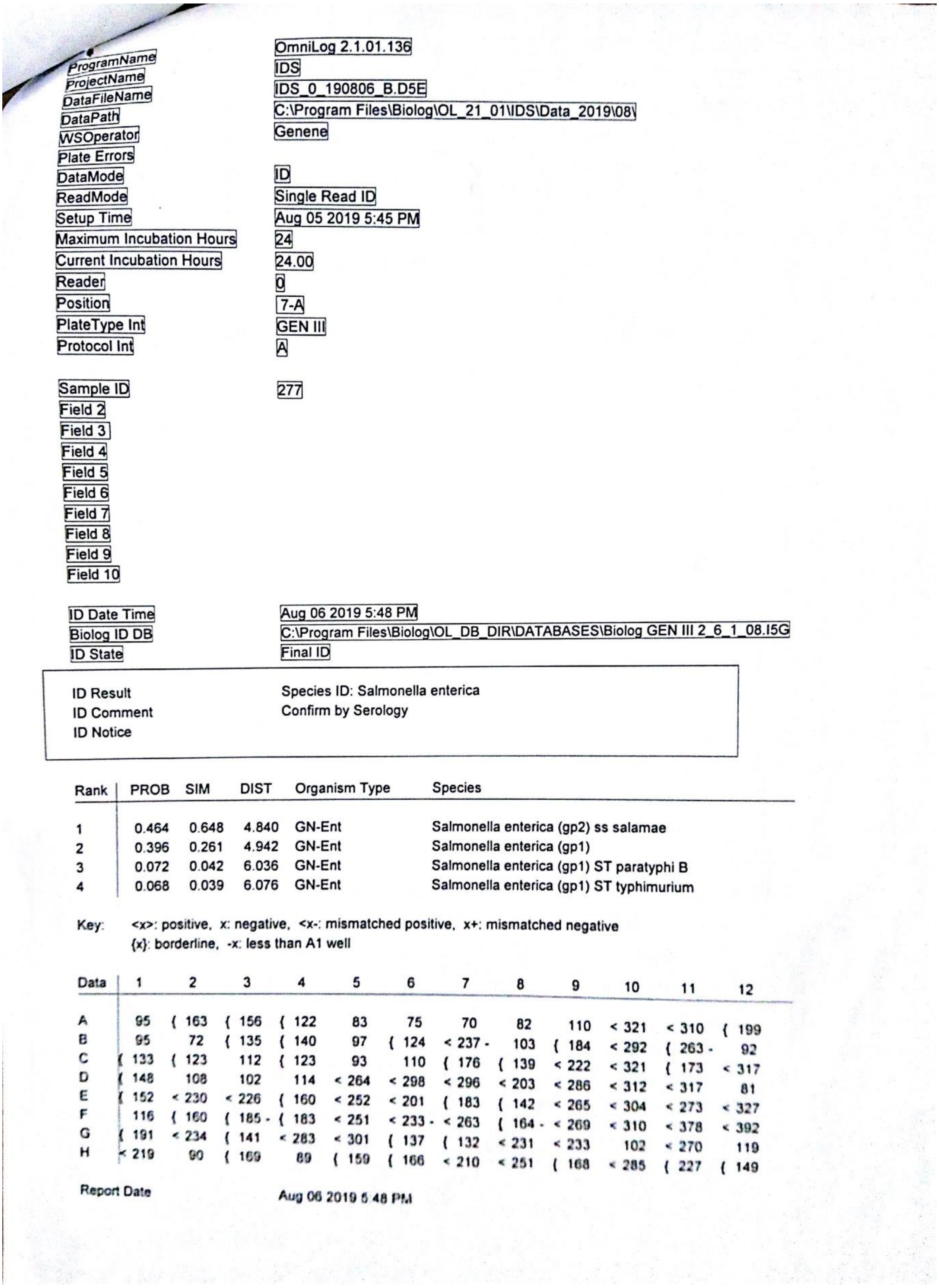
gramName	OmniLog 2.1.01.136
ProjectName	IDS
DataFileName	IDS_0_190806_B.D5E
DataPath	C:\Program Files\Biolog\OL_21_01\IDS\Data_2019\08\
WSOperator	Genene
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Aug 05 2019 5:45 PM
Maximum Incubation Hours	24
Current Incubation Hours	24.00
Reader	0
Position	6-B
PlateType Int	GEN III
Protocol Int	A
Sample ID	237
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	
ID Date Time	Aug 06 2019 5:48 PM
Biolog ID DB	C:\Program Files\Biolog\OL_DB_DIR\DATABASES\Biolog GEN III 2_6_1_08.I5G
ID State	Final ID

ID Result	Species ID: Salmonella enterica
ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.450	0.641	4.882	GN-Ent	Salmonella enterica (gp2) ss salamae
2	0.262	0.167	5.230	GN-Ent	Salmonella enterica (gp1)
3	0.195	0.122	5.418	GN-Ent	Salmonella enterica (gp1) ST paratyphi B
4	0.093	0.055	5.896	GN-Ent	Salmonella enterica (gp1) ST typhimurium

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	102	{ 151	{ 186	{ 151	92	84	73	87	112	< 340	< 341	{ 225
B	101	62	{ 142	{ 140	{ 180 -	{ 139	{ 128	111	< 202	< 319	{ 232	110
C	{ 142	{ 148	{ 124	{ 137	97	97	{ 180	{ 163	< 232	< 323	{ 212	< 334
D	{ 150	111	114	{ 130	< 245	< 286	< 298	< 205	< 290	< 307	< 321	103
E	105	< 223	< 207	{ 162	< 239	{ 188	{ 157	{ 155	< 295	< 318	< 299	< 284
F	75	106 +	{ 155	{ 173	< 212	< 221 -	< 218	{ 144	< 307	< 320	< 425	< 391
G	< 259	{ 185	{ 147	< 270	< 301	< 198	107	< 235	< 206	112	{ 266	123
H	< 200	75	{ 122 +	76	103 +	118	{ 185	< 251	113	< 278	{ 223	141



ProgramName	OmniLog 2.1.01.136
ProjectName	IDS
DataFileName	IDS_0_190806_B.D5E
DataPath	C:\Program Files\Biolog\OL_21_01\IDS\Data_2019\08\
WSOperator	Genene
Plate Errors	
DataMode	ID
ReadMode	Single Read ID
Setup Time	Aug 05 2019 5:45 PM
Maximum Incubation Hours	24
Current Incubation Hours	24.00
Reader	0
Position	7-A
PlateType Int	GEN III
Protocol Int	A

Sample ID	277
Field 2	
Field 3	
Field 4	
Field 5	
Field 6	
Field 7	
Field 8	
Field 9	
Field 10	

ID Date Time	Aug 06 2019 5:48 PM
Biolog ID DB	C:\Program Files\Biolog\OL_DB_DIR\DATABASES\Biolog GEN III 2_6_1_08.I5G
ID State	Final ID

ID Result	Species ID: Salmonella enterica
ID Comment	Confirm by Serology
ID Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.464	0.648	4.840	GN-Ent	Salmonella enterica (gp2) ss salamae
2	0.396	0.261	4.942	GN-Ent	Salmonella enterica (gp1)
3	0.072	0.042	6.036	GN-Ent	Salmonella enterica (gp1) ST paratyphi B
4	0.068	0.039	6.076	GN-Ent	Salmonella enterica (gp1) ST typhimurium

Key: <x>: positive, x: negative, <x-: mismatched positive, x+: mismatched negative
{x}: borderline, -x: less than A1 well

Data	1	2	3	4	5	6	7	8	9	10	11	12
A	95	{ 163	{ 156	{ 122	83	75	70	82	110	< 321	< 310	{ 199
B	95	72	{ 135	{ 140	97	{ 124	< 237 -	103	{ 184	< 292	{ 263 -	92
C	{ 133	{ 123	112	{ 123	93	110	{ 176	{ 139	< 222	< 321	{ 173	< 317
D	{ 148	108	102	114	< 264	< 298	< 296	< 203	< 286	< 312	< 317	81
E	{ 152	< 230	< 226	{ 160	< 252	< 201	{ 183	{ 142	< 265	< 304	< 273	< 327
F	116	{ 160	{ 185 -	{ 183	< 251	< 233 -	< 263	{ 164 -	< 269	< 310	< 378	< 392
G	{ 191	< 234	{ 141	< 283	< 301	{ 137	{ 132	< 231	< 233	102	< 270	119
H	< 219	90	{ 169	89	{ 159	{ 166	< 210	< 251	{ 168	< 285	{ 227	{ 149

Report Date Aug 06 2019 5:48 PM