ADDIS ABABA UNIVERSITY
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CHARACTERIZATION OF ADHESION AND INVASION VIRULENCE FACTORS IN *STREPTOCOCCUS PNEUMONIAE*, *SHIGELLA*, AND *SALMONELLA* SPECIES ISOLATED FROM ETHIOPIAN PATIENTS

By

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ADDIS ABABA, ETHIOPIA

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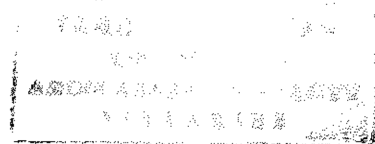

CHARACTERIZATION OF ADHESION AND INVASION VIRULENCE FACTORS IN *STREPTOCOCCUS PNEUMONIAE*, *SHIGELLA*, AND *SALMONELLA* SPECIES ISOLATED FROM ETHIOPIAN PATIENTS

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, ADDIS ABABA UNIVERSITY, IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGY

By

TEFERI ESHETU

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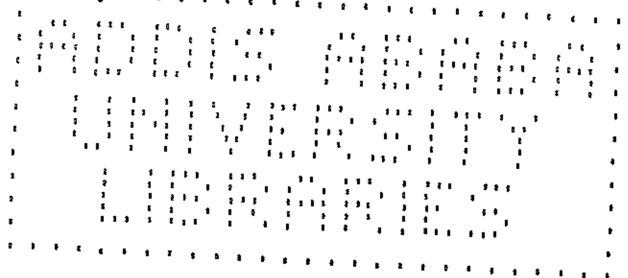

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Abstract

Pathogenic bacteria have multiple virulence factors that act in concert to cause damaging effects in the host body. In this work few of the virulence factors interactions between pathogenic bacteria and human epithelial cells have been studied. Therefore, tissue adherence and invasion as well as haemagglutinating abilities of 50 strains of *Streptococcus pneumoniae*, 25 *Shigella* spp. and 25 *Salmonella* spp. isolated from patients were studied.

The tissue adherence ability of these bacterial isolates was detected using cultured human epithelial cells (HEp-2 cells). In addition to the tissue adherence assay agglutination ability of the bacteria against six erythrocyte types was tested to find out if the same factors are involved in haemagglutination and adhesion to HEp-2 cells. The results of these tests showed that haemagglutination and tissue adherence abilities of the pathogenic bacteria are different. All *Salmonella* and *Shigella* isolates except one had adherence ability to HEp-2 cells. Unlike their adherence ability to HEp-2 cells, the haemagglutination result showed that only 13 strains of the *Salmonella* spp. and 17 strains of the *Shighella* spp. are haemagglutinating. Similarly, 43 (86%) of *Streptococcus pneumoniae* were able to adhere to HEp-2 cells and among these only 9 strains showed non-haemagglutinating ability. In the *S. pneumoniae* isolates, there were 6 strains that do not adhere to HEp-2 cells, and one was found to be neither haemagglutinating nor adhering to HEp-2 cells.

The tissue invasion ability of the pathogenic bacteria was determined with a factor of 2-hr intracellular growth incubation time and addition of exogenous gentamicin. The invasiveness ability of each strain was determined as percentage of bacteria recovered from initial inoculum quantity of bacteria added to HEp-2 cell monolayer. From the invasiveness results all the *Shigella* and *Salmonella* isolates were able to invade the cultured epithelial cells with invasion results ranging between 1.26 and 32.93 %. In the case of *Streptococcus pneumoniae* isolates, only 12 out of the 50 strains were able to invade the cultured HEp-2 cells with invasion percentages ranging between 0.04 and 2.11%. The remaining 38 strains could not invade the HEp-2 cells.

1. Introduction

During evolution pathogenic bacteria have developed different ways to meet the requirements of living in various niches (Lugtenberg, 1985). As means of survival in host tissues, microorganisms have different virulence determinants that allow them to evade host immune responses (Sherris and Ryan, 1994). A successful pathogen multiplies either when conditions are favorable or at other times may struggle to survive within a complex often hostile environment. Eventually, the pathogen with its pathogenic strategy results in successful transmission to a new host or secures its colonization of the primary host (Falkow *et al.,* 1992). Moreover, pathogens are typically armed with a number of virulence factors and thus pathogenicity involves multifactorial phenotypes (Falkow, 1994).

Virulence factors can be defined as components of a pathogen that damage the host, and can include components either essential for the survival of the pathogen or not. The components can comprise microbial products that permit the pathogen to cause diseases (Casadevall and Pirofski, 1999). There are many genes required for virulence in bacteria, which are in discrete DNA segments such as 'Pathogenicity islands'. The acquisition of such genes is sufficient for a bacterium to become a virulent one (Hacker *et al.,* 1997). Due to variation in the composition of virulence genes the chemistry of the microbial surface would also vary. The variation can be the major distinction between pathogenic and non-pathogenic bacteria (Smith, 1977).

Important virulence factors of Gram-negative and Gram-positive pathogens are plasmid encoded. However, other virulence determinants are located on the chromosome, where they are associated in so-called 'virulence blocks' or 'virulence cassettes'. Particular regions of chromosomally encoded virulence-associated genes have also been termed 'Pathogenicity islands' (Pais) (Blum *et al.,* 1994). Virulence genes of pathogenic bacteria, which code for toxins, adhesins, invasins, or other virulence factors, may be located on transmissible genetic elements such as transposons, plasmids, or bacteriophages (Hacker *et al.,* 1997). Bacterial pathogens tightly regulate the expression of genes required for virulence. There are more than 100 bacterial proteins which are upregulated within cells, and approximately 40 of these appear to be unique to the intracellular environment (Pfeifer *et al.,* 1999).

During bacterial infection mechanisms for establishment within a suitable niche, nutrient acquisition, and avoidance of immune clearance act together and cause overt diseases (Miller *et al.,* 1989). The first major step of interaction between a pathogenic microorganism and its host entails attachment to host cell surfaces (Falkow, 1994). Furthermore, colonization of the host tissue surfaces is the most critical point in the process of infection for pathogens requiring the uptake of essential exogenous nutrients. Among the required nutrients iron is essential for bacterial growth. However, free iron is present at only very low level (~ 10^{-8} M) at sites where colonization occurs because the iron is complexed with high affinity iron sequestering molecules (Bullen, 1981). Consequently, a critical component of the virulence factors of microorganisms is their ability to obtain iron from their hosts (Duchesne *et al.,* 1999).

By adherence, pathogenic bacteria colonize human hosts through interactions between specific bacterial adhesins and respective binding sites on the host cell surfaces (Finlay and Cossart, 1997). Adherence is also an important characteristic especially for bacteria that live on mucosal surfaces during their infectious process (Walker, 1998). However, for a majority of bacterial and viral infections, which enter the body through mucosal surfaces, adherence is the first step in pathogenesis (Jakobsen, 1999a). For example, *Streptococcus pneumoniae,* initially colonizes the human nasopharynx and can cause infections of the lower respiratory tract infections and bacteremia (Kim *et al.,* 1999).

In addition to their ability of adherence host cell invasion is a specialized strategy for survival and multiplication used by a number of microorganisms. The microorganisms may enter either professional phagocytes, such as neutrophils and macrophages, or enter non-phagocytic cells, such as epithelial and endothelial cells (Falkow, 1994). Hence, virulence may depend upon the degree of invasiveness, the route or mechanism of invasion, the biological sequelae to invasion, or a combination of these (Douce *et al.,* 1991). Pathogens often coordinately regulate the expression of virulence factors via environmental signals. In this respect, for many pathogens, host cell invasion occurs by triggering of an endocytic process initiated by binding of the bacteria to specific receptors on the target tissue (Spence *et al.,* 1997). Thus, identification of the mechanisms of the interactions may help define domains on both bacterial and host tissues, which are critical for establishing diseases. This in turn provides a basis for the development of novel therapeutic or preventive strategies (Prasadarao *et al.*, 1999). In addition, studies of factors that involve in virulence are important in one way or another in the field of molecular biology of pathogenic bacteria. In general the studies of virulence factors have important

practical implications such as vaccine developments or serve as diagnostic tools (Law, 1988).

Studies on factors that involve in pathogenesis during bacterial infections are limited in Ethiopia. Aberra Geyid (1995) studied different virulence factors of diarrhoegenic *Escherichia coli* strains that were causes of acute and persistent diarrhea among Ethiopian infants.

General Objective

The general aim of the present work is to carry out study of virulence factors of some important pathogenic bacteria isolated from Ethiopian patients (*Streptococcus pneumoniae, Shigella,* and *Salmonella* species).

Specific Objectives

The specific objectives of this work are:

1) To investigate the ability of adherence to epithelial cells in the pathogenic bacteria.
2) To investigate the virulence factor invasion of epithelial cells in the pathogenic bacteria using cultured epithelial cells.
3) To analyze presence of association between the ability to agglutinate erythrocytes and adhesion to epithelial cells.
4) To compare the adherence and invasion characteristics of the bacteria among the groups.

2. Literature Review

2.1. The Virulence Factors of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a Gram-positive, oval diplococcus with its axis end to end, giving the individual bacterium a bullet or lancet shape. It grows best at temperatures 35 to 37° C and can grow under atmospheric conditions ranging from aerobic to strictly anaerobic. On blood agar the pneumococcus produces round glistering 0.5 to 2.0 mm colonies surrounded by a zone of alpha-hemolysis (Ryan and Falkow, 1994a).

The pneumococcus is encapsulated with polysaccharide (PS), which is the main virulence factor. The polysaccharide protects the bacterium from phagocytosis following opsonization by polysaccharide-specific antibodies and complement proteins (Vidarsson *et al.,* 1994). The bacterium is capable of considerable interstrain heterogeneity as evidenced by its ability to express at least 90 unique types of the capsular polysaccharides. In addition, the virulence of the pneumococcus is highly sensitive to relatively small differences in amounts of capsular polysaccharides between strains of the same type (Weiser and Kapoor, 1999).

In addition to interstrain variation there is a marked intrastrain variation in most isolates. The variation is explained as differences between opaque and transparent phenotypes when colonies are viewed on translucent surfaces rather than on blood agar (Weiser *et al.,* 1994). In comparison to opaque forms, the transparent variants have greater amounts of cell wall teichoic acid (associated with C-polysaccharide) and an altered distribution of proteins that are anchored to the choline moiety on the teichoic acid (Rosenow *et al.,*

1999). Opaque pneumococci express from 2.5-to 22-fold more cell wall associated capsular polysaccharide, as measured by capture enzyme-linked immunosorbent assay, than do transparent variants of the same strain. The increased expression of capsular polysaccharide by opaque pneumococci (i) hinders cytoadherence, which may account for their inefficiency at colonization of the nasopharynx, and (ii) increases resistance to opsonophagocytosis, which may explain their greater virulence in a murine model of sepsis (Kim *et al.,* 1999).

Streptococcus pneumoniae is the most important bacterial cause of pneumonia, meningitis, and otitis media in young children, in the elderly, and in patients with chronic medical conditions or immunosuppressive illnesses, particularly AIDS (Siber, 1994). The bacteria can also cause bacteremia (sepsis) especially in elderly people and in young children though it enters the body through the respiratory mucosa (especially the nasopharynx) (Jakobsen *et al.,* 1999a).

Severe pneumococcal infection and its complications result partly from the direct actions of pneumococcal virulence determinants and the corresponding immune responses to the various pneumococcal components. Pneumococcal virulence determinants and the corresponding immune responses produce four key effects: adhesion, invasion, inflammation, and shock. Several of the virulence determinants have been proposed as vaccine candidates (Gillespie and Balakrishnan, 2000).

It is observed that secretory IgA at mucosal surfaces inhibits the adherence and invasion of mucosal pathogens, as a result mucosal immune responses against pneumococci are

considered to be beneficial. Furthermore, mucosal immunization has additional benefits in that it induces sufficient systemic immune responses and generates immunological memory (Jakobson *et al.,* 1999b). The already existing 23-valent-pneumococcal polysaccharide vaccine was formulated to prevent invasive infections in the elderly and high risk populations. However, the effectiveness of the vaccine for population of adults over 65 years old remains controversial (Rubins *et al.,* 1999). In addition, the vaccine is poorly immunogenic in young children (Jakobson *et al.,* 1999b). Thus, seeking effective preventive means for *streptococcus pneumoniae* infections is given priority in this era (Rubins *et al.,*1999).

2.1.1. Adhesion to and Invasion of Host Tissues by *S. pneumoniae*

Pneumococcal phase variation between opaque and transparent variants has been implicated for the variation in the ability of pneumococci to colonize the nasopharynx in an infant rat model (Weiser *et al.,* 1994). Transparent variants are able to colonize the nasopharynx more easily than opaque variants. This attribute is in keeping with their greater capacity for adherence to various cells, e.g. human buccal cells, and human lung epithelial cells (Gillespie and Balakrishnan, 2000).

A two step model of pneumococcal adherence has been established. Pneumococci first target host cells by binding surface glycoconjugates; such as Gal NAc-β 1,4-Gal and Gal NAc-β 1,3-Gal. A second adherence step is shown to lead to invasion and is observed upon activation of the human cells by proinflammatory cytokines (Cundell *et al.,* 1995c).

Adherence in the nasopharynx involves only recognition of *N*-acetyl-D-glucoseamine β1-3 galactose (Glc NAc β1-3 Gal) glycoconjugate receptors on buccal epithelial cells. In contrast, adherence to resting lung cells (pneumocytes and vascular endothelial cells) involves two classes of receptors containing *N*-acetyl-D-galactosamine linked to either β1,3 or β1,4 galactose (Gal NAc β1,3 Gal and Gal NAc β1,4 Gal) (Cundell *et al.*,1995a).

Pneumococci are also able to adhere to platelet-activating factor (PAF) receptors on host cells via their phosphorylcholine determinant. PAF receptors have been identified in a number of tissues and cells including lung, brain, and leukocytes (Cundell *et al.*, 1995b). The adherence of pneumococci is enhanced by treatment of target cells with interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α). The increased adherence is due to enhanced appearance of new specificity for *N*-acetyl-D-glucoseamine (Glc Nac) and platelet-activating factor (PAF) receptors as shown on activated pneumocytes and vascular endothelial cells (Cundell *et al.*, 1995a).

Choline-binding protein A (CbpA): the surface of *S. pneumoniae* is decorated with a family of choline-binding proteins (CBPs) that are non-covalently bound to the phosphorylcholine of the teichoic acid in the cell wall. One of the CBPs is the choline-binding protein A (CbpA), which is identified as an adhesin and a determinant of virulence. CbpA is a 75-kDa surface exposed protein. Two examples of other CBPs are Pneumococcal surface protein A (PspA) and the major autolysine (Lyt A) (Rosenow *et al.*, 1999). CbpA is present on the pneumococcal surface via non-covalent association with the choline of the cell wall and appears to promote increased pneumococcal attachment to cytokine activated human cells (Ring *et al.*, 1998).

Pneumococcal adherence to cells has been shown *in vivo* and *in vitro* to be affected by reversible phase variation marked by changes in morphology from opaque to transparent phenotype and vice versa (Cundell *et al.,* 1995a). In comparison to noninvasive opaque forms, transparent pneumococci present more cell wall choline, the natural ligand for the platelet-activating factor (PAF) receptor. As a result transparent pneumococci express more CpbA and interact with cytokine activated host cells that favors adhesion and subsequent invasion (Ring *et al.,* 1998).

Pneumococcal surface adhesin A (PsaA): is a 37-kDa surface lipoprotein essential for pneumococcal virulence. PsaA is encoded by *psaA* gene, which is part of the *psa* locus that also contains three other open reading frames (ORFs).The ORFs encode an adenosine triphosphate (ATP)-binding protein (PsaB), a hydrophobic membrane protein (PsaC) and another protein (PsaD) which has homology with periplasmic thiol peroxidase of *Escherchia coli* (Gillespie and Balakrishnan, 2000).

The role of PsaA as pneumococcal adhesin is identified by the finding that $PsaA^-$ strains had significantly less *in vitro* adherence to cell lines than $PsaA^+$ strains. Also it has been shown that *psaA* gene mutant type-2 strain of *S. pneumonia* has significantly reduced virulence for mice. PsaA is also a protective immunogen in mice as a purified protein (Berry and Paton, 1996).

The most important event in pneumococcal infection is invasion of tissues in the lower respiratory tract. This ability of tissue invasion provides a route of transmission across the alveolar space into the blood stream and across the blood–brain barrier into the menings (Gillespie and Balakrishnan, 2000). In addition, the ability of *S. pneuomniae* to readily gain access to the blood circulation from the alveolar space is believed to occur due to a strong interaction with vascular endothelial cells (EC) of the alveolar capillaries (Cundell *et al.*,1995a).

Pneumococci can not enter resting vascular endothelial cells *in vitro*. This condition was shown by the survival of only 0.1% of pneumococcal inoculum upon exposure to exogenous gentamicin. In contrast, activation of endothelial cells by cytokines results in the entry of 2-3% of the pneumococci within 30 minutes. Internalization of pneumococci by activated cells can be largely prevented (i.e. decreased by ~87%) by treatment of the cells with PAF receptor antagonists (Cundell *et al.*, 1995c). In addition, in an *in vitro* test pneumococci were incubated with human umbilical vein endothelial cells, and IL-1 and TNF-α cytokines. After the incubation the pneumococci were seen preferentially adhered to the borders of the cells. Subsequent to their adherence the pneumococci were taken up rapidly by the endothelial cells because bacteria were present inside vacuoles with in 1hr of incubation (Geelen *et al.*, 1993).

2.1.2. Enzymes of *S. pneumoniae* as Virulence Factors

Autolysin: it is *lytA* gene encoded major autolysin (*N*-acetylmuramoyl-L-alanin amidase) of *S. pneumoniae*. It is a member of a widely distributed group of cell wall-degrading enzymes (Whatmore and Dowson, 1999).

The involvement of *lytA* gene product autolysin in virulence has been shown by that isogenic *lytA* mutants have been found to be significantly less virulent than the parent strains in animal models. When mutant strains were inoculated into mouse lung in a model of pneumonia, the strains were cleared rapidly and could not invade the blood stream (Canvin *et al.,* 1995). Mechanisms for the roles of autolysin in pathogenesis have been postulated. Autolysin may play a role in virulence by mediating the release of bacterial cell wall components that are shown to be highly inflammatory in animal models. In another way autolysin plays role in pathogenesis by mediating bacterial cell lysis and the subsequent release of virulence factors, such as pneumolysin that is not actively exported from the bacterial cell (Whatmore and Dowson, 1999).

Hyaluronidase: this could facilitate pneumococcal invasion by degrading connective tissues. The importance of this enzyme in virulence has been demonstrated by that pneumococcal strains with higher hyaluronidase activity breach the blood-brain barrier and disseminate more effectively (Gillespie and Balakrishnan, 2000). Also it has been shown that addition of exogenous hyaluronidase to strains, which did not produce the enzyme were able to disseminate and breach the blood-brain barrier in mice. Hence, it was concluded that high hyaluronidase activity is the most important factor contributing to the development of pneumococcal meningitis (Kostyukova *et al.,* 1995).

Neuraminidase: the enzyme is secreted during logarithmic phase of the bacterial growth. It cleaves terminal molecules of N-acetylneuramic acid (NANA) from glycoproteins and gangliosides. So that, if secreted into cerebrospinal fluid (CSF) it cleaves CSF proteins, gangliosides, and the glycoproteins of neurons and other central nervous system tissues. The damages to these components result in clinical manifestations of neural dysfunction (O' Toole *et al.,* 1971). In addition, the enzyme cleaves sialic acid residues from a wide variety of molecules damaging host tissues. This action may serve to expose receptors for pneumococcal adhesins facilitating both colonization and invasion (Gillespie and Balakrishnan, 2000).

Production of neuraminidase by *S. pneumoniae* results in exposure of red blood cell T-antigen, resulting in hemolysis, thrombocytopenia, and acute renal failure in hemolytic uremic syndrome (HUS) in children (Pan *et al.,* 1995). Hemolytic uremic syndrome (HUS) associated with infection by neuraminidase producing *S. pneumoniae* usually presents with pneumoniae and results in high mortality rate (Erickson *et al.,* 1994).

2.1.3. Other Virulence Factors of *S. pneumoniae*

Pneumolysin: it is a 53-kDa thiol-activated cytolysin produced by virtually all clinical isolates of pneumococci. It is a proven virulence factor of *S. pneumoniae.* Its cytolytic activity involves interaction with cholesterol in target cell membranes and insertion into the lipid bilayer, followed by oligomerization to form transmembrane pores. Eventually this activity brings about host cell lysis (Berry *et al.,* 1999).

During infection the cytolytic properties of pneumolysin enables it to cause damage on a wide variety of host cells, including bronchial epithelial cells, alveolar epithelial cells and pulmonary endothelium. Its detrimental effects on bronchial epithelium result in slowing of the ciliary beat, impairing the ability of mucociliary escalator to clear particles effectively (Rayner *et al.*,1995). Pneumolysin also has toxic effects in rat lungs and rabbit corneas when supplied *in vivo.* Also from *in vitro* tests it lysed polymorphonuclear leukocyte (PMNL) and platelets. It has also inhibitory effects on the polymorphonuclear leukocytes' respiratory burst activity *in vitro* (Sato *et al.,* 1996). The involvement of pneumolysin in direct lung damage is suggested by the finding that phospholipase-A in pulmonary artery endothelium is strongly activated by pneumolysin. Once the phospholipase-A is activated it breaks down a wide variety of Cell-membrane phospholipids (Rubins *et al.,* 1994)

In addition to its activity of cell lysis, pneumolysin is the primary component of live pneumococci stimulating Nitric Oxide (NO) production in macrophages (Braun *et al.*, 1999). The production of NO during inflammatory response deleterious effects. The deleterious effects occur during sepsis acute inflammation, including circulatory and organ failure in septic shock (Braun *et al.,* 1999). It has been also observed that pneumolysin is capable of directly activating the classical complement pathway. It activates the complement pathway in the absence of specific antibody, with a concomitant reduction of serum opsonic activity (Paton *et al.,* 1984).

Pneumococcal surface protein A (PspA): it is an 84-kDa protein containing several distinct domains. Among the domains the C-terminal end of PspA is a choline-binding

component made up of 10 repeats of 20 amino acids each. It is responsible for the attachment of PspA to pneumococcal surface (Gillespie and Balakrishnan, 2000).

According to Tu *et al.*, (1999) $PspA^+$ and $PspA^-$ strains of pneumococci were used to examine the effect of PspA on virulence both *in vivo* and *in vitro*. From the *in vivo* and *in vitro* data the mechanisms by which PspA contributes to virulence is by interfering with deposition of the complement protein C3b. This condition prevents the formation of the alternative pathway amplification by C3 convertases. This process also leads to reduction in opsonophagositosis of the pneumococcus.

C-polysaccharide (Capsular polysaccharide): it has been established that the polysaccharide capsule of the pneumococci is an important virulence factor. This was shown by the production of mutant strain, lacking detectable capsular polysaccharide, from an encapsulated parent strain. The uncapsulated mutant had greatly reduced virulence in a mouse model compared to the parental strain (Watson and Musher, 1990).

The polysaccharide capsule of *S. pneumoniae* has an important role in generating inflammatory mediators and consequent pathological changes. Injection of cell wall preparation or isolated capsular antigen has been shown to generate strong inflammatory response in chinchilla rabbits (Tuomanen *et al.*, 1985). Also it is thought that the components of the complement cascade generated by interaction with C-polysaccharide are crucial in generation of an inflammatory reaction in the alveoli, the menings, and middle ear. Also there is higher trend of mortality in patients with detectable C-polysaccharide in their serum, pneumococci with high cell wall turnover induce greater inflammatory response (Gillespie *et al.*, 1997).

In another observation *S. pneumoniae* strains were seen to bind the complement regulatory protein factor H via the C-polysaccharide. The binding results in the inhibition of complement activation. Also the binding of *S. pneumoniae* to factor H results in resistance to complement- dependent opsonophagositosis, which is an essential part of host defense against the pneumococci (Neeleman *et al.*, 1999).

2.2. The Virulence Factors of *Shigella* spp.

Shigella spp. are Gram-negative enteric bacteria that continue to cause significant cases of diarrhoea and dysentery worldwide (Way *et al.*, 1999). *Shigella* spp. are closely related to *E. coli* biochemically and antigenically. The genus is divided into four species, or groups on the bases of differences in O-antigens and some biochemical reactions. The species (groups) are *Shigella dysentriae* (A), *Shigella flexineri* (B), *Shigella boydii* (C), and *Shigella sonnei* (D). All but *S. sonnei* are further subdivided into a total of more than 30 individual serotypes (Ryan and Falkow, 1994b). *Shigella dysentriae* (group A), *Shigella flexineri* (group B), *Shigella boydii* (group C), and *Shigella sonnei* (group D)-include 10, 6, 18, and 1 serotypes respectively (Echeverria *et al.*, 1991).

Shigella spp. cause disease by first invading the epithelial cells of the colonic mucosa and then spreading intra- and intercellularly. The intercellular dissemination produces inflammation and ulceration, resulting in diarrhoea or dysentery (Hartman *et al.*, 1999). Microbiological surveys, in areas where diarrhoeal disease is endemic, have implicated *Shigella* spp. as etiologic agents in at least 20% of diarrhoeal cases. *Shigella flexineri* 2a is usually the prevalent species and serotype in these areas (Coster *et al.*, 1999).

In *Shigella* spp. loci encoding type III secretory apparatus (system) components have been identified. In general type III systems are complex membrane-bound structures dedicated to the delivery of virulence effectors from the bacterial cytoplasm to eukaryotic cell surfaces or intracellular environments. The type III secretory system is also referred to as contact dependent, as it can be activated in some cases by direct pathogen-host cell interaction (Schuch and Maurelli, 1999).

2.2.1. Adhesion to and Invasion of Host Tissue by *Shigella* spp.

In shigellosis, bacterial invasion of colonic epithelial cells (CECs) is one of the most significant events responsible for the clinical manifestation of the disease. Also adhesion of the bacteria to the epithelial cells before internalization is a prerequisite. Attachment of *Shigella* to mammalian cells is often mediated through sugar-lectin interaction (Guhatha *et al.*, 1992). From *in vitro* experiment it has been shown that strains of *S. flexineri* serotype-1b adhere to guinea-pig colonic cells and strains of *S. flexineri* serotype-5 to HeLa cells (Qadri *et al.*, 1991). In the intestine *Shigella* spp. adhere and subsequently invade enterocytes through the basolateral membrane. Internalized bacteria subsequently spread within the infected cells by organizing host cell actin into cytoskeleton-based motor (Coster *et al.*, 1999). In the invasion process the type III secretion pathway of *Shigella* secretes a set of bacterial gene products, called invasion plasmid antigen (Ipa) proteins. During invasion the proteins trigger epithelial cell membrane ruffling process that is responsible for mediating bacterial entry (Schuch and Maurelli, 1999).

Within the intracellular environment shigellae multiply and begin elaborating an intercellular spreading phenotype. The spreading phenotype propels the bacteria within cellular protrusions resulting in passage into adjacent uninfected cells (Schuch and Maurelli, 1999). The ability to spread intercellularly is dependent upon the activity of one protein, IcsA, which is born by the large virulence plasmid of *Shigella* spp. IcsA is a 120 kDa outer membrane protein which is asymmetrically localized to the old pole of the bacteria (Sandlin and Maurelli, 1999). It has been recognized that the IcsA protein is a recruiter for cytosolic nucleators of filamentous actin. The recruited actin is found concentrated at the distal poles of septating shigellae. The resulting comet-like tail provides a motive force for the bacteria within the cytoplasm of infected cells. Then the mobilized bacteria spread into adjacent epithelial cells via membrane protrusion (Vasselon *et al.,* 1992). The multistep process, which involves the internalization of shigellae by epithelial cells, followed by intracellular growth, intra- and intercellular spread, results in host cell death (Schuch and Maurelli, 1997).

As it has been studied in *S. flexineri* virulence is conferred in part by products of loci encoded on a 220-kb virulence plasmid. Many of the plasmid-encoded genetic determinants of virulence are localized within a 31kb " invasion region" which encompasses over 30 genes. Among the products of this loci are secreted effectors of invasion process (four Ipa proteins) as well as proteins dedicated to their secretion (Mix and Spa proteins) (Schuch and Maurelli, 1999).

2.2.2. *Shigella* Toxin as Virulence Factor

Shiga toxin is a potent toxin produced by *Shigella dysentriae* type-1 strain. This protein toxin of the strain is the virulence factor of the organism (Brown *et al.,* 1991). The toxin has four biologic activities that are cytotoxicity, enterotoxicity, neurotoxicity, and inhibition of protein synthesis in the host. It consists of two polypeptide chains, an A-chain (molecular weight, 32, 225Dalton) and a B-chain (molecular weight, 7, 691Dalton). These two polypeptides associate with a stoichiometry of one A and five B subunits to form the holotoxin (Donohue-Rolfe *et al.,* 1991).

In the Shiga-toxin the B-chain mediates binding of the holotoxin to susceptible host cells. Isolated, renatured B subunit, but not A subunit of the toxin binds to HeLa cells. Shiga-toxin binds to Gal-α1-4 Gal-containing glycolipids by its B-subunit. During enterotoxicity the B subunit binds to Gb3 receptors on the villus cells. Gb3 is a P blood group-active substance composed of trisaccharide Gal-α1-4 Gal-β1-4 Glc linked to the lipid moiety ceramide as shown in a rabbit model (Jacewicz *et al.,* 1986). Also by targeting the villus cells, Shiga-toxin inhibits Na^+ absorption with out Cl^- secretion. This situation appears to be due to the expression of substantial quantities of the toxin-binding glycolipid Gb3 by villus cells. The binding of the toxin to the villus cells impairs the transport function of the cells. Thus, reduction of transport of Na^+ and presence of unchanged Cl^- secretion would lead to net fluid accumulation at the mucosal side of the intestine (Keusch *et al.,* 1991).

The A-subunit of the Shiga-toxin is responsible for the biochemical effect of the toxin i.e. inhibition of protein synthesis by host cells. The A-subunit involves in cleaving the *N*-

glycosidic bond of adenine at nucleotide position 4324 in the 28s rRNA of the 60s ribosomal subunit (Donohue-Rolfe *et al.,* 1991). Since Shiga toxin acts on the ribosome while inhibiting protein synthesis, it must enter the cytoplasm of the target cells. The entry of Shiga-toxin is strongly dependent on divalent cathion presence. So that physiologically occurring Ca^+ transport channels are required for the entry of Shiga-toxin into cells. It has been demonstrated by electron microscopy that Shiga-toxin was taken up from coated pits into endosomes, lysosomes, and golgi region (Brown *et al.,* 1991).

Neurologic disturbances are the most frequent complications of acute gastroenteritis caused by *Shigella.* Shiga-toxin has been implicated in neurotoxicity, as its administration caused paralysis and enhancement of seizures (a neurological complication) in mice and rabbits. The proinflammatory cytokines TNF-α and IL-1β may be involved in the disease because they can affect many functions including neurotransmission. There are also several pathways by which TNF-α and IL-1β can affect brain function (Yuhas *et al.,* 1999).

2.2.3. The Lipopolysaccharide (LPS) of *Shigella* spp. as Virulence Factor

The lipopolysaccharide in *Shigella,* as in other gram-negative bacteria, contributes to virulence providing resistance to the host defence such as phagocytosis and antibody's bactericidal activity. The LPS molecule consists of covalently linked components: lipid-A, a core polysaccharide, and an O-antigenic polysaccharide chain (Okamura *et al.,* 1983).

During infection the LPS in the host's body induces wide spectrum of non-specific pathophysiologic reactions, known as endotoxic reactions such as fever, hypotension, and leukopenia, followed by leukocytosis. These effects are attributable to the lipid-A component of the LPS. These conditions were shown by the synthesis of lipid-A analogue that has been tested both *in vitro* and *in vivo* tests. The data from these tests indicated that lipid A of *Shigella* is capable of eliciting in the host all the pathophysiologic reactions of endotoxin (Kumazawa *et al.,* 1988).

2.3. The Virulence Factors of *Salmonella* spp.

Members of the genus *Salmonella* are ubiquitous pathogens that can infect humans and their livestock, wild animals, reptiles, birds, and even insects. However, most of the human diseases are caused by only about ten different serotypes of *Salmonella* (Ryan and Falkow, 1994b). The group classified as *Salmonella enterica* posses different serovares that are capable of infecting humans and other hosts. Disease manifestations are results of complex interactions between the infecting *S. enterica* serovar and the host species. For example, *S. enterica* serovar Typhimurium (*Salmonella typhimurium*) causes disease both

in mice and humans, yet the nature of the infection is distinctly different in each host. While other *S. enterica* serovares are adapted to specific hosts, one of them is *S. enterica* serovar Typhi (*Salmonella typhi*) the etiologic agent of human typhoid fever. *S. typhi* infects only humans under natural conditions and avirulent in non primate hosts (Morrow *et al.*, 1999).

In general, *Salmonella* spp. are facultative intracellular pathogens which are capable of causing different disease manifestations, including enteric fever, bacteremia, and gastroenteritis. Following oral ingestion, *Salmonella* penetrates mucosal epithelium of the small intestine, by interacting with columnar epithelial cells and microfold cells that overlay the peyer's patches (Henderson *et al.*, 1999). *Salmonella* after crossing the gut mucosa can also invade the host by colonizing the gut-associated lymphoid tissue (GALT), as well as the spleen and liver (Benyacoub *et al.*, 1999).

All serovars of *Salmonella enterica* encode a type III protein secretion system within a Salmonellae-Pathogenicity-Island-1 (SPI-1) at centisome 63 of their chromosome. This system mediates the translocation of a battery of bacterial proteins in to the host cells, which stimulate or interfere with host cellular functions (Eichelberg and Galán, 1999). This type III secretion system is also involved in the initiation of programmed cell death (apoptosis) in macrophages (Chen *et al.*, 1996). In addition, *Salmonella enterica* serovares have virulence plasmids. The virulence plasmids of several *Salmonella enterica* serovars invariably carry the *spv* operon, which plays a role in the virulence of the host strain. The size of these plasmids varies with each serovar, ranging from 50 to 285 kb. The level of closeness of the plasmids among the serovars runs in descending order, from the virulence

plasmid of Typhimurium (pSTV) to that of Entritidis (pSEV), then to that of Choleraesuis (pSCV), and finally to that of Dublin (pSDV) (Chu *et al.,* 1999).

Deletion of various virulence genes can render *Salmonella* avirulent. For instance, a mutation in *invH,* a gene located on SPI-1 (Salmonella Pathogenicity Island-1), results in the attenuation of the pathogen during oral infection of calves. Also a mutation in *hilA*, a positive regulator of genes on SPI-1, reduces the ability of *S. typhimurium* to cause fluid accumulation in bovine ligated ileal loops, which is a good model of *Salmonella* caused diarrhea in human (Tsolis *et al.,* 1999).

2.3.1. Adhesion to and Invasion of Host Tissue by *Salmonella* spp.

Enteric pathogens experience different environmental changes when they enter their hosts by the oral route, e.g. low pH, increased temperature, low CO_2 tension, high osmolarity, and nutrient deprivation. In the case of *S. typhi* it has been shown that maximal expression of virulence determinants for adherence to intestinal epithelial cells in the distal ileum is favoured by high osmolarity (Tartera and Metcalf, 1993). In addition, an essential step in the development of diseases due to *Salmonella* spp. is the entry of the bacteria into non phagocytic cells including those that line the intestinal epithelium (Zierler and Galán, 1995).

Salmonella entry into host cells is a result of multistep process. The events that trigger internalization require a battery of bacterial proteins that are exported by the type III protein secretion system. The type III protein secretion systems are usually encoded by

genes that are clustered together (Eichelberg and Galán, 1999). Contact of *Salmonella* spp. with host cells results in the assembly of peritrichous appendages called Invasomes in the bacteria. Invasomes assembly requires a functioning type III secretion system. In addition to Invasomes a virulence protein , InvJ, is secreted which facilitates bacterial internalization into host cells (Zierler and Galán, 1995). The ability to cross the intestinal epithelial barrier is also an important determinant of *Salmonella* virulence. This phenomenon is mediated first by the entry into several cell types, including enterocytes, M cells, and macrophages (Procyk *et al.,* 1999).

A large number of genetic loci required for the entry of *Salmonella* into host cells have been identified by several laboratories. Most of host cell entry loci are clustered in discrete region of the *Salmonella* chromosome at centisome 63. Invasion loci located elsewhere in the chromosome have also been identified (Galán, 1996). It is now recognized that as much as 35-40 kb of DNA encompassing a contiguous region of the *Salmonella* chromosome at centisome 63 encode determinants required for entry into host cells. Nucleotide sequence analysis of this region carried out to date has revealed the presence of at least 28 genes (Mills *et al.,* 1995). At least 17 genes are found in *inv, spa,* and *prg* loci that encode the contact dependent type III protein secretion system in *Salmonella* (Wattiau *et al.,* 1996). The *spa* locus has been described as being specific to *Salmonella* spp.. It was also demonstrated that two genes of this locus, *spaP* and *spaS,* play an important role in bacterial entry. Additional genes *spaO, spaQ,* and *spaR* in *spa* locus play essential role in bacterial internalization, since mutation in each one of these genes rendered *S. typhimurium* deficient for entry into cultured epithelial cells (Collazo and Galán, 1996).

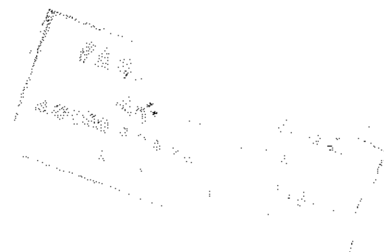
A number of proteins encoded in the 63-centisome region of *Salmonella* chromosome may either assist the secretion process of the type III protein secretion pathway or act as modulators of the function of putative signaling molecules. For example, specific cytoplasmic chaperones assist the export of target proteins that involve in invasion (Wattiau *et al.*, 1996). In the same manner in *S. typhimurium* internalization appears to require an array of bacterial secreted proteins. They are essential to the induction of host cell signal transduction pathways that lead to host cell membrane ruffling and subsequent bacterial uptake (Gewirtz *et al.*, 1999).

In addition to Salmonellae-Pathogenicity-Island-1 (SPI-1) at centisome 63, Salmonellae-Pathogenicity-Island-2 (SPI-2) is also identified at centisome 31 of the chromosome. SPI-2 is required for bacterial systemic spread and survival within phagocytic cells (Medina *et al.*, 1999). In addition to the ability of *Salmonella* to invade epithelial cells, surviving within macrophages is essential virulence factor. This ability may represent a major factor in determining host restriction. *Salmonella* can also induce apoptosis in macrophages, which is a factor that may further contribute to virulence (Lowe *et al.*, 1999).
Eventually, salmonellae interaction with epithelial cells can be modeled using cultured epithelial cells. Such models have been used to identify and understand the bacterial, host, and environmental factors that involve in invasion. For example, non-invasive mutants of *S. typhimurium* for cultured cells exhibit virulence defects in mice that is consistent with an inability to cross the intestinal mucosa (Johnston *et al.*, 1996).

2.3.2. The Lipopolysaccharide (LPS) of *Salmonella* spp. as Virulence Factor

The LPS of *Salmonella* spp. consists of three structural domains: an inner moiety, Lipid A, embedded in the outer membrane and adjoined by the Core Oligosaccharide (central part of the LPS), and then an outer generally hydrophilic, O-antigenic repeating polysaccharide unit (Nnalue *et al.*, 1992). Wild-type strains synthesize LPS with long polysaccharide chains, the so-called S-form (smooth LPS). Whereas in R-form (rough LPS) strains, biosynthesis of the O-polysaccharide and in some cases the Core Oligosaccharide is defective. Consequently, R-form strains synthesize LPS, generally termed R-chemotype or R-form LPS with shorter saccharide chains (Kirikae *et al.*, 1999).

Like other members of Enterobacteriaceae the LPS of *Salmonella* is a major virulence factor and an important antigen for protective immune response. In *Salmonella,* the core region of the LPS is highly conserved unlike the O-antigen. The >2000 serotypes of *Salmonella* share only two closely related core types (Nnalue, 1999). In addition to its immunogenicity the LPS plays a major role in the pathogenesis of septic shock and a variety of other clinical disorders (Nnalue *et al.*, 1992). During typhoid fever caused by *S. typhi* proinflammatory cytokines, such as IL-1β and TNF-α that are induced by LPS, have been shown to play important role in the development of fever and other symptoms associated with systemic inflammatory response syndromes (SIRS). Hence, high concentration of LPS associated with high bacteremia might lead to a sever form of SIRS referred to as endotoxic shock because of induction of high concentration of cytokines by LPS (Wyant *et al.*, 1999).



2.4. Haemagglutination

The ability of bacteria to agglutinate red blood cells from humans and a number of animal species is a good measure for the presence haemagglutinins. Moreover, haemagglutination technique provides a means for detecting and classifying specific adhesins (Parry and Rooke, 1985). Also fimbriae that involve in adhesion to epithelial cells could be demonstrated by haemagglutination (Grund and Stolpe, 1992). For example, in *Haemophilus influenzae* haemagglutinating pili promote adherence to a broad range of human cell-lines (Krasan *et al.,* 1999). There are several types of haemagglutinating fimbrial adhesins that have been recognized to date. Among the bacterial surface adhesins that mediate haemagglutination are type-1 fimbrial adhesins. They mediate the so-called mannose-sensitive haemagglutination (MSHA), which give an agglutination of erythrocytes that is inhibitable by D-mannose (Nichols *et al.,* 1990). Still there are other adhesins recognized as mannose-resistant haemagglutinins (MRHA), which give an agglutination of erythrocytes that is not inhibitable by D-mannose (Sussman, 1985). Erythrocytes can occur in natural variants differing in the composition of their surfaces. Hence, they give specific receptors for bacterial adherence (Korhonen and Finne, 1985).

In addition, virulence determinants such as smooth forms of LPS are known to involve in haemagglutination in pathogens like *Shigella* spp. (Qadri *et al.*, 1991). In a search for haemagglutinins an extracellular haemagglutinating slim layer was identified in *S. dysentriae* 1, *S. flexineri* 2a, *S. boydii* 12, and *S. sonnei* 1. The slim layer extracted from the surface of these *Shigella* strains was able to agglutinate erythrocytes. However, the cell

surface haemagglutinin is a loosely bound slim layer, which is expressed in Casamino Acids-Yeast Extract (CYE) broth medium. The slim layer was also shown to be different from LPS (Haque *et al.*, 1995).

3. Materials and Methods

3.1. Bacterial Strains

For this work both clinically and laboratory confirmed pathogenic bacterial strains (*Shigella* spp., *Salmonella* spp., and *Streptococcus pneumoniae* strains) were received from the Clinical Bacteriology Laboratory of Ethiopian Health and Nutrition Research Institute (EHNRI). The Gram-negative bacteria, *Shigella* and *Salmonella* species are isolates from patients with enteric diseases and were preserved at -70^0C after isolation. For the assays 25 strains of *Shigella* spp. and 25 strains of *Salmonella* spp. were used. All the strains were confirmed by culture, biochemical tests, and serological test at the time of isolation from patients. Also the Gram-positive bacteria, *Streptococcus pneumoniae* strains, are isolates from patients with acute respiratory tract infection (pneumonia) and were preserved at -70^0C after the isolation. The pneumococcal strains were confirmed in the laboratory by their alpha-haemolysis characteristic, optochin application with zone of inhibition, bile solubility test, and slidex pneumokit (Biomerieux) test. A total of 50 strains were used for all the assays in this work.

Prior to application for tests strains of *Salmonella* spp., *Shigella* spp. and *Streptococcus pneumoniae* were recultured. Strains of *Shigella* and *Salmonella* were grown on SS-agar as well as on MacConkey agar at 37^0C overnight. After the reculturing the strains were selected and kept in tryptone soya yeast (TSY) broth with 20% glycerin in eppendorfs at -70^0C to be used for each test. *Streptococcus pneumoniae* strains were recultured on blood agar at 37^0C overnight. After reculturing the strains were selected and kept in tryptone soya yeast (TSY) broth with 20% glycerin in eppendorfs at -70^0C.

3.2. Haemagglutination

For haemagglutination assay human erythrocytes of type A, B, AB, O, and erythrocytes of guinea-pig and rabbits were used. For the test the erythrocytes were separated from citrated blood with low speed-centrifugation. Then the erythrocytes were washed three times with phosphate buffered-saline (PBS (Difco); pH 7.2) and suspended to 3% (v/v) in PBS (pH 7.2).

For the test strains of *Shigella* spp. and *Salmonella* spp. stored at -70^{0}C were subcultured and grown overnight at 37^{0}C on MacConkey agar medium (bioMérieux). From the agar the bacteria were inoculated into Brain Heart Infusion (BHI; Oxoid) broth in 10ml volume and grown overnight at 37^{0}C without shaking. Also *Streptococcus pneumoniae* strains were grown on blood agar medium overnight at 37^{0}C. Colonies from the agar medium were inoculated into BHI broth and grown overnight at 37^{0}C without shaking. From each group bacteria grown in the broth medium were sedimented by centrifugation at 3000rpm for 10 min. After sedimentation the bacteria were washed three times with PBS (pH 7.2) and suspended in PBS to a concentration of ~5x10^{10} bacteria/ml. Bacteria concentration was determined by counting using haemocytometer. From each bacterial suspension 25μl volume was taken and mixed with either 25μl of saline or 25μl D-mannose-saline (1% w/v) in round-bottomed wells of polyvinyl microtitration plates (96-well trays). The bacteria suspension in each well was mixed using a rotary shaker for 5 min especially to promote the interaction of bacteria with D-mannose. Immediately after the mixing aliquot (25μl) of 3% (v/v) erythrocyte suspension was added to each well from each type of erythrocyte and mixed with the bacteria. The erythrocytes were also added to wells that

did not contain bacteria but had either saline only or D-mannose-saline as control. After addition of the erythrocytes microtitration trays were sealed with parafilm tape. Mixing of the erythrocytes with the bacteria was done on a rotary shaker for 10min at room temperature. After the mixing the trays were incubated at 4^0C for 1 hr. After the incubation haemagglutination results were determined using a magnifying glass mirror (Titertek Microtitration equipment) from the bottom of the microtitration trays. Results were read as strong (+++), or moderate (++), and as weak (+) or negative (-) for both mannose sensitive and mannose resistant haemagglutination (Sussman, 1985).

3.3. Adhesion and Invasion Assays

3.3.1. Tissue Culture (HEp-2 Cell) Maintenance

HEp-2 cells (human pharyngeal carcinoma cells) were used for both bacterial adherence and invasiveness assays. HEp-2 cells were chosen because of their wide spread use and ready availability. The initial HEp-2 cell gift was from "Polio Diagnostic Laboratory" at the Ethiopian Health and Nutrition Research Institute (EHNRI). Cells were routinely grown in Falcon Tissue Culture Flasks (75cm^2; Becton Dickinson) in complete RPMI-1640 (Sigma) tissue culture growth medium. The complete growth medium was prepared with the addition of fetal calf serum in 10%(v/v) proportion, 2 g/l sodium bicarbonate, antibiotic suspension 1%(v/v) (10, 000 U/ml penicillin G and 10, 000 μg/ml Streptomycin sulfate). Flasks were incubated at 37^0C in a 5% CO_2 incubator until cells grow to the required level. Cells in the flasks were always grown to confluence level.

For the assays of adhesion and invasion confluent monolayers of HEp-2 cells in flasks were washed twice with Mg^{2+} and Ca^{2+} free phosphate buffered-saline (PBS; pH 7.2). After the washing two milliliters of 0.25% (w/v) trypsin (Gibco) was added to each monolayer. The trypsin was poured off after 1 min. The monolayers were then incubated at 37^0C (in a 5% CO_2 incubator) until cells began to detach (5-10 min). After the incubation five milliliters of RPMI-1640 (Sigma) growth medium without antibiotic was added and cells were resuspended. A 1.5-ml portion of the suspended cells was then added to 15-ml of growth medium (with antibiotic) in tissue culture flask ($75cm^2$) and incubated at 37^0C in 5% CO_2 incubator for further growth. The remaining portion of the suspended cells was diluted to $5x10^5$ cells/ml and 10^6 cells/ml in complete growth medium without antibiotic to be used for each assay of invasion and adhesion respectively. This procedure was repeated for required HEp-2 cells during each test.

3.3.2. Tissue Culture Test for Adherence

The adherence ability and patterns of 25 *Shigella* spp., *Salmonella* spp. and 50 *Streptococcus pneumoniae* strains on HEp-2 cultured cells were studied as described by Cravioto *et al.* (1991) with some modification. For the test 2 ml of suspended HEp-2 (10^6 cells/ml) cells in RPMI-1640 complete growth medium without antibiotic were added into 4cm-petridish containing a 22-mm sterile glass cover slips. Then the dishes were incubated at 37^0C for 48 hr in 5 % CO_2 incubator. After growth for 48 hr on the glass cover slips the HEp-2 cell monolayer in each dish was washed twice with Earl's Balanced Salts Solution (EBSS; calcium chloride, magnesium sulfate, potassium chloride, sodium

bicarbonate, sodium chloride, sodium phosphate monobasic. H_2O) and prepared for bacterial inoculation.

For the test *Shigella* and *Salmonella* strains were plated on MacConkey agar overnight at 37^0C. For broth culture bacteria from the agar medium were inoculated into 5ml-volume brain heart infusion broth in screw cap tubes. Broth cultures were grown overnight at 37^0C without shaking. *S. pneumoniae* strains were also grown on blood agar overnight at 37^0C. For broth culture bacteria were inoculated into 5ml-volume brain heart infusion (Oxoid) broth and allowed to grow overnight at 37^0C. The broth culture was sedimented by centrifugation and washed with PBS (pH 7.2) three times. The washed bacteria were suspended in PBS with 0.5%(w/v) D-mannose to 10^8 bacteria/ml. The bacteria were then incubated for 10min at 37^0C before inoculation to HEp-2 cell monolayers. Then one milliliter of the diluted culture was added to each washed monolayer in the petridishes. Then the petridishes were agitated at 300rpm on a rotary shaker for 5min to allow contact of the bacteria and HEp-2 cells. The dishes were then incubated for one hour at 37^0C in 5% CO_2 incubator (Cravioto *et al.*, 1991).

After one hour of incubation for adherence, the monolayers were washed thoroughly 4 times with EBSS (pH 7.2) to remove non adhered bacteria. The monolayers were then covered with methanol and left for 10min. The methanol was removed and replaced with newly prepared 10% (v/v) Giemsa-stain for 45min. Then the cover slips in the petridishes were washed with water to remove the stain. The cover slips were mounted on glass slides after passing through acetone, aceton-xylene (50/50 v/v), aceton-xylene (33/66 v/v) and finally xylene. The cover slips mounted on the glass slides were viewed under oil

immersion at x1000 magnification under light microscopy. The patterns of bacterial adherence were determined by studying at least 10 fields per slide. The results were classified as LA (Localized Adherence), DA (Diffused Adherence), AA (Aggregative Adherence), or NA (No Adhesion) following definitions by Law (1994).

3.3.3. Tissue Culture Test for Invasiveness

For invasion assay HEp-2 cells were suspended in RPMI-1640 complete growth medium without antibiotic. From the suspension 1ml of $5x10^5$ cells/ml was added into each well of 24-well tissue-culture trays (Becton Dickinson) was added. The trays were incubated for 48 hr at 37^0C in 5% CO_2 incubator. The resulting HEp-2 cell monolayers in each well were confluent. Then the monolayers in the wells were washed twice with EBSS (pH 7.2) before bacteria inoculation for invasion (Douce *et al.*, 1991).

Overnight cultures of *Shigella* spp., *Salmonella* spp., and *Streptococcus pneumoniae* grown in 5ml-volume brain heart infusion (BHI) broth at 37^0C were sedimented by centrifugation and washed three times with PBS (pH 7.2). Immediately after the bacteria were washed and ready for inoculation an infection medium was prepared. It was prepared from 70ml of Earl's Balanced Salts Solution (EBSS), 10ml brain heart infusion broth, and 20ml heat inactivated fetal calf serum (60^0C, 2hr). Then the washed bacteria were suspended in the infection medium to a concentration of 10^7 bacteria/ml. One milliliter of the suspended bacteria was added to HEp-2 cell monolayers in each well. After the addition of the bacteria, the tissue culture-trays were agitated on a rotary shaker for 10min at 300rpm at room temperature. After the centrifugation the trays were incubated for 2-hr

at 37^0C in 5% CO_2 incubator. This was infection time for the bacteria. After the infection time the infection medium was removed and the monolayers were washed thoroughly four times with EBSS (pH 7.2). Then 1 ml of fresh RPMI-1640 complete growth medium with gentamicin (200 μg/ml) was added to the monolayer in each well. The medium with the antibiotic was added to allow replication of intracellular bacteria and killing of extracellularly adhered bacteria. Then the tissue-culture trays were incubated further for 2 hr in 5% CO_2 incubator for intracellular growth of the bacteria. After the incubation the growth medium was removed and the monolayers were then washed three times with EBSS (pH 7.2). After the washing one milliliter of Triton X-100 (1% v/v in PBS) was added to each well and left for 10 min to release intracellular bacteria by the HEp-2 cells lysis.

After the HEp-2 cells lysis the suspension in each well of the tissue-culture trays was mixed vigorously by the aid of sterile micropippet tips to disperse aggregates of bacteria before counts are determined. To determine the number of recovered bacteria double dilutions were prepared from each well and inoculated onto agar medium. *Shigella* strains and *Salmonella* strains were inoculated on MacConkey agar. *S. pneumoniae* strains were inoculated on blood agar medium. The inoculation was done using 1-μl calibrated loop for colony count. After overnight growth at 37^0C the number of bacteria on the agar plates were determined using colony counter (Fisher Accu-Lite Colony Counter). Then the number of viable bacteria in each well of the tissue-culture trays recovered after Triton X-100 cell lysis was determined from the colony count results. Finally invasion index for each strain was determined as percentage of live bacteria recovered from the initial inoculum after HEp-2 cells lysis by Triton X-100. This procedure was also done for

selected strains from each group with a four-hour intracellular growth incubation time with gentamicin. The 4-hr intracellular growth incubation was done to see the impact of the gentamicin during the four hour incubation.

4. Results

4.1 Haemagglutination

A total of 50 strains of *Streptococcus pneumoniae,* 25 strains of *Shigella* spp., and 25 strains of *Salmonella* spp. were tested for their ability to agglutinate different erythrocyte species. Each strain was tested against six types of erythrocytes (human red blood cell groups A, B, AB and O, guinea-pig red blood cell, and rabbit red blood cell). Each test was done in the absence and presence of D-mannose because agglutination of erythrocytes by type-1 pili in the bacteria can be detected by with or without the addition of D-mannose.

The haemagglutination results of *Streptococcus pneumoniae* strains are shown in Table 1. The results are recorded as MSHA (mannose-sensitive haemagglutination) or MRHA (mannose-resistant haemagglutination) for those strains agglutinating only in the absence or presence of D-mannose, respectively. Out of the 50 strains tested 35 (70%) were able to agglutinate all the six types of erythrocytes. Whereas 5 (10%) of the strains were able to agglutinate one, two, or four types of the tested erythrocytes. Among the tested strains 10 (20%) could not agglutinate any one of the erythrocyte species at all. All the agglutinating strains showed mannose-resistant haemagglutination. The detailed results of haemagglutination for the strains of *S. pneumoniae* in comparison with their results of HEp-2 cell adhesion are presented in Appendix 1.

Haemagglutination results of strains of *Shigella* spp. are shown in Table 2. Each strain was tested with the six types of erythrocytes. Out of the 25 strains tested 17 (68%) of the strains gave positive agglutination results. Out of the 17 haemagglutination positive

strains, 12 showed agglutination with only rabbit red blood cells. On the other hand, 3 strains showed agglutination against red blood cells of both guinea pig and rabbit. The remaining 2 strains could agglutinate all the six types of erythrocytes. All the haemagglutinating strains have shown mannose-resistant haemagglutination except four strains that showed both mannose-sensitive and mannose-resistant haemagglutination. The detailed results of haemagglutination for strains of *Shigella* in comparison with their results of HEp-2 cell adhesion are presented in Appendix 2.

Table 3 shows haemagglutination results of strains of *Salmonella* that include *S. typhi* strains that are cause of typhoid fever (Morrow *et al.*, 1999) and other types of strains. Out of the 25 strains tested 13 (52%) were able to give positive haemagglutination results, of which 12 were able to agglutinate only rabbit red blood cells. Only one strain showed agglutination of all the six erythrocyte types (human A, B, AB, and O red blood cell group, guinea-pig RBC and rabbit RBC). The haemagglutination results were all mannose-resistant. The detailed results of haemagglutination for each strain of *Salmonella* spp. in comparison to their results of HEp-2 cell adhesion are presented in Appendix 3.

Table 1. Haemagglutination results of *Streptococcus pneumoniae* (n=50) isolates against six erythrocyte types

	MRHA with erythrocyte of						MSHA with erythrocyte of					
	Human				Ra	Gp	Human				Ra	Gp
	A	B	AB	O			A	B	AB	O		
Number of *S. pneumoniae* strains	35	35	36	36	37	40	0	0	0	0	0	0

Note: *S. pneumoniae* = *Streptococcus pneumoniae*, MRHA = mannose-resistant haemagglutination, MSHA = mannose-sensitive haemagglutination, Ra = rabbit, Gp =guinea-pig

Table 2. Haemagglutination results of *Shigella* spp. (n=25) against six erythrocyte types

Strains of*	MRHA against erythrocytes of						MSHA against erythrocytes of					
	Human				Ra	Gp	Human				Ra	Gp
	A	B	AB	O			A	B	AB	O		
Shigella A (n=6)	0	0	0	0	3	0	0	0	0	0	0	'0
Shigella B (n=18)	1	1	1	1	13	1	1	1	1	1	0	4
Shigella C (n=1)	0	0	0	0	0	1	0	0	0	0	0	0
Total	**1**	**1**	**1**	**1**	**16**	**2**	**1**	**1**	**1**	**1**	**0**	4

Note: MRHA= mannose-resistant haemagglutination MSHA= mannose-sensitive haemagglutination, Ra = rabbit, Gp = guinea pig, * = the serogroups of *Shigella* are listed in Appendix 2.

Table 3. Haemagglutination results of *Salmonella* spp. (n=25) against six erythrocyte types

Strains of*	MRHA against erythrocytes of						MSHA against erythrocytes of					
	Human				Ra	Gp	Human				Ra	Gp
	A	B	AB	O			A	B	AB	O		
S. typhi (n=6)	0	0	0	0	0	0	0	0	0	0	0	0
Salmonella A (n=1)	0	0	0	0	1	0	0	0	0	0	0	0
Salmonella B (n=7)	0	0	0	0	5	0	0	0	0	0	0	0
Salmonella C (n=9)	1	1	1	1	5	1	0	0	0	0	0	0
Salmonella D (n=2)	0	0	0	0	2	0	0	0	0	0	0	0
Total	**1**	**1**	**1**	**1**	**13**	**1**	**0**	**0**	**0**	**0**	**0**	**0**

Note: *S. typhi* = *Salmonella typhi*, MRHA = mannose-resistant haemagglutination, MSHA = mannose-sensitive haemagglutination, Ra = rabbit, Gp = guinea pig, * = the serogroups of *Salmonella* are listed in Appendix 3.

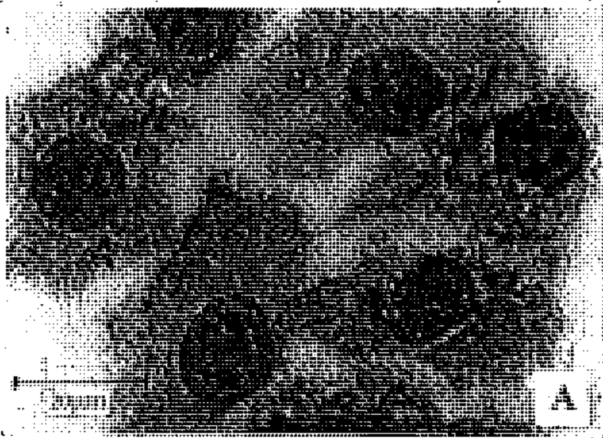
4.2. Adhesion to Cultured HEp-2 Epithelial Cells

Strains of *Streptococcus pneumoniae, Shigella* spp. and *Salmonella* spp. were tested for their ability of adherence on cultured HEp-2 epithelial cells. Results were determined after Giemsa staining and microscopic examination of the epithelial cell monolayers. Majority of the strains from each group gave positive adherence results while some could not adhere. Table 4 shows the results of adherence of *S. pneumoniae* strains, *Shigella* strains, and *Salmonella* strains on HEp-2 cells. The organisms in the three groups showed three patterns of adherence with varying frequencies. In addition, figure 1 shows representative adherence results of *Shigella* spp., *Salmonella* spp. and *Streptococcus pneumoniae* on HEp-2 cells photographed from prepared slides.

Table 4. Adherence results of *Shigella* spp., *Salmonella* spp., and *Streptococcus pneumoniae* isolates on HEp-2 cells.

Strains of:	Results of Adherence				Total
	LA	AA	DA	NA	
S. pneumoniae	15 (30%)	17 (34%)	11 (22%)	7 (14%)	50
Shigella spp.	19 (76%)	3 (12%)	2 (8%)	1 (4%)	25
Salmonella spp.	6 (24%)	16 (64%)	3 (12%)	0 (0%)	25

Note: *S. pneumoniae* = *Streptococcus pneumoniae*, LA = localized adherence,
AA = aggregative adherence, DA= diffused adherence, NA = no adhesion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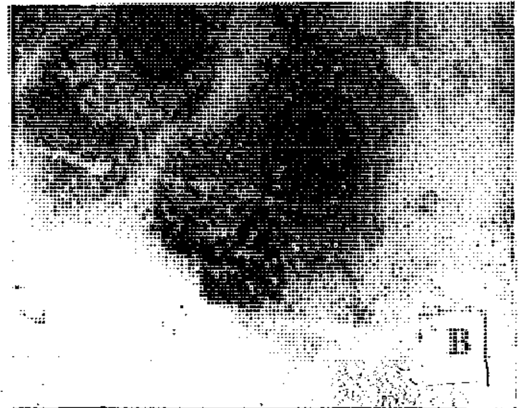
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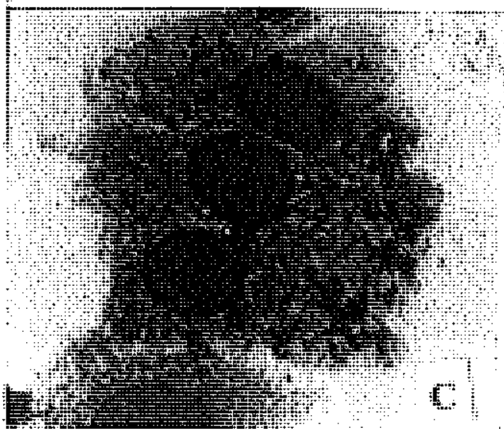
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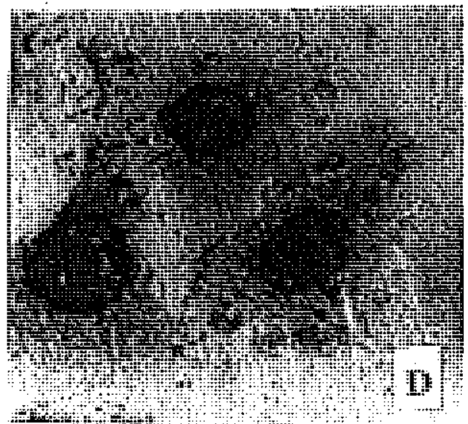
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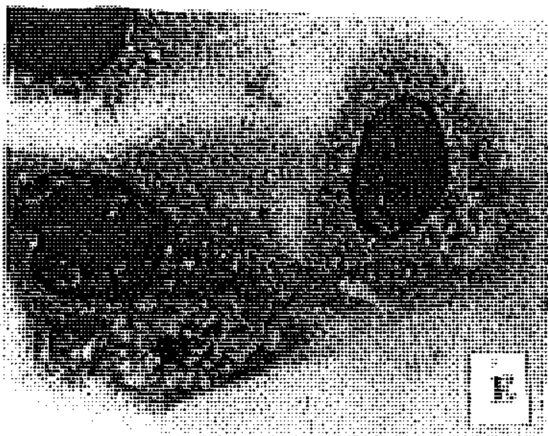
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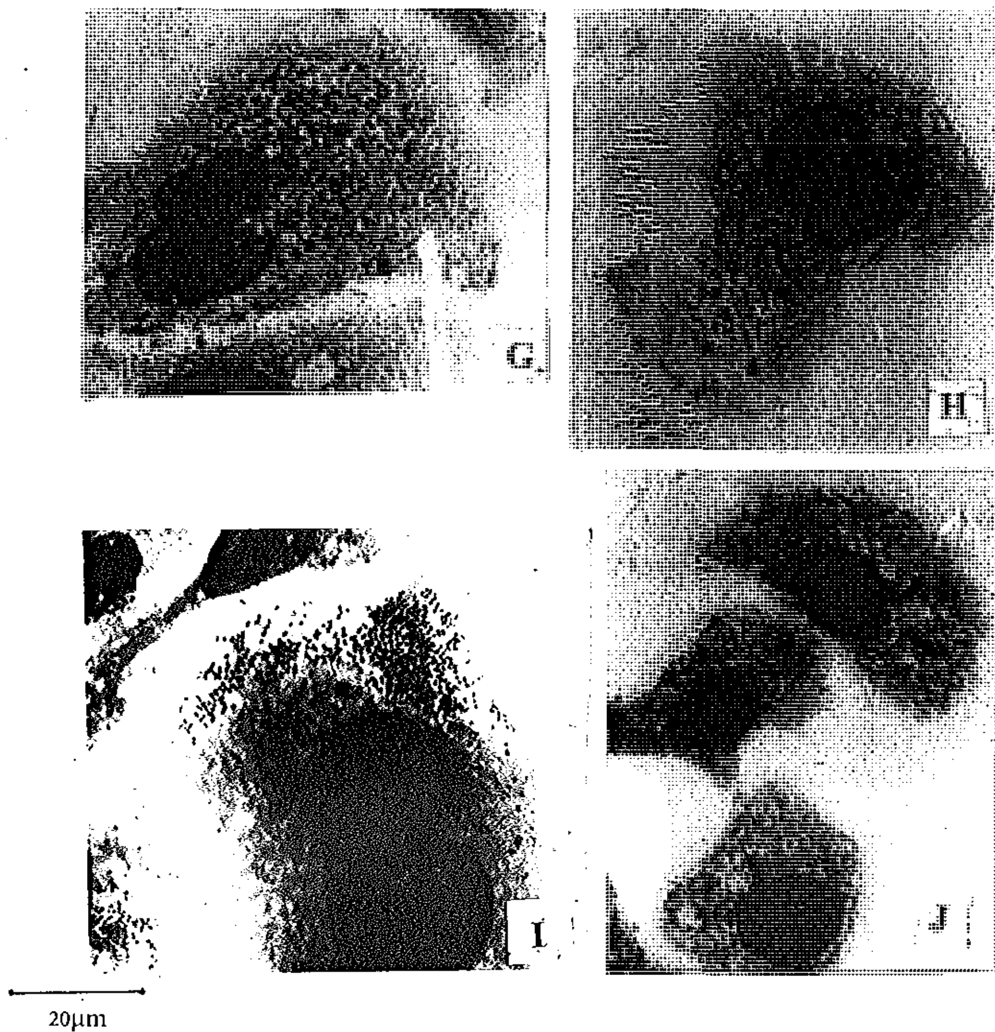

Figure 1. The adherence of ***Streptococcus pneumoniae*, *Shigella*** spp., and ***Salmonella*** spp. on HEp-2 cells. A = uninfected HEp-2 cell monolayer, B= localized adherence (LA) of a *Streptococcus pneumoniae* strain, C = aggregative adherence (AA) of a *Streptococcus pneumoniae* strain, D = diffused adherence (DA) of a *Streptococcus pneumoniae* strain, E = localized adherence (LA) of a *Shigella* spp., F = aggregative adherence (AA) of a *Shigella* spp., G = diffused adherence (DA) of a *Shigella* spp., H = localized adherence (LA) of a short bipolar *Salmonella* spp., I = aggregative adherence (AA) of a short bipolar *Salmonella* spp., J = diffused adherence (DA) of a short bipolar *Salmonella* spp.

4.3. Invasion of HEp-2 Epithelial Cells

Invasiveness of the pathogenic bacteria or their ability to enter human epithelial cells was measured using cultured HEp-2 epithelial cells. All the strains of *Shigella* spp. and *Salmonella* spp. were positive for invasion with variable percentages of bacteria recovered (invasion percentage). Whereas out the 50 strains of *Streptococcus pneumoniae* tested only 12 were positive for invasion. Table 5 shows descriptive statistics of invasion results of *S. pneumoniae, Shigella* species and *Salmonella* species.

Invasion results of *Streptococcus pneumoniae* strains (invasion percentages) are shown in figure 2. The minimum invasion percentage is 0.00 % while the maximum invasion percentage is 2.11%. Figure 3 shows invasion results of the Gram-negative enteric pathogens, strains of *Shigella* spp. The results cover a minimum percentage of invasion 1.26% from a strain to a maximum percentage of invasion 32.93% to another strain. Figure 4 shows invasion results of the other gram-negative enteric pathogens, *Salmonella* strains. The maximum percentage of invasion for *Salmonella* strains is 11.80% and minimum percentage is 1.30%.

Invasion results for both of the enteric pathogens *Shigella* spp. and *Salmonella* spp. are shown together (Figure 5). The average values of invasion percentages for *Shigella* spp. and *Salmonella* spp. are 8.91% and 5.19% respectively (Table 5). Figure 6 represents comparison of invasion results for six strains of *Salmonella* spp. between 2-hr and 4-hr intracellular growth incubation period with gentamicin. Figure 7 also represents comparison of invasion results for six strains of *Shigella* spp. between 2-hr and 4-hr

intracellular growth incubation with gentamicin. Figure 8 represents comparison of invasion results, for 10 strains of *Streptococcus pneumoniae*, between 2-hr and 4-hr intracellular growth period with exogenous gentamicin treatment. For the three groups of bacteria the 2-hr and the 4-hr incubation time tests were done at different times.

Table 5. Some descriptive statistics of HEp-2 cells invasion results of the pathogenic bacteria

Species	N	Mean Inv. %	Std. Deviation	Std. Error	Minimum Inv. %	Maximum Inv. %
S. pneumoniae	50	0.17	0.44	0.06	0.00	2.11
Shigella spp.	25	8.91	7.44	1.49	1.26	32.93
Salmonella Spp.	25	5.19	2.83	0.57	1.30	11.78

Note: Inv. % = Invasion percentage

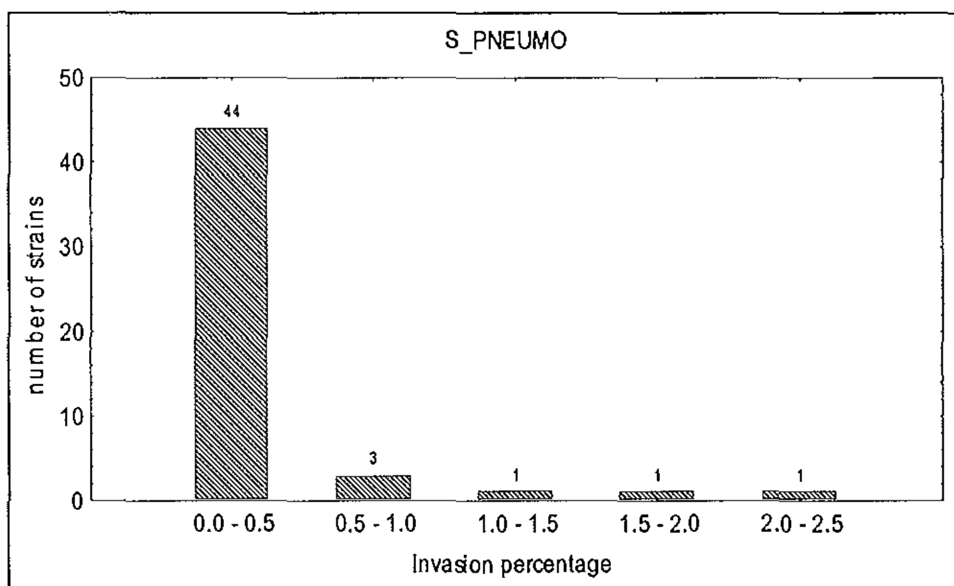

Figure 2. *Streptococcus pneumoniae* HEp-2 cell invasion result

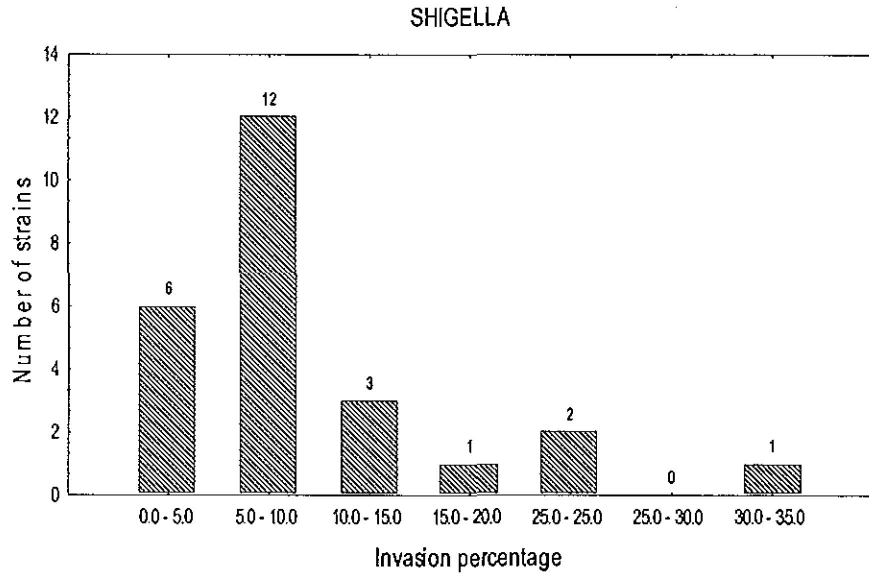

Figure 3. *Shigella* spp. HEp-2 cell invasion result

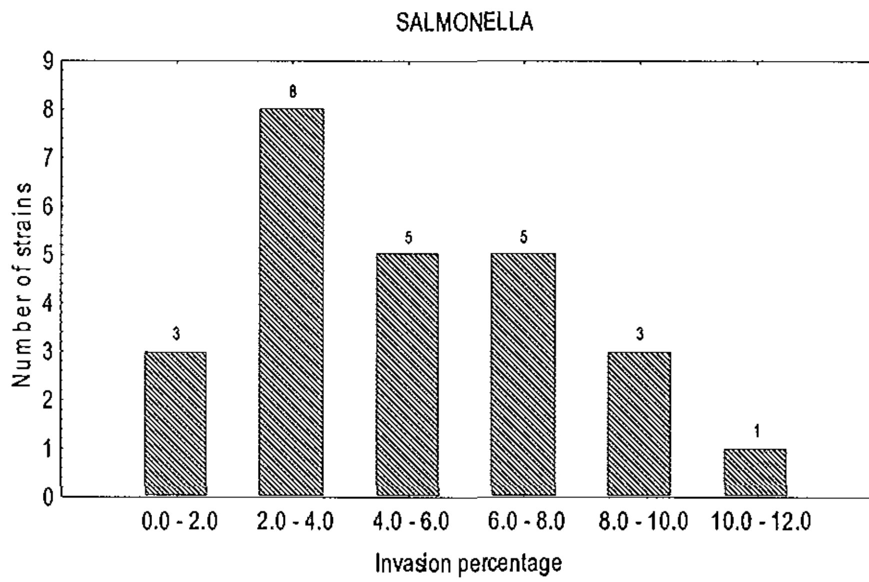

Figure 4. *Salmonella* spp. HEp-2 cell invasion result

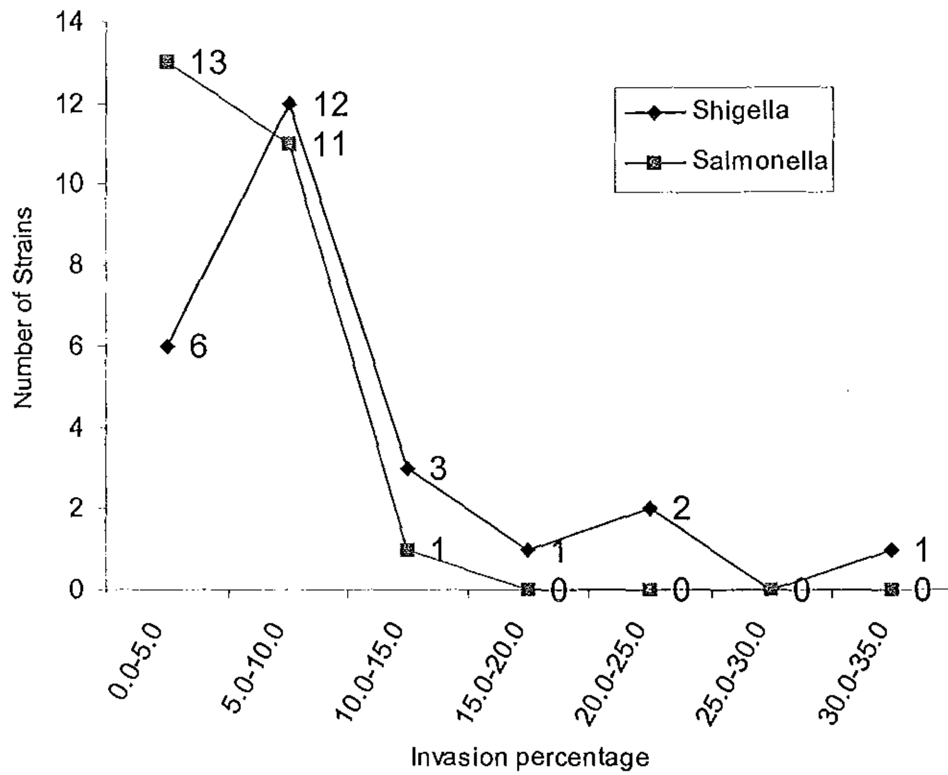

Figure 5. Comparison of *Shigella* spp. and *Salmonella* spp. Invasion Results

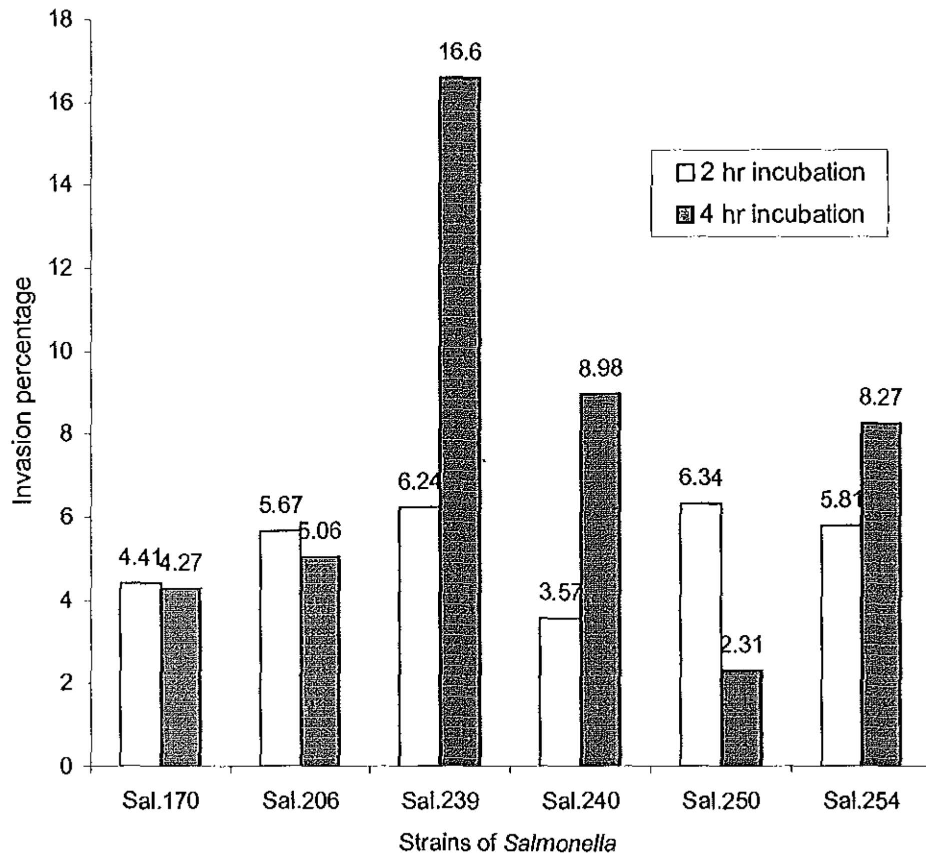

Figure 6. Comparison of percentages of bacteria recovered after a 2-hr and 4-hr intracellular growth incubation with gentamicin for six *Salmonella* spp.

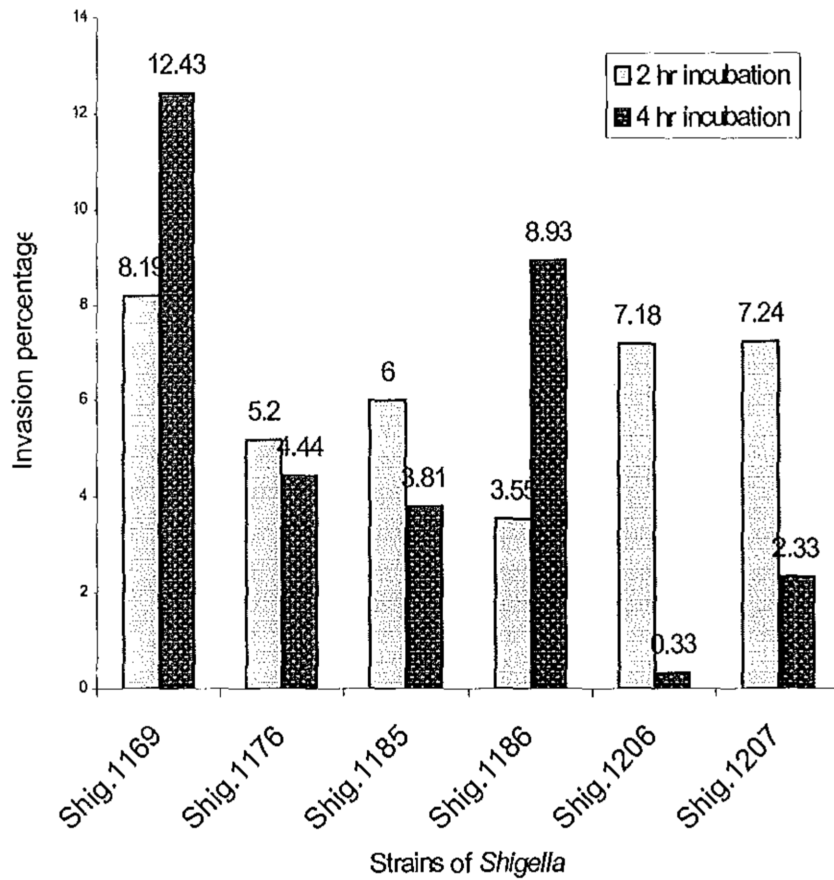

Figure 7. Graph showing comparison of percentages of bacteria recovered after a 2-hr and 4-hr intracellular growth incubation with gentamicin for six *Shigella* spp.

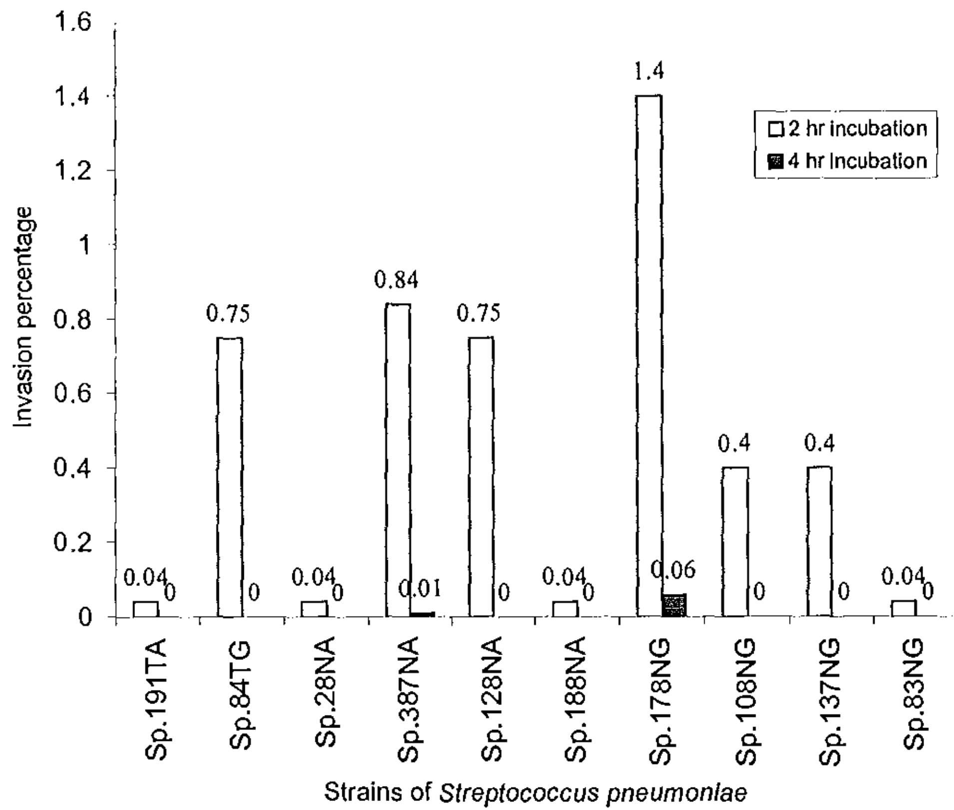

Figure 8. Comparison of invasion results after a 2-hr and 4-hr intracellular growth incubation period with gentamicin for *S. pneumoniae* strains

5. Discussion

Bacteria that are pathogenic to mammalian hosts adhere to the surface of host cells and remain extracellularly localized or enter into the cells. On the other hand mutant strains defective for the synthesis of the microbially encoded adherence factors are either unable to colonize appropriate host tissues, unable to compete with resident microbial flora, or attenuated for virulence relative to isogenic wild-type strains (Isbereg, 1991). Here in the present work strains of *Streptococcus pneumoniae*, *Shigella* spp. and *Salmonella* spp. showed different adherence ability to cultured HEp-2 cells.

In the case of *Streptococcus pneumoniae* majority of the strains (86%) adhered to the HEp-2 cells but some of them could not adhere. The adhered bacteria's binding is either strong or weak. This variation in their ability to adhere to the epithelial cells may depend on both phenotypical and genotypical factors. The extent of adhesion of the bacteria may also depend on avidity of the interaction between receptors on the epithelial cells and the bacterial adhesins (Isbereg, 1991). In the natural infection process most pneumococci are found adhered in the nasophrynx. However, data in this work shows there are seven strains out of the total fifty strains tested that could not adhere to the cultured HEp-2 cells. One important factor that can be presumed as a reason for the loss of adherence is pneumococcal phase variation unless they are mutants. The phase variation is between opaque and transparent phenotypes. The transparent phenotypes are efficient colonizers (Weiser *et al.*, 1994; Cundell *et al.*, 1995a; Gillespie and Balakrishnan, 2000). Furthermore, it is indicated that transparent variants bind to GlcNAc β 1-3 Gal on

epithelial cells between 10^2- and 10^6 –fold more efficiently than their opaque counter parts (Cundell *et al.,* 1995c).

The data on haemagglutination showed that forty strains of *S. pneumoniae* were able to agglutinate erythrocytes from human (A, B, AB, and O blood group), guinea pig, and rabbit whereas ten strains could not haemagglutinate any one of the erythrocytes. All the forty strains showed mannose-resistant haemagglutination. In the mannose-resistant haemagglutination the addition of D-mannose couldn't inhibit agglutination of the erythrocytes by *S. pneumoniae* indicating that the haemagglutinis are non pili-1 adhesins. Isberg (1991) has indicated this concept that a variety of Gram-negative bacteria possess different pili but Gram-positive bacteria do not have. Here we are uncertain to determine whether the same factors are involving in haemagglutination and adhesion to HEp-2 cells because nine of the ten non-haemagglutinating strains were positive for adhesion to HEp-2 cells. Since the haemagglutination and the adhesion assays are done at different times, the pneumococcal phenotypic phase variation could be implicated as a reason for the inconsistent results in haemagglutination and adhesion. This condition can also be correlated to the result that six out of the haemagglutinting strains were negative for adhesion to HEp-2 cells.

In *Shigella* spp. isolates the adherence ability to epithelial cells has been shown by their adhesion to cultured HEp-2 cells. From the 25 isolates tested for adhesion 24 of them were able to adhere to HEp-2 cells. They adhered either in a localized, aggregative, or diffused manner over the surfaces of the HEp-2 cells with the majority showing localized adherence pattern. Only one strain was unable to adhere to HEp-2 cells; however, it had

the ability to agglutinate rabbit erythrocyte in mannose-resistant manner. The adherence results showed that the factors involved in the adhesion to HEp-2 cells are non type-1 pili adhesins because the adherence of the *Shigella* spp. occurred in the presence of D-mannose (Sussman, 1985).

On the other hand out of the 24 *Shigella* spp. that are positive for adhesion to HEp-2 cells eight strains were unable to agglutinate any of the six erythrocytes. The remaining 16 isolates were positive for haemagglutination with rabbit erythrocyte in mannose-resistant manner. However, few of them were able to agglutinate human and guinea pig erythrocytes besides the rabbit erythrocyte also involving in mannose-sensitive haemagglutination. This condition indicates that the same factors are not involved in the haemagglutination and adhesion to HEp-2 cells among the *Shigella* spp. isolates.

In the case of *Salmonella* spp. isolates all the 25 strains tested are able to adhere to HEp-2 cells. They adhered either in a localized, aggregative, or diffused manner on the surface of the HEp-2 cells with the majority of them showing the aggregative pattern. All the isolates adhered to HEp-2 cells in the presence of D-mannose. This condition shows that the bacterial adhesins involved are not type-1 fimbrial (pili) adhesins (Nichols *et al.,* 1990). The haemagglutination results of *Salmonella* spp. showed that only 13 strains are agglutinating. These strains could agglutinate rabbit erythrocyte in the mannose-resistant manner. However, one of the 13 strains agglutinated human erythrocytes (A, B, AB, and O blood group) and guinea pig erythrocyte besides the rabbit erythrocyte. The results observed in haemagglutination and adhesion to HEp-2 cells among the *Salmonella* spp. indicate that there are different factors involved.

The invasion of host cells by *S. pneumoniae*, *Shigella* spp. and *Salmonella* spp. isolated from patients was measured using cultured HEp-2 cells. All the bacteria were tested by the same parameter for their invasiveness. In the current study quantitative data for invasiveness are obtained for each strain tested from the three groups. In the case of *Streptococcus pneumoniae*, only 12 out of the total 50 strains are invasive for the cultured HEp-2 cells. The remaining 38 strains are not invasive. The invasion rates for the 12 invading strains range between 0.04% and 2.11%. These values are percentages of bacteria survived from the initial inoculum after being exposed to external gentamicin, so that, the values show the efficiency of the bacteria to enter into the HEp-2 cells. The rates of invasion obtained are very low indicating the inefficient invasiveness of these pneumococci strains (Cundell *et al.,* 1995).

Likewise Cundell *et al.* (1995b) have characterized the invasiveness of *Streptococcus pneumoniae* isolates with cultured monolayers of human vascular endothelial cells. The result obtained from their work was that only 0.1% of the initial pneumococcal inoculum can be recovered upon exposure to exogenous gentamicicn. These workers used the same parameter as the one used in this work to measure invasiveness. They have also indicated that the classically 'invasive' bacteria *Shigella* spp. and *Salmonella* spp. have invasion rates > 2%-3% of the initial inoculum. Hence, it can be shown that *Streptococcus pneumoniae* can not be considered invasive for naive (not activated) cultured epithelial cells as compared to *Shigella* spp. and *Salmonella* spp. Geelen *et al.* (1993) have also mentioned that pneumococci can not enter resting endothelial cells. On the contrary, these workers have shown that activation of the cultured epithelial cells by proinflammatory

cytokines (IL-1 and TNF-α) resulted in the entry of 2%-3% of the pneumococci within 30 minutes.

The 2-hr invasion results were compared using one way ANOVA among the groups of the organisms. Appendix 4 shows analysis of the significance of variation in invasion results between *Shigella* and *Salmonella* species. There is significant variation between the invasion results ($P< 0.05$). Appendix 5 shows analysis of the significance of variation in invasion results between *Shigella* spp. and *S. pneumoniae*. There is highly significant variation between the invasion results ($P< 0.001$). Appendix 6 shows analysis of the significance of variation in invasion results between *Salmonella* spp. and *S. pneumoniae*. There is highly significant variation between the invasion results ($P< 0.001$).

Furthermore, results of tissue invasion after a 2-hr and 4-hr incubation with gentamicin are characterized for ten *S. pneumoniae* strains. As it is shown in fig. 8 the ten strains have invasion percentages ranging between 0.04% and 1.40% for the 2-hr incubation. However, after a 4-hr incubation with gentamicin only 2 strains (Sp.387NA and Sp.178NG) were recovered with invasion percentages 0.01% and 0.06%, respectively. The remaining eight strains were not recovered after the 4-hr incubation with gentamicin. The results for the 4-hr incubation, 0.01% and 0.06%, were significantly lower than the results for the 2-hr incubation, 0.84% and 1.4%, respectively, for the two strains. These data show the possibility that increase of incubation time beyond 2 hr may result HEp-2 cells' membrane damage, so that, gentamicin may enter into the cells and kill intracellular bacteria. This condition may occur due to accumulation of the *S. pneumoniae* factors that damage cell membranes (Berry *et al.*, 1999; Gillespie and Balakrishnan, 2000).

The invasiveness study of *Shigella* spp. showed that all the tested 25 strains are invasive. The invasion rates for the strains range between 1.26% and 32.93%. This shows invasiveness depends on the characteristic of the individual strain because the same initial inoculum (10^7 bacteria/ml) was used to infect cultured HEp-2 cells. From these isolates only three of them resulted in percentages of invasion below 2.00% with an average value of 1.61%. Thus these isolates were determined as poorly invasive. The remaining 22 isolates showed invasion rates greater than 2.00% with an average value of invasion percentages 10.23%. According to Cundell *et al.* (1995c) ' invasive' species such as *Salmonella* and *Shigella* have invasion percentages > 2%-3% of the initial inoculum of bacteria added to tissue monolayer for invasion. Hence, the 22 *Shigella* isolates are determined as invasive from the HEp-2 cell invasion study.

The *Salmonella* spp. invasiveness study showed that all the 25 isolates are invasive for the cultured HEp-2 cells. The invasion rates for the isolates range between 1.30% and 11.78%. Three isolates have invasion percentages below 2.00% with an average value of 1.66%. Hence, these three isolates were determined as poorly invasive. The remaining 22 isolates showed invasion percentages greater than 2.00% with an average value 5.68%. Thus, these isolates were determined as invasive. The *Salmonella* spp. on the average showed less invasion rates as compared with *Shigella* spp., however, invasiveness depends on the individual strain. Finlay and Falkow (1990) have indicated that *Salmonella* spp. invade intestinal epithelial cells and multiply within the cells to a lesser extent than *Shigella* spp.

For both *Shigella* spp. and *Salmonella* spp. isolates invasion characteristics with 2-hr and 4-hr intracellular growth incubation with exogenous gentamicin were assessed. Six isolates from each group were selected and compared for the 2-hr and 4-hr incubation invasion results. The invasion results obtained are not evenly distributed as shown from the two groups (fig. 6 and fig. 7). As shown in fig. 7 out of the six *Shigella* spp. tested four of them decreased their invasion percentages after the 4-hr incubation with gentamicin as compared to the 2-hr incubation results. While the remaining two isolates showed increase of percentages of invasion after the 4-hr incubation with gentamicin. The decrease in the percentage of invasion is significant in some of the strains. For example, a strain Shig.A.1206 that has 7.18% invasion percentage for the 2-hr incubation resulted 0.33% after the 4-hr incubation test. Thus, the biological significance of the quantitative data obtained using the 4-hr incubation with gentamicin wouldn't be reliable for measure of invasiveness.

On the other hand out of the six *Salmonella* spp. tested three of them resulted decreased invasion percentages after the 4-hr incubation with gentamicin compared with the 2-hr incubation invasion results (fig. 6). The other three showed increases in the percentages of invasion after the 4-hr incubation with gentamicin as compared to the 2-hr incubation invasion results. The increase of invasion percentages after the 4-hr incubation shown by some of the isolates are possibly due to the replication of the bacteria inside HEp-2 cells being protected from the gentamicin. On the contrary the intracellular replication of some fast growing, cytotoxic or virulent strains during the 4-hr incubation may result in damage of HEp-2 cells and allow entry of gentamicin to kill the intracellular bacteria. Hence, entry of gentamicin reduces the recovery of viable intracellular bacteria (Douce *et al.,* 1991).

6. Conclusion

Bacterial adhesion to epithelium of mucosal surfaces is an essential step in the pathogenesis of many infections including enteric and respiratory tract infections. So that adhesion to epithelial cells is an important virulence factor. Different adhesion factors of bacterial pathogens are studied to date. Nevertheless, characterization of such important factors is difficult *in vivo*. In this study adhesion of the pathogenic bacterial isolates to cultured HEp-2 cells has enabled us to characterize their adherence ability.

The *S. pneumoniae, Shigella* and *Salmonella* species adherence ability to the cultured HEp-2 cells was not inhibitable by D-mannose. So that type-1 fimbrial adhesins did not mediate the adherence of the bacteria. The adhesion of the *Shigella* spp. is worth noting in that there is one isolate non-adherent to the cultured HEp-2 cells which is positive for haemagglutination and HEp-2 cell invasion. In the case of *Streptococcus pneumoniae* isolates majority of them, 86% (43/50), are adherent to HEp-2 cells with the remaining 14% (7/50) did not adhere. This characterization of adherence ability has generated the information to further analyze the *S. pneumoniae* factors that involved in the adhesion to HEp-2 cells.

Almost all the haemagglutination activities by the bacterial isolates are mannose-resistant which imply that the haemagglutinins are non type-1 fimbrial adhesins. Association between agglutination of erythrocytes and adhesion to HEp-2 cells could not be shown in this study. The observations that some of the bacteria are haemagglutinating but not adherent to HEp-2 cells and the vice versa show the lack of association between

haemagglutination and adhesion. So that the adherence ability of the pathogenic bacteria to epithelial cells can not be measured, alternatively, using the six groups of erythrocytes.

The current study has shown the tissue invasion ability of the pathogenic bacteria, using cultured HEp-2 cells. Ability of the entry of invasive bacteria into human epithelial cells can be measured by quantifying invasion rates. The condition that bacteria located within mammalian cells (invasive) are not killed by a variety of aminoglycoside antibiotics and externally adhered bacteria are killed rapidly has helped to quantify invasiveness. The quantification of the invasiveness of the Gram-negative bacteria *Shigella* spp. and *Salmonella* spp. has shown that all the isolates are invasive. On the contrary *S. pneumoniae* isolates are not 'invasive' as compared to the *Shigella* spp. and *Salmonella* spp. invasiveness based on the parameter used in this work to measure invasiveness.

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8. Appendices

Appendix 1. Haemagglutination and HEp-2 cell adhesion results of 50 *S. pneumoniae* strains

Strain	Strain No.	Human red blood cell group				Ra-RBC	Gp-RBC	HEp-2 Cell Adherence
		A	B	AB	O			
S. pneumoniae	Sp.191TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.316NA	(-)	(-)	(-)	(-)	(-)	(-)	LA
>>	Sp.178NG	(-)	(-)	(-)	(-)	(-)	(-)	AA
>>	Sp.110TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.108NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	SP.102NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.47TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.84TG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.82NG	MRHA[c]	MRHA[c]	MRHA[c]	MRHA[c]	MRHA[b]	MRHA[b]	LA
>>	Sp.28NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.137NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.574NA	(-)	(-)	(-)	(-)	(-)	(-)	LA
>>	Sp.452NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA[*]
>>	Sp.281TA	(-)	(-)	(-)	(-)	(-)	(-)	AA
>>	Sp.500TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.558TA	(-)	(-)	(-)	(-)	(-)	(-)	DA[*]
>>	Sp.35NA	(-)	(-)	(-)	(-)	(-)	(-)	LA
>>	Sp.60TG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA
>>	Sp.193TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.18NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.144TG	(-)	(-)	(-)	(-)	(-)	MRHA[c]	AA
>>	Sp.109NG	(-)	(-)	(-)	(-)	(-)	MRHA[c]	DA[*]
>>	Sp.63NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	NA
>>	Sp.83NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.79NA	(-)	(-)	(-)	(-)	(-)	MRHA[c]	DA[*]
>>	Sp.162NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	NA

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Strain	Strain No.	Human red blood cell group				Ra-RBC	Gp-RBC	HEp-2 Cell Adherence
		A	B	AB	O			
S. pneumoniae	Sp.191TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.316NA	(-)	(-)	(-)	(-)	(-)	(-)	LA
>>	Sp.178NG	(-)	(-)	(-)	(-)	(-)	(-)	AA
>>	Sp.110TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.108NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	SP.102NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.47TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.84TG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.82NG	MRHA[c]	MRHA[c]	MRHA[c]	MRHA[c]	MRHA[b]	MRHA[b]	LA
>>	Sp.28NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.137NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.574NA	(-)	(-)	(-)	(-)	(-)	(-)	LA
>>	Sp.452NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA[*]
>>	Sp.281TA	(-)	(-)	(-)	(-)	(-)	(-)	AA
>>	Sp.500TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.558TA	(-)	(-)	(-)	(-)	(-)	(-)	DA[*]
>>	Sp.35NA	(-)	(-)	(-)	(-)	(-)	(-)	LA
>>	Sp.60TG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA
>>	Sp.193TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.18NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.144TG	(-)	(-)	(-)	(-)	(-)	MRHA[c]	AA
>>	Sp.109NG	(-)	(-)	(-)	(-)	(-)	MRHA[c]	DA[*]
>>	Sp.63NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	NA
>>	Sp.83NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.79NA	(-)	(-)	(-)	(-)	(-)	MRHA[c]	DA[*]
>>	Sp.162NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	NA

Appendix I. (Continued)

S. pneumoniae	Sp.325NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	NA
>>	Sp.85NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA[*]
>>	Sp.195NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.125NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.31NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.56NA	(-)	(-)	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.504NA	MRHA[b]	MRHA[b]	MRHA[c]	MRHA[c]	MRHA[b]	MRHA[c]	LA
>>	Sp.387NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.683NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA
>>	Sp.146NG	(-)	(-)	(-)	(-)	(-)	(-)	DA[*]
>>	Sp.512NA	(-)	(-)	(-)	(-)	MRHA[a]	MRHA[b]	LA
>>	Sp.100NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	NA
>>	Sp.483T.	MRHA[a]	MRHA[a]	MRHA[illegible]	MRHA[a]	MRHA[a]	MRHA[illegible]	NA
>>	Sp.153NG	(-)	(-)	(-)	(-)	(-)	(-)	NA
>>	Sp.142NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	NA
>>	Sp.143TG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	LA
>>	Sp.182TA	(-)	(-)	(-)	(-)	(-)	(-)	AA
>>	Sp.29NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA[*]
>>	Sp.335TA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.494NG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.128NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA[*]
>>	Sp.172NA	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	AA
>>	Sp.126TG	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	MRHA[a]	DA[*]
>>	Sp.188NA	(-)	(-)	(-)	(-)	(-)	(-)	LA

NOTE: MRHA= mannose-resistant haemagglutination, a= strong (+++) haemagglutination, b= moderate (++) haemagglutination, c= weak (+) haemagglutination, (-)= no haemagglutination, LA= localized adherence, AA= aggregative adherence, DA= diffused adherence, NA= no adhesion, *= weak adherence. Ra-RBC= rabbit red blood cell, Gp-RBC= guinea-pig red blood cell, *S. pneumoniae*= *Streptococcus pneumoniae*

Appendix 2. Haemagglutination and HEp-2 cell adhesion results of 25 *Shigella* strains

Strains	Strain No.	Human red blood cell group				Ra-RBC	Gp-RBC	HEp-2 cell adherence
		A	B	AB	O			
Shigella A	Shig.1206	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	LA
Shigella A	Shig.1222	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	LA*
Shigella A	Shig.1225	(-)	(-)	(-)	(-)	(-)	(-)	LA
Shigella A	Shig.1214	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	AA
Shigella A	Shig.1207	(-)	(-)	(-)	(-)	(-)	(-)	LA
Shigella A	Shig.1223	(-)	(-)	(-)	(-)	(-)	(-)	LA
Shigella B	Shig.2	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	LA
Shigella B	Shig.1177	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	LA
Shigella B	Shig.1198	MRHA^{b}	MRH[illegible]	MRHA^{b}	MRHA^{b}	MRHA[illegible]	MRHA^{b}	LA
Shigella B	Shig.1202	(-)	(-)	(-)	(-)	MRHA^{a}	(-)	LA
Shigella B	Shig.3	(-)	(-)	(-)	(-)	MRHA^{a}	MSHA^{a}	DA*
Shigella B	Shig.13	(-)	(-)	(-)	(-)	MRHA^{b}	MSHA^{c}	LA
Shigella B	Shig.1169	(-)	(-)	(-)	(-)	MRHA^{b}	MSHA^{c}	LA
Shigella B	Shig.7	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	DA
Shigella B	Shig.1208	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	AA
Shigella B	Shig.1186	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	LA
Shigella B	Shig.1199	(-)	(-)	(-)	(-)	(-)	(-)	LA
Shigella B	Shig.1175	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	LA
Shigella B	Shig.1200	(-)	(-)	(-)	(-)	(-)	(-)	LA
Shigella B	Shig.1212	(-)	(-)	(-)	(-)	(-)	(-)	LA
Shigella B	Shig.1205	(-)	(-)	(-)	(-)	(-)	(-)	LA
Shigella B	Shig.1185	(-)	(-)	(-)	(-)	MRHA^{b}	(-)	LA
Shigella B	Shig.1173	(-)	(-)	(-)	(-)	(-)	(-)	LA
Shigella B	Shig.15	MSHA^{c}	MSHA^{c}	MSHA^{c}	MSHA^{c}	MRHA^{b}	MSHA^{a}	AA
Shigella C	Shig.1176	(-)	(-)	(-)	(-)	(-)	MRHA^{b}	NA

NOTE: MRHA= mannose-resistant haemagglutination, MSHA= mannose-sensitive haemagglutination, a= strong haemagglutination, b= moderate haemagglutination, C= weak haemagglutination, (-)= no haemagglutination, LA= localized adherence, AA= aggregative adherence, DA= diffused adherence, NA= no adhesion, *= weak adherence, Ra-RBC= rabbit red blood cell, Gp-RBC= guinea-pig red blood cell

Appendix 3. Haemagglutination and HEp-2 cell adhesion results of 25 *Salmonella* strains

Strains	Strain No.	Human red blood cell group				Ra-RBC	Gp-RBC	HEp-2 cell adherence
		A	B	AB	O			
S. typhi	Sal.224	(-)	(-)	(-)	(-)	(-)	(-)	AA
S. typhi	Sal.236	(-)	(-)	(-)	(-)	(-)	(-)	AA
S. typhi	Sal.222	(-)	(-)	(-)	(-)	(-)	(-)	AA
S. typhi	Sal.250	(-)	(-)	(-)	(-)	(-)	(-)	AA
S. typhi	Sal.206	(-)	(-)	(-)	(-)	(-)	(-)	DA*
S. typhi	Sal.238	(-)	(-)	(-)	(-)	(-)	(-)	LA
Salmonella A	Sal.170	(-)	(-)	(-)	(-)	MRHA[c]	(-)	AA
Salmonella B	Sal.239	(-)	(-)	(-)	(-)	MRHA[c]	(-)	AA
Salmonella B	Sal.200	(-)	(-)	(-)	(-)	(-)	(-)	LA
Salmonella B	Sal.264	(-)	(-)	(-)	(-)	MRHA[c]	(-)	DA
Salmonella B	Sal.168	(	(-)	(-)	(-)	MRHA[c]	(-)	L,
Salmonella B	Sal.211	(-)	(-)	(-)	(-)	(-)	(-)	AA
Salmonella B	Sal.230	(-)	(-)	(-)	(-)	MRHA[c]	(-)	DA
Salmonella B	Sal.229	(-)	(-)	(-)	(-)	MRHA[b]	(-)	AA
Salmonella C_1	Sal.262	(-)	(-)	(-)	(-)	(-)	(-)	LA
Salmonella C_1	Sal.234	(-)	(-)	(-)	(-)	(-)	(-)	AA
Salmonella C_1	Sal.248	(-)	(-)	(-)	(-)	MRHA[c]	(-)	AA
Salmonella C_1	Sal.254	(-)	(-)	(-)	(-)	(-)	(-)	AA
Salmonella C_1	Sal.237	(-)	(-)	(-)	(-)	MRHA[c]	(-)	AA
Salmonella C_1	Sal.259	MRHA[b]	MRHA[b]	MRHA[b]	MRHA[b]	MRHA[b]	MRHA[b]	AA
Salmonella C_2	Sal.161	(-)	(-)	(-)	(-)	MRHA[c]	(-)	AA
Salmonella C_1	Sal.269	(-)	(-)	(-)	(-)	(-)	(-)	LA
Salmonella C_1	Sal.244	(-)	(-)	(-)	(-)	MRHA[b]	(-)	AA
Salmonella D	Sal.213	(-)	(-)	(-)	(-)	MRHA[b]	(-)	LA
Salmonella D	Sal.240	(-)	(-)	(-)	(-)	MRHA[b]	(-)	AA

NOTE: MRHA= mannose-resistant haemagglutination, a= strong haemagglutination, b= moderate haemagglutination, c= weak haemagglutination, (-)= no haemagglutination, LA= localized adherence, AA= aggregative adherence, DA= diffused adherence, *= weak adherence, *S. typhi*= *Salmonella typhi*

Appendix 4. One way ANOVA test between invasion results of *Shigella* and *Salmonella* spp.

Test	df	Mean square	F	Sig. (P-value)*
Between group	1	172.42	5.45	0.024
Within group	48	31.66		

Note: *= invasion results compared between the two groups ($P < 0.05$)

Appendix 5. One way ANOVA test between invasion results of *S. pneumoniae* and *Shigella* spp.

Test	df	Mean square	F	Sig. (P-value)*
Between group	1	1274.82	69.62	0.00
Within group	73	18.31		

Note: *= invasion results compared between the two groups ($P < 0.001$)

Appendix 6. One way ANOVA test between invasion results of *S. pneumoniae* and *Salmonella* spp.

Test	df	Mean square	F	Sig. (P-value)*
Between group	1	421.98	152.49	0.00
Within group	73	2.77		

Note: *= invasion results compared between the two groups ($P < 0.001$)

Declaration

I, the undersigned, declare that this thesis is my own work and it has not been presented in other Institutions for a similar degree or other purpose.

TEFERI ESHETU	July 2, 2001	[signature]
Name	Date	Signature

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

CHARACTERIZATION OF ADHESION AND INVASION VIRULENCE FACTORS IN *STREPTOCOCCUS PNEUMONIAE*, *SHIGELLA*, AND *SALMONELLA* SPECIES ISOLATED FROM ETHIOPIAN PATIENTS

By

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