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COLLEGE OF HEALTH SCIENCES
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DEPARTMENT OF CLINICAL MICROBIOLOGY,

CHARACTERIZATION OF NON-TYPHOIDAL *SALMONELLA* ISOLATED FROM PATIENTS ATTENDING AGONA SWEDRU MUNICIPAL HOSPITAL

A THESIS SUBMITTED TO THE DEPARTMENT OF CLINICAL MICROBIOLOGY, IN FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF PHILOSOPHY IN CLINICAL MICROBIOLOGY

BY

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DECLARATION

The experimental work described in this thesis was carried out at the Department of Clinical Microbiology, KNUST. This work has not been submitted for any other degree.

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ABSTRACT

Introduction: There is increasing occurance of salmonellosis with observed emergence of high level resistance to antimicrobial agents leading to the difficulty in treating infected patients with invasive infections worldwide. The study aims at determining the *Salmonella species* causing diarrhoea in Agona Swedru Municipal Hospital, as diarrhoea ranked the third common complaints in the hospital.

Methods: Clinical samples comprising stool (n=380), blood (n=378) and urine (n=375) were cultured for *Salmonella* and other bacteria. The *Salmonella* isolates were serotyped based on Kauffmann and White scheme. Antimicrobial susceptibility testing was performed on all the isolates using the disc diffusion Kirby-Bauer method. Double-disk synergy test was used to test the isolates for Extended Spectrum β-Lactamase production. The *Salmonella* isolates were fingerprinted by Pulsed-Field Gel Electrophoresis (PFGE) to determine their genetic relatedness.

Results: Thirteen (13) *Salmonellae* were isolated from the diarrhoeal patients stool samples (n=380). Among the *Salmonella* serovars identified, *Salmonella* Enterica (69.2%; n=9/13) predominated, followed by *Salmonella* Choleraesuis (23.1%; n=3/13) and *Salmonella* Paratyphi B (7.7%; n=1/13). The most common pathogen isolated other than *Salmonella* was *Escherichia coli* (63.6%; n=21/33), followed by *Staphylococcus aureus* (18.2%; n=6/33), *Citrobacter species* (9.1%; n=3/33), *Proteus mirabilis* (6.1%; n=2/33) and *Pseudomonas aeruginosa* (3.0%; n=1/33). *Salmonella* isolates showed varied resistance to antimicrobials tested, with resistance proportions being Cotrimoxazole (9/13 (69.2%), Tetracycline (3/13, 23.1%), Amoxicillin (2/13, 15.4%), Ampicillin (2/13, 15.4). All the isolates were sensitive to Ceftriaxone, Ciprofloxacin, Amikacin, Gentamicin, AmoxicillinClavulanic acid, Chloramphenicol and Meropenem. None of the *Salmonella*

isolates were multidrug resistant (MDR) and none produced Extended Spectrum β-Lactamase. Extended Spectrum β-Lactamase production was varied among other isolates. The *Salmonella* Choleraesuis isolates though were same species, were of different PFGE types. **Conclusion:** Prevalence of *Salmonellae* among patients attending Agona Swedru Municipal Hospital was 3.4%. Most of the *Salmonellae* were non-Typhoidal *Salmonellae*. Proportion of isolates resistant to antibiotics was low. There are possibly different *S.* Choleraesuis isolates circulating in Agona Swedru and its environs.

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Chapter 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Salmonellae are members of Enterobacteriaceae family and are facultative anaerobic Gram negative rod bacteria pathogens in both humans and animals (Velge *et al*., 2012). *Salmonella* infections are often transmitted through consumption of food contaminated with animal and or human faeces containing the *Salmonella* organism (Addis *et al*., 2011). Foods such as meat, vegetables, poultry products, milk products, fish and others have been shown as media spreading *Salmonella* to human beings (Kariuki *et al*., 2006a). *Salmonella* Typhi is known to cause typhoid (enteric) fever whilst *Salmonella* Paratyphi serotypes also cause paratyphoid (enteric) fevers. *Salmonella* Typhi and *Salmonella* Paratyphi (A, B, and C) serotypes are all solely human pathogens (Pui *et al*., 2011). Non-Typhoidal *Salmonella* (NTS) serotypes have emerged as a significant and common food-borne pathogen
throughout the world (Addis *et al*., 2011).

The recorded frequencies of typhoid fever are steadily less in developed nations whilst nontyphoidal infections have gone up globally and hence an important public health problem worldwide (Pui *et al*., 2011). Annual recorded report estimates typhoid fever cases worldwide to be sixteen (16) million and gastroenteritis cases are projected to be 1.3 billion (Pui *et al*., 2011) with highest prevalence in children (Ifeanyichukwu *et al*., 2013). Deaths worldwide due to *Salmonella* are estimated to be three (3) million (Pui *et al*., 2011). Nontyphoidal *Salmonellae* (NTS) infections result in gastroenteritis and the infection usually resolves without antimicrobial treatment (Kariuki *et al*., 2006a). Some

NTS serovars, such as, *Salmonella* Dublin, *Salmonella* Panama, *Salmonella* Heidelberg, *Salmonella* Choleraesuis and *Salmonella* Enteritidis have a tendency to cause extra-intestinal or invasive infections such as higher rates of bacteremia, meningitis and mortality in humans (Sirichote *et al*., 2010). The NTS causing invasive infections extends outside the gastrointestinal tract into blood and other organs of the body resulting in generalized infections (Pui *et al*., 2011).

The most frequently isolated non-typhoidal *Salmonellae* serotypes identified to infect humans are *Salmonella* Enteritidis *and Salmonella* Typhimurium (Kariuki *et al*., 2006a). Infections with multi-drug resistant non-typhoidal *Salmonellae* (NTS) in African children are rated among the most important diseases that lead to frequent deaths (Kariuki *et al*., 2006b). In a study conducted in Nairobi, Kenya, high prevalence of non-typhoidal *Salmonellae* isolates obtained were *Salmonella* Typhimurium (54.9%; n=106) and that was followed by *Salmonella* Enteritidis (33.2 %; n=64). Multiple antibiotic resistance strains were reported among 34.2% of the isolates (Kariuki *et al*., 2006a). In Malawi, epidemics of invasive NTS have been described as associated with the emergence of multidrug resistance (Gordon *et al*., 2008).

In Ghana, public health interest is more centered on HIV infections, malaria and tuberculosis, while the burden of generalized blood-stream disease remains inadequately studied (Nielsen *et al*., 2012). Death due to diarrhoea is expected annually to be 5000 among children within five years of age (Monney *et al*., 2013). In spite of this, only a small number of studies have been conducted in Ghana to give in-depth knowledge and relate to the source of diarrhoea illness (Monney *et al*., 2013). Invasive bacterial diseases

are underestimated since there are few hospitals or health facilities that are well-equipped to performed culture needed to isolate bacterial pathogens and to diagnose and provide susceptible drug for treatment of bacteremia (Crump *et al*., 2011; Nielsen *et al*., 2012). A study conducted in Ghana reported 129 Non-Typhoidal *Salmonella* (NTS) isolates from blood culture and it accounted for 53.3% of invasive bacterial isolates obtained (Nielsen *et al*., 2012). Multidrug resistance (MDR) strains were reported among the *Salmonella* isolates (Nielsen *et al*., 2012). The incident of salmonellosis is worldwide and high level resistance by *Salmonella* serovars to antimicrobials had lead to re-emergence of untreatable infections caused by *Salmonellae* (Newman *et al*., 2011; Pui *et al.,* 2011).

In developing countries such as Ghana, poor sanitation conditions and poor personal hygiene may lead to increased contamination of food and water (Addis *et al*., 2011) which may enhance increased spread and prevalence of *Salmonella* infections (Kuubiere *et al*., 2014). This study will determine genetic relatedness of *Salmonellae* circulating in Agona Swedru environment and the antimicrobial resistance pattern of those isolates including that of other bacteria pathogens that will be obtained from stool, blood and urine in the study.

1.2 AIM

The aim of the study is to characterize *Salmonellae* infecting patients in Agona Swedru Municipal Hospital.

1.3 OBJECTIVES

1. To culture samples (stool, urine, and blood) obtained from patients for *Salmonella* and other pathogens.

2. To determine the serotypes and the antimicrobial susceptibility patterns of the *Salmonella* isolates and other pathogens obtained.
3. To perform Pulse Field Gel Electrophoresis (PFGE) on the *Salmonella* isolates, to document their fingerprints.

1.4 PROBLEM STATEMENT.

The increased reports of diarrhoea cases at Agona Swedru Municipal Hospital in Ghana are a cause for concern since non-typhoidal *Salmonella* is a leading cause of diarrhoea and the incidence of diarrhoea due to non-typhoidal *Salmonella* has increased worldwide (Adam *et al*., 2007; Addis *et al*., 2011; Pui *et al*., 2011). In addition, reports on emergence and spread of antibiotic resistant invasive NTS represent a significant health risk worldwide and threaten the lives of infected persons, but the risk burden differs in different communities.

The scope of this risk at the area of this study Agona Swedru remains unknown.

1.5 JUSTIFICATION

Agona Swedru Government Hospital in Ghana from January to July 2010 recorded 227 cases of gastroenteritis and in the same period in 2011, cases of gastroenteritis increased to 472 (Annual Report, 2011). The increase in gastroenteritis cases at the hospital require further investigation as Non-Typhoidal *Salmonellae* (NTS) are the common and major cause of gastroenteritis worldwide (Adam *et al*., 2007).

Though Salmonellosis is usually self-limiting, antimicrobial treatment is required for individuals with severe salmonellosis or for individuals with invasive infections. Microbial resistance against the first-line antibiotics (Ampicillin, Chloramphenicol and Trimethoprim/Sulphamethoxazole) justify the testing of the antimicrobials for their resistance patterns.

1.6 STUDY HYPOTHESIS.

Salmonellosis is a major public health burden and caused mainly by serovars of Non-Typhoidal *Salmonella* as reported worldwide.

Chapter 2

LITERATURE REVIEW

2.1 GRAM NEGATIVE BACTERIA.

Gram-negative bacteria have a cytoplasmic membrane, a thin layer of peptidoglycan and an external membrane made of lipid-protein bilayer membrane consisting of phospholipids, lipopolysaccharides (LPSs) and proteins which includes porins (Beveridge, 1999). The porins assemble into pores to facilitate bacteria nutrition via diffusion and dissemination of water. The outer membrane separates the external environment of the bacteria from the periplasm. The periplasm serves as reservoir for virulence factors, binding proteins, enzymes (degradative and detoxifying) and others (Beveridge, 1999). The medically important Gram negative bacteria can be grouped as shown in Figure 2.1.

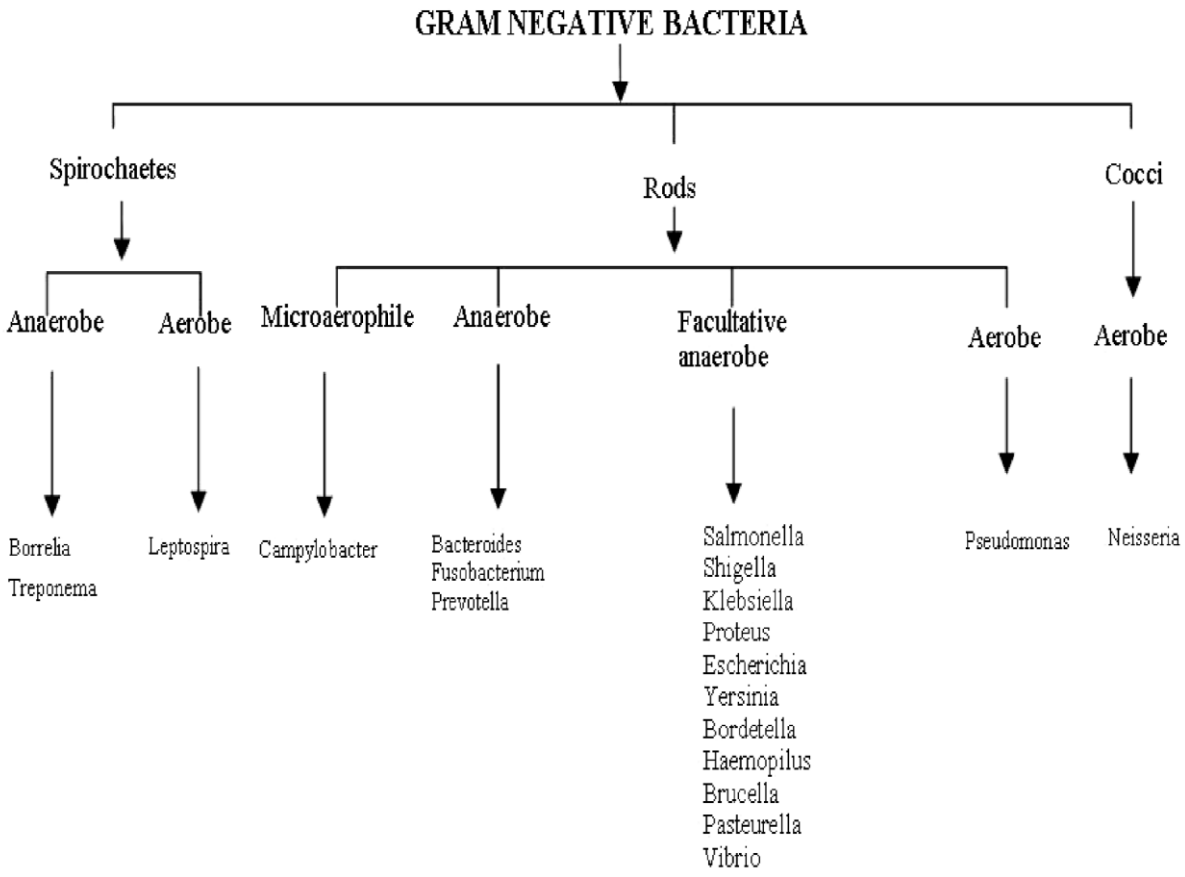

Figure 2.1: Basic grouping of medically important Gram negative bacteria (Prepared with the knowledge from Cheesbrough, 2006).

The largest group of medically important Gram negative bacteria that causes wide range of diseases belongs to the family Enterobacteriaceae (see Figure 2.2). The Enterobacteriaceae comprised of all the facultative aerobic and aerobic bacteria, Gram-negative non-spore forming rods and ferment lactose with acid and gas production (Hemraj *et al*., 2013). Members of this group can be identified based on their cultural characteristics, morphological test (Gram reaction), biochemical test, sugar fermentation test, sensitivity pattern and serological test (Hemraj *et al*., 2013).

The biochemical test and sugar fermentation test results that can be used to identify some medically important Gram negative bacteria are shown in Table 2.1.

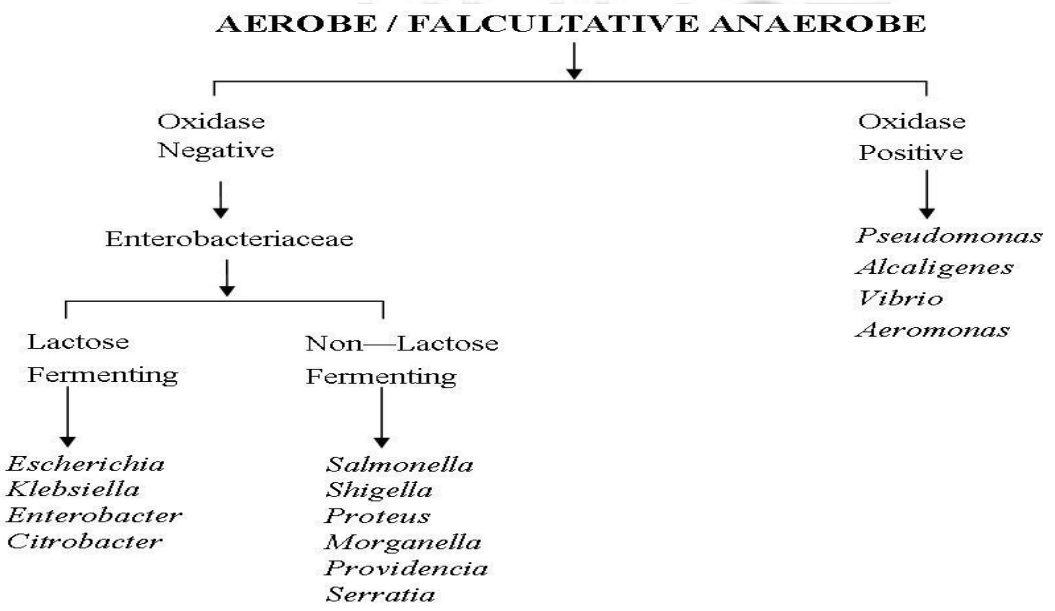

Figure 2.2: Basic grouping of the Enterobacteriaceae (Prepared with the knowledge from Cheesbrough, 2006).

Table 2.1: Biochemical test and sugar fermentation test results used to identify some members of the family Enterobacteriaceae.

Bacteria Isolates	**Ox.**	**Cit.**	**Fermentation**				**Mot.**	**Ind.**	**Urea**	**KIA**			
			Glu.	**Lact.**	**Suc.**	**Man.**				**Medium Slope**	**Butt**	**H_2S**	**G**
			+	+	+/-	+							
Escherichia coli	-	-	+				+	+	-	Y	Y	-	+
Shigella species	-	-	+	-	-	+/-	-	+/-	-	R	Y	-	-
Salmonella Typhi	-	-	+	-	-	+	+	-	-	R	Y	+	-
S. Paratyphi A	-	-	+	-	-	+	+	-	-	R	Y	-	+
Salmonella species	-	+/-	+	-	-	+	+	-	-	R	Y	+	+/-
Citrobacter freundii	-	+	+	+	+/-	+	+	-	+/-	R/Y	Y	+/-	+
Klebsiella species	-	+	+	+	+	+	-	-	+	Y	Y	-	+
Enterobacter species	-	+	+	+	+/-	+	+	-	-	Y	Y	-	+/-
Serratia marcescens	-	+	+	+/-	+	+	+	-	+/-	R/Y	Y	-	+/-
Proteus vulgaris	-	+/-	+	-	+	-	+	+	+	R	Y	+	+/-
Proteus mirabilis	-	+	+	-	+/-	-	+	-	+	R	Y	+	+
Morganella morganii	-	-	+	-	-	-	+	+	+	R	Y	-	+/-
Providencia species	-	+	+	-	+/-	+/-	+	+	+/-	R	Y	-	+/-
Yersinia enterocolitica	-	-	+	-	+	+	+	+/-	+	R	Y	-	-

Key: Ox. = Oxidase test, Cit. = Citrate test, Glu. = Glucose, Lact.=Lactose, Suc. = Sucrose, Man. = Mannitol, Mot. = Motility, Ind. = Indole test, R = Red-pink (alkaline reaction), Y = Yellow (acid reaction), H_2S = Hydrogen sulphide production (blackening), G = Gas. (Prepared with the information from Cheesbrough, 2006 and Hemraj *et al*., 2013).

2.2 HISTORY OF *SALMONELLA.*

In the era between 1701 and 1800, attention was drawn on *Salmonella* as the possible cause of enteric fever. Budd William first identified enteric fever among sick persons in 1873 (Abatcha *et al*., 2014). Afterwards, Karl Eberth revealed cylindrical bacteria in lymph nodes and spleen of someone who suffered enteric fever. *Salmonella* was successfully cultured in Germany by George Gaffky (Abatcha *et al*., 2014). That led to the isolation of *S*. Choleraesuis from the porcine bowel. In 1886, Damon Elder Salmon and Theobald Smith illustrated the organism of pig disease (Abatcha *et al*., 2014).

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The most famous outbreak of *Salmonella* infection was Mary Mallon as "Typhoid Mary" as shown in Figure 2.3 (Pui *et al.,* 2011). In 1906 it was reported that Mary Mallon was a healthy carrier of *Salmonella* typhi. She transmitted the disease to one hundred and twenty two (122) people who were some members of the families she served as a cook. Five (5) of the infected people died (Soper, 1939; Marineli *et al*., 2013). In 1990, the genus name "Salmonella" was suggested by French bacteriologist, Joseph Léon Marcel Ligniéres in honour of an American veterinary pathologist by name Dr. Daniel Elmer Salmon although many scientists had contributed to the discovery of the "*Salmonella"* organism (Marineli *et al*., 2013; Abatcha *et al*., 2014).

Figure 2.3: Mary Mallon as "Typhoid Mary" in the local newspaper of the era of typhoid (Adapted from Marineli *et al*., 2013).

2.3 THE GENUS *SALMONELLA.*

2.3.1 Surface Structures

2.3.1.1 Lipopolysaccharide (LPS)

The *rfb* centre of the bacterial chromosome posseses group of genes that code for enzymes which make and arrange sugars to form polysaccharides that make the side chains of lipopolysaccharide (Fierer and Guiney, 2001). The independent variations in the antigens possessed by flagella (H) and *Lipopolysaccharide* (O) make it feasible to type *Salmonellae* and other bacteria strains serologically (Kauffmann-White scheme) (Fierer and Guiney, 2001). *Salmonella* has its central arrangement of lipopolysaccharide (lipid A) mainly preserved (Fierer and Guiney, 2001). The polysaccharide polymorphism has enabled the placement of *Salmonellae* into groups (Fierer and

Guiney, 2001; CDC, 2011).

2.3.1.2 Flagellum

The bacterial flagellum is a complex structure that allows movement and survival of bacteria (Oliveira *et al*., 2011). Flagella promote bacteria cell adhesion and invasion and hence are essential structures for pathogens. *Salmonella* Typhimurium possess around its cell about (six) 6 to (ten) 10 flagella that are situated peritrichously (Oliveira *et al*., 2011). Most *Salmonellae* possess sets of two distinct genes of flagellin, and only one gene is expressed at a time. This allows serotyping of different strains (Baker *et al*., 2007).

2.3.1.3. Fimbriae

The long fimbriae are among the several classes of fimbriae made by *Salmonellae* (Fierer and Guiney, 2001). The fimbriae assist *Salmonella* adhere to Peyer's patches (Fierer and Guiney, 2001). Long polar fimbriae (Lpf) expressions of *Salmonella* undergo stage changes. *Salmonellae* interchange from showing long polar fimbriae (Lpf) to not expressing it. The existence of *lpf* genes in *S.* Enteritidis and *S.* Typhimurium are greatly preserved and therefore expressed long polar fimbriae protein cross react immunogenically. *S*. Typhimurium can stop the production of surface proteins which are immunogenic in nature and also have many different and redundant fimbriae (Fierer and Guiney, 2001).

2.3.2. Virulence Genes

2.3.2.1 Salmonella Pathogenicity Islands (SPI)

Bacteria acquire genetic information via horizontal gene transfer and are considered the most efficient evolutionary mechanism of bacteria (Hansen-Wester and Hensel, 2002). Bacteria from nonpathogenic ancestors become pathogenic by the integration of DNA segments containing clusters of virulence genes from pathogenic bacteria (Silva *et al*., 2012). These segments or regions of bacterial genomes containing clusters of virulence genes are called Pathogenicity islands (Hansen-Wester & Hensel, 2002). The function, size and structure of *Salmonella* Pathogenicity islands and their location in *Salmonella* are distinctly diverse. The majority of *Salmonella* Pathogenicity islands have turned out to be part of *S. enterica* core set of genes and they code for species-specific traits (Silva *et al*., 2012). The effector proteins of type III secretion system and their effect on host cell are shown in Table 2.2 and Table 2.3. Not less than five Pathogenicity islands (SPI) in *Salmonella* Typhimurium that give precise virulence characters have been reported (Marcus *et al*., 2000). Pathogenicity

island 1 (SPI-1) is important for invasion of nonphagocytic cells (Giacomodonato *et al*., 2007).

Table 2.2: Effector proteins of *Salmonella* Pathogenicity Island 1 with related type III secretion system.

Effector proteins	**Outcome on host cell**
SopA	Immune cell enrollment, fluid discharge
SopB	Cytoskeleton rearrangement, neutrophil recruitment, fluid secretion
SopC	Neutrophil enrollment, fluid discharge
SipA	Reorganization of cytoskeleton/neutrophil staffing
SipB	Actin nucleation / change location of other effectors
SipC	Change location of other effectors
SopD	Neutrophil staffing, fluid emission
SopE	reorganization of cytoskeleton
SptP	reorganization of cytoskeleton

Adopted from Kaur and Jain, (2012).

Table 2.3: The effector proteins of *Salmonella* Pathogenicity Island 2 related type III secretion system.

Effector proteins	Changes on host cell
SseJ	Dynamics in *Salmonella*-containing vacuole membrane or acyl transferase
SseF	Asist construction of filament by *Salmonella*
PipB	Target to *Salmonella* induced filaments
SopD2	Late endosomes or targeting to *Salmonella* induced filaments
SspH2	Cytoskeleton reorganization
SseG	Asist construction of filament by *Salmonella*
SifA	Keep reliability of *Salmonella*-containing vacuole membrane
SifB	Target *Salmonella* induced filaments
SseI	Reorganization of cytoskeleton
SrfT	Programmed cell death
SpiC	Interrupt vesicular transportation

Adopted from Kaur and Jain, (2012).

2.3.2.2 Salmonella Genomic Island 1 (SGI-1)

Salmonella genomic island 1 (SGI-1) is a chromosomal islet located in many different serotypes of *Salmonella* (Levings *et al*., 2005). SGI-1 with 43 kb has forty four open reading frames (ORFs) with several having no similarity to already identified genes order (Silva *et al*., 2012). The first part of chromosomal islet express similarity to

genes related to plasmid. The SGI-1 contains resistance to antibiotics genes (Levings *et al*., 2005; Silva *et al*., 2012), and it is known to confer resistance to beta-lactam antibiotics, including others such as streptomycin, spectinomycin, sulfonamides, chloramphenicol, florfenicol and tetracycline (Silva *et al*., 2012).

2.3.2.3 Salmonella virulence encoded by plasmid

Plasmid is a single circular mobile genetic element that transfers genes laterally from one bacteria cell to another (Heuer *et al*., 2008). Like in other bacteria, plasmids in *Salmonellae* bear genes that enhance *Salmonellae* response to unfavorable environmental conditions. Plasmids can easily acquire genes and can lose it easily as well (Silva *et al*., 2012). *Salmonellae* bear plasmids that carry determinants or genes for virulence and resistance to anti-infective agents (Silva *et al*., 2012).

2.4. CLASSIFICATION AND TAXONOMY OF THE GENUS *SALMONELLAE.*

*Salmonella*e belong to the family *Enterobacteriaceae* and the genus *Salmonellae* (Velge *et al*., 2012). In the modern classification of *Salmonella*, a phylogenetic tree is attained through sequence analysis of the differences in 16S rRNA among different species of *Salmonella* (Pui *et al.,* 2011). Based on this rRNA sequences analysis, *Salmonella* serotypes are grouped into two species; *enterica* and *bongori* (Pui *et al.,* 2011). The *Salmonella enterica* is divided into 6 subspecies that are assigned with taxonomic names and occasionally shortened by Roman numeral which are placed in brackets. Designating *Salmonella* serotypes by formula are done using the Roman numeral (CDC 2011). The six (6) subspecies of *Salmonella enterica* with their corresponding Roman numerals designations in brackets are; *Salmonella enterica*

subspecies *enterica* (I), *Salmonella enterica* subspecies *salamae* (II), *Salmonella enterica* subspecies *arizonae* (IIIa), *Salmonella enterica* subspecies *diarizonae* (IIIb), *Salmonella enterica* subspecies *houtenae* (IV), *Salmonella enterica* subspecies *indica* (VI) **(**CDC**,** 2011**)**. Strains of *Salmonella* that fit into *Salmonella enterica* subspecies *enterica* and cause disease in human beings represented not less than 99% (Pui *et al.,* 2011). *Salmonella bongori* is a different species from *Salmonella enterica*, for convenience and simplicity, these species are occasionally called "subspecies V" (CDC, 2011). The *Salmonella species* and subspecies and their usual habitat are shown in

Table 2.4.

Table 2.4: *Salmonella* species, subspecies, serotypes and their typical habitats based on Kauffmann-White scheme.

species and subspecies of *Salmonella*	**Serotypes numbers**	**typical habitat**
S. enterica subspecies *Enterica* (I)	1454	Homeotherms
S. enterica subspecies *Salamae* (II)	489	Poikilotherms and environment
S. enterica subspecies *Arizonae* (IIIa)	94	Poikilotherms and environment
S. enterica subspecies *Diarizonae* (IIIb)	324	Poikilotherms and environment
S. enterica subspecies *Hautenae* (IV)	70	Poikilotherms and environment
S. enterica subspecies *Indica* (VI)	12	Poikilotherms and environment
S. bongori (V)	20	Poikilotherms and environment
Total	**2,463**	

Adopted from Brenner *et al*., (2000).

Sero-typing which is dependent on immuno-reactivity of *Salmonella* surface structures, the "O" (Somatic) and "H" (flagella) antigens distinguishes *Salmonella species* further than the level of subspecies (CDC, 2011). The serovars designation is based on Kauffmann-White Scheme conventions (CDC, 2011). Kauffmann-White scheme categorizes *Salmonella* based on 3 main antigenic determinants made of somatic O antigens, flagella H antigens, and capsular K antigens virulence (Vi) (Pui *et al.,* 2011). Serotypes of *Salmonella* subspecies I are given names such as Typhimurium,

Enteritidis, and Typhi. *Salmonella* Enteritidis for instance will be called *Salmonella enterica* subsp. *enterica* serovar Enteritidis. Typical antigenic formula format that is designated to all *Salmonella* serotypes is as follows: Subspecies [space] O antigens [colon] Phase 1 H antigen [colon] Phase 2 H antigen (if present). Note that, a negative sign (-) is placed if not present. For instance, the antigenic formula of *Salmonella enterica* subspecies *enterica* serovar Typhimurium is: I 4,5,12:i:1,2. Some antigenic formulas of *Salmonella enterica* serotypes are shown in Table 2.5. Isolates of *Salmonella* serotypes are not less than 2,500. There is frequent identification of newer serovars (CDC, 2011). The serovar name is capitalized because it is not a species name. Since the routine application of the naming formula is cumbersome, *Salmonellae* are simply named by their Genus name and serotype name. For instance, *Salmonella enterica* subspecies enterica serovar Typhimurium is simply named *Salmonella*

Typhimurium. The Typhimurium is capitalized and not italicized.

Table 2.5: Antigenic formula for a number of *Salmonella enterica* serotypes based on Kaufmann-White scheme.

Serotype	O-Antigen(s)	H1-antigen(s)	H2-antigen(s)
S. Gallinarum	1, 9, 12	-	-
S. Dublin	1, 9, 12 [Vi]	g, p	-
S. Enteritidis	1, 9, 12	[f], g, m, [p]	[1, 7]
S. Virchow	6, 7	R	1, 2
S. Typhimurium	1, 4, 5, 12	I	1, 2
S. Infantis	6, 7, 14	R	1, 5

Adopted from Imen *et al*., (2012).

2.5 PATHOGENICITY OF *SALMONELLA.*

The processes involved in *Salmonella* pathogenicity include pathogenesis, transmission and colonization of the host (Velge *et al*., 2012). These processes are illustrated mostly in rodents whilst illustration in humans and farm animals still remain inadequate (Velge *et al*., 2012). The variety of factors that *Salmonella* pathogenesis depends on include host immune standing, dosage of inoculated organism and genetic make-up of both disease causing bacteria and host (Beyene, 2008; Velge *et al*., 2012). Although, the dosage of the causative organism needed to cause disease is not known, it is expected to be about 10^6 *Salmonella* bacteria (Cianflone, 2008). In instances such as patients with gastric hypoacidity, ingesting smaller amount of bacteria between 10^2 and 10^3 bacteria can result in illness (Cianflone, 2008). Hypoacidity is often seen in infants, patients with pernicious anaemia, or antacid usage (Cianflone, 2008). The amount of *Salmonella* ingested is inversely proportional to incubation time (Cianflone, 2008). The larger the dosage

ingested the more severe disease manifestations (Cianflone, 2008). Additional factors appeared to increase host vulnerability to *Salmonella* diseases. These include old age and diversity of immune-suppressive situations, for instance the human immunodeficiency syndrome, rheumatological conditions, malignancy, diabetes mellitus, reticuloendothelial obstruction, corticosteroids usage and other drugs (Cianflone, 2008). Iron overload and chronic granulomatous disease are linked to elevated threat of those diseases (Cianflone, 2008).

Salmonella infections are usually initiated by taking in water or food contaminated with faeces of an infected person or animal (Muller *et al*., 2012). The common foods implicated in *Salmonella* disease transmission include uncooked or partially cooked poultry foods, uncooked milk products and partially cooked meat (Muller *et al*., 2012).

The *Salmonella* organism is taken into the stomach following ingestion, and then gets to the intestine usually distal ileum and proximal colon (Muller *et al*., 2012). In the intestine, *Salmonella* organisms attach to the intestinal wall and then translocate into epithelial cells of the intestine. The organism then enters enterocytes and Microfolds cells of Peyer's patches at the apical side. The organism migrates to the baso-lateral side and exocytosis takes place in the lamina propria interstitial space (Velge *et al*., 2012).

S. Typhimurium, for instance, gets in contact with epithelial cells of the intestine and tranfers effector proteins into cytoplasm of the host cell (Santos *et al*., 2003). In the epithelial cell cytosol, the bacteria effector proteins alter host cell signaling pathways, leading to changes in host gene expression and cytoskeleton. Subsequently, the bacteria enter the host cell (Santos *et al*., 2003).

Salmonella bacteria sense its surroundings within the host small intestine lumen and enables type III secretion system 1 genes to be expressed (Velge *et al*., 2012). Consequently, the secretory tool is organised at the membrane of the bacteria. The *Salmonella* pathogen then binds to the host cell surface (Velge *et al*., 2012). This bacteria-cell contact activates the translocon and through its affinity for cholesterol is introduced into host cell membrane which enhances translocation or secretion of bacteria effectors into the host cell (Velge *et al*., 2012). The bacteria are capable to resist host cell killing effects. It then multiplies to cause disease.

2.5 IMMUNITY.

The humoral and cellular immune systems play an important task in the defense mechanism of the host's gut against *Salmonella* infection (Sarker and Gyr 1992). The gastric juice has a low pH of less than 3.0 and this contributes to its bactericidal properties (de Jong *et al*., 2012). Gastric acidity reduces the number of *Salmonellae* organism in the stomach so that fewer or no organism enters the intestine. This prevents *Salmonellae* colonization of the stomach and the small intestine through the lysosyme produced by the epithelium of the intestinal tract (de Jong *et al*., 2012). Lysozyme is known to lyse invading bacteria. Initially, the invasion of solitary isolated lymphoid tissues and Peyers Patches by *Salmonella* induces immense inflammatory response that draws in neutrophils, inflammatory monocytes, neutrophils, dendritic cells and macrophages. In non-Typhoidal *Salmonella* infections, neutrophils are believed in preventing the spread of bacteria from the intestine to tissues of other organs. Neutrophils also detain and reduce replication of bacteria instantly after bacteria entry (Albiger *et al*., 2007). It is for these reasons, that there is increased risk

of bacteremia and extracellular growth of bacteria in patients with neutrophils numbers reduced or depleted (Albiger *et al*., 2007).

The host develops robust protective immunity against *Salmonella* bacteria despite its intracellular nature (Griffin and McSorley, 2011). This involves the activities of both B and T cells. In the region of 3 to 6 hours of *Salmonella* infection, CD4 T- cell activation specific to *Salmonella* takes place in the Payers Patches. The role of CD4 T cells is to clear primary *Salmonella* infection and for acquired resistance to secondary infection (Griffin and McSorley, 2011). B cells on the other hand are required for protection against secondary infection and are not necessary for resolving primary *Salmonella* infection (Griffin and McSorley, 2011).

2.7 EPIDEMIOLOGY OF THE GENUS *SALMONELLA.*

In many countries, there is distinct increased in the prevalence of *Salmonella* disease in humans over the years (Cheng-Hsun *et al*., 2004). In the United States of America, every year, non-Typhoidal *Salmonella* species affect about 2 million to 3 million people and deaths ranges between 500 and 2000 (Altekruse *et al*., 1997; Cheng-Hsun *et al*., 2004).

In Europe, the prevalence of Non-Typhoidal *Salmonella* diseases varied from 4 per 100,000 inhabitants in Norway to 2,741 per 100,000 inhabitants in Bulgaria (de Jong and Ekdahl, 2006). Northern European countries recorded the lowest burden of Salmonellosis except Poland that recorded high incidence of Salmonellosis. The higher burden of Salmonellosis was located in southern area of Europe.The higher

burden of Salmonellosis was recorded in the eastern European countries than that recorded in the Western Europe (de Jong and Ekdahl, 2006). *Salmonella* Enteritidis was the most common serovars and accounted for 67% of cases. This is followed by *Salmonella* Typhimurium with 9% of cases. There was variation in cases of S. Enteritidis from 25% in Iceland up to 98% in Latvia (de Jong and Ekdahl, 2006). *Salmonella enterica* subspecies *enterica* serovar Enteritidis originated from poultry products and raw shell eggs products (de Jong and Ekdahl, 2006).

In the United Kingdom, outbreak of *Salmonella* Enteritidis phage type (PT) 4 occurred in 1993. Several strategies that were initiated to manage the outbreak involved education on food safety, vaccinating farm animals against Salmonellosis and enactment of laws (O'Brien, 2013). The measures introduced saw a significant reduction of the incidence of S. Enteritidis salmonellosis (O'Brien, 2013).

A study conducted by Cajetan *et al*., (2013) in children less than 5 years in Federal Capital Territory Abuja, Nigeria showed that out of 400 children who participated, 9 were positive for *Salmonellae* isolates giving a prevalence of 2.3%. The 9 S*almonella* isolates identified were typed into three serovars: five isolates were *Salmonella* Zanzibar, three were *Salmonella* Brancaster, and one was *Salmonella* Enteritidis. All the isolates were Non-Typhoidal *Salmonellae*. Symptoms reported by subjects were acute gastroenteritis, which included non bloody diarrhoea (97%), abdominal pains (7.2%), nausea (2.0%) and vomiting (1.7%). *Salmonella* isolates which were recovered from children who were on antimicrobial therapy was 3.1%.

In another study in Nigeria, Kano State (Abdullahi, 2010) 41 *Salmonella* isolates were isolated from stool samples of 300 children representing 13.7% prevalence.

Salmonella Typhimurium predominated among the isolates with 7.7% (n=23). That was followed by *Salmonella* Typhi with 4.0% (n=12) and *Salmonella* Paratyphi (2.0%, n=6). Out of the 41 Salmonella isolates, 24 (8.0%) were recovered from males and 17 (5.7%) were from females (Abdullahi, 2010).

Akinnibosun and Nwafor (2015) also isolated 4 *Salmonellae* from 50 children at University of Benin Teaching Hospital, Nigeria. The *Salmonellae* isolated were all sensitive to Chloramphenicol, Amoxicillin and Nalidixic acid.

Earlier than 1997, the prevalence of *Salmonella* Typhimurium was 75% among NonTyphoidal *Salmonella* bloodstream infections in Kenya whilst infections due to *Salmonella* Enteritidis amounted to 4.8% (Kariuki *et al*., (2006a). However, *Salmonella*

Enteritidis bloodstream infections prevalence increased gradually to 40%. Kariuki *et al*.,

(2006a) attributed the higher rate of *Salmonella* Enteritidis infections to increased dependence on chicken eggs for protein in Kenya and hence increased rearing of chicken at home. The study by Kariuki *et al*., (2006a) had 193 NTS isolated from 2246 children admitted with fever. The *Salmonellae* isolated from their study identified *Salmonella* Typhimurium as the most frequently isolated serovar representing 54% (n=106). This was followed by *Salmonella* Enteritidis with 33.2% (n=64) and other NTS serotypes amounted to 23 (11.9%).

In another study by Kariuki *et al*. (2006b), 332 children who had NTS diarrhoea or bacteraemia and reported to hospital in Kenya were recruited to study characterization of community acquired Non-Typhoidal *Salmonellae*. The number of females (184)

was more in that study. *Salmonella* Typhimurium isolated was 59% (n=196) and *Salmonella* Enteritidis was 28.3% (n=94) with both being the most isolated NonTyphoidal *Salmonellae* obtained. The other 42 were serotyped as *S*. Braenderup, *S*.

StPaul, *S*. Indiana, *S*. Haifa, *S*. Choleraesuis and *S*. Dublin (Kariuki *et al*., 2006b).

Salmonella Typhimurium and *Salmonella* Enteritidis were implicated in invasive Non-Typhoidal *Salmonella* disease in children which on average accounted for 87% of Non-Typhoidal *Salmonella* isolates in Kenya (Kariuki and Onsare, 2015). Kariuki and Onsare (2015) analyzed 192 Non-Typhoidal *Salmonella* isolates that were obtained from blood and stool samples of children who were on admissions in Kenya during 2000–2013. The isolates comprised of 114 *Salmonella* Typhimurium and 78 *Salmonella* Enteritidis.

Kwambana-Adams *et al*., (2015) had 203 *Salmonella* isolates obtained from 190 patients from 2005 to 2015 in Gambia. Out of the 203 isolates, 52% (n=106) were obtained from blood and 39% (n=79) from faeces. *Salmonellae* were also obtained from cerebrospinal fluid, aspirates, wounds, urine and abscesses. *Salmonellae* prevalence in blood cultures was reported to be 0.8% (106/13 905). That in stool among children under the age of 5 years was also reported to be 2% (30/1938). In their study (Kwambana-Adams *et al*., 2015), the Non-Typhoidal *Salmonella* isolates predominated with 86% (131/152) and *Salmonella* Typhi accounts for 14% (21/152) of the isolates. Out of 102 typed NTS isolates, *Salmonella* Typhimurium formed 40% (41), *Salmonella* Enteritidis accounted for 10% (10/102) and *Salmonella* Arizonae made up 3% (3/102).

Beyene (2008) isolated 65 *Salmonellae* from 1225 children in Addis Ababa and Jimma, Ethiopia from January 2006 to June 2008. A total of 48 *Salmonella* strains from stock cultures that were collected from January, 2004 to December, 2005 were added to Beyene (2008) study. *Salmonella* isolates that were recovered from stool were 77 and that from blood was 36. *Salmonella* Concord dominated a total of 12 different

Salmonella serotypes that the 113 *Salmonella* isolates were grouped.

Investigation into childhood acute diarrhoea in Ouagadougou, Burkina Faso (Bonkoungou *et al*., 2013) had 24 (9%) *Salmonellae* isolated from 283 cases that were studied and one isolate of *Salmonella* was isolated from 60 control cases. S. Typhimurium accounted for 24% of the isolates. The different serotypes that were identified amounted to 15. *S.* Typhimurium represented 24% of the total *Salmonella* isolates. That was followed by *S.* Cubana with 16% and 12 % for *S.* Muenster. The other serotypes including *S*. Typhi represented 4% each of the total *Salmonellae* that were isolated (Bonkoungou *et al*., 2013).

A study in Gourcy and Boromo, Burkina Faso had 6% prevalence of *Salmonellae* from 400 children's stool samples that were studied from July 2009 to June 2010 (Dembélé *et al*., 2014). The isolates were serotyped into 14 serovars with *S*. Typhimurium dominating with 37.6% (n = 9). That was followed by *S*. Poona (n = 2; 8.1%) and *S*. Virchow (n = 2; 8.1%).

Boni-Cissé *et al*., (2012) studied 62 *Salmonella* isolates that they obtained within the period of 2005 to 2009 in Abidjan, Cote d'Ivoire. Non-Typhoidal *Salmonellae* were the most prevalent isolates representing 76.1% and *Salmonella* Typhi accounted for 23.9%. *Salmonella* Typhimurium was 37% and *Salmonella* Enteritidis formed 16% of the isolates (Boni-Cissé *et al*., 2012).

A study by Akoua-Koffi *et al*., (2015) isolated 66 bacteria pathogens from 293 blood cultures in Bouaké, central Côte d'Ivoire within the period of June 2012 to September 2014. Gram negative bacteria accounted for 93.9% of the isolates. *Klebsiella pneumonia* was found to be the most occurring pathogen representing 8.5% (n=25), *Salmonella* Enterica accounted for 5.8% (n=17) of the isolates followed by *Enterobacter species* with 3.8% (n=11) and *Staphylococcus aureus* 1.4% (n=4).

2.8 *SALMONELLA* INFECTION IN GHANA.

Salmonellae was the second common isolate that accounted for 21.6% (n=24) of the one hundred and eleven (111) pathogens that were recovered from four hundred and seventy two (472) children's blood cultures at the Korle-Bu Teaching Hospital Pediatrics Department, Ghana, from December 1993 to March 1994 (Wilkens *et al*., 1997). The majority of the *Salmonellae* isolated were Non-Typhoidal *Salmonella*. S. Enteritidis (59%; n=14) was identified as major NTS isolates (Wilkens *et al*., 1997).

A study by Saba *et al*., (2013) reported prevalence of *Salmonellae* to be 3.9% (n=4) from ninety one (91) stool samples in 2010 at Tamale Teaching Hospital, Ghana. The serotypes identified in their study were *S.* Urbana*, S.* Ouakam, *S.* Stanleyville, and *S.*

Senftenberg (Saba *et al*., 2013).

Feglo *et al*., (2004) isolated six (6) *Salmonellae* from 258 food handlers stool samples in Kumasi, Ghana. That gave prevalence of *Salmonellae* in food handlers to be 2.3%. Out of the six (6) *Salmonella* isolates, three (3) were Non-Typhoidal *Salmonella* isolates and the other three (3) were *Salmonella* Typhi (Feglo *et al*., 2004).

A total of one hundred and twenty eight (128) Non-Typhoidal *Salmonella* were recovered from one thousand and thirty two (1,032) blood cultures from children who were admitted to Agogo Presbyterian Hospital in Ashanti Region, Ghana between September 2007 and July 2009 (Schwarz *et al*., 2010). Nielsen *et al*., (2012) recovered 129 NTS from 1,196 blood cultures of hospitalized children at Agogo Presbyterian Hospital. The NTS isolated represented 53.3% of the pathogens isolated from that study
(Nielsen *et al*., 2012).

In 2001 and 2002 Groß *et al*., (2011) in their comparative studies isolated 100 *Salmonellae* from the blood cultures of 409 patients in rural hospitals in Ghana. *S*. Typhi represented 40.7%, Non-Typhoidal *Salmonellae* 27.6% and S. Paratyphi were 0,7% of the total bacteria pathogens isolated in that study (Groß *et al*., 2011). Again, they isolated 24 *Salmonellae* from 258 patients in 2009. *S*. Typhi accounted for 31.3% and Non-Typhoidal *Salmonellae* 18.8% of the entire bacteria pathogens isolated (Groß *et al*., 2011).

A total of 1,456 hospitalized children under 15 years of age blood cultures were studied by Marks *et al*., (2010) at Agogo Presbyterian Hospital, Ghana from September, 2007 to November, 2008. The prevalence of *S*. Typhi isolated among 298 blood positive cultures in that study amounted to 12.4% (n=37). The other pathogens that were isolated include Non-typhoidal *Salmonellae*, *Staphylococcus aureus* and *Streptococcus pneumonia (*Marks *et al*., 2010).

A retrospective study into *Salmonella* bacteraemia from January 2010 to December 2013 at Korle-Bu Teaching Hospital, Ghana, reported *Salmonella* bacteraemia to be 6.5% (n=181/2768) (Larbi *et al*., 2014). Non-Typhoidal *Salmonellae* was the isolate that occurred most frequently with 63.5% (n = 115/181) and Typhoidal *Salmonella* accounted for 36.5% (n = 66/181) (Larbi *et al*., 2014).

Non-Typhoidal *Salmonellae* (n=29; 57%) and *Staphylococcus aureus* (n=15; 29%) were the most common organisms that caused sepsis in 251 children who had symptoms suggestive of severe malaria at Komfo Anokye Teaching Hospital Children's ward (Evans *et al*., 2004).

Awua-Boateng (2007) study in 2005 at Komfo Anokye Teaching Hospital, Kumasi, Ghana, had the prevalence of *Salmonellae* among positive blood cultures to be 9.5% (n=372/3,908) and 3.1% (n=372/11,809) out of the total blood cultures. Infections in children up to 12 years of age formed the most (79.6%; n=296) and infection in males (53.7%; n=159) dominated in that study (Awua-Boateng, 2007). Out of the 296 *Salmonellae* that were isolated, 87.2% were identified to be Non-Typhoidal

Salmonellae and *S*. Typhi represented 12.8 % (Awua-Boateng, 2007).

Agona Swedru Municipal hospital lacked the facility to perform laboratory investigations that involves isolation of bacteria and antibiotics sensitivity testing. Due to that, there were no data on *Salmonellae* found at the study area. Patients suspected to be infected with *Salmonella* at the study area were treated empirically with ciprofloxacin and ceftriaxone (Annual report, 2011).

2.9 MECHANISM OF ANTIBIOTIC RESISTANCE.

The mechanisms by which bacteria are either inherently resistant or acquire resistance to antibiotics may be categorized as damage or alteration in the antibiotics, propelling the antibiotics out from the bacteria using the efflux pumps, substitution or alteration in the site of action by the antimicrobial agent and reduction in cell membrane penetrability (Miko *et al*., 2005; Abatcha 2014). Generally, gene mutations of antibiotics site of action or horizontal or vertical acquisition of resistance genes through transposons, integrons, plasmids etc leads to antibiotic resistance in microorganism

(Miko *et al*., 2005; Abatcha 2014).

2.10 ANTIMICROBIAL RESISTANCE PATTERN OF *SALMONELLAE*

In 2001-2002, S. Typhi isolated by Groß *et al*. (2011) in Ghana were found to be 93.3% resistant to Ampicillin, 86.7% resistant to cotrimoxazole, 88.3% Chloramphenicol, 1.7% to Cefuroxime but were all sensitive to Gentamicin and ciprofloxacin. The non-typhoidal *Salmonellae* isolated showed 100% resistance to Ampicillin, 90% resistance to cotrimoxazole, 82.5% to chloramphenicol and 20% to

Cefuroxime (Groß *et al*., 2011).

In 2009, Groß *et al*. (2011) found all the S. Typhi that were isolated resistant to Chloramphenicol, Ampicillin and Cotrimoxazole, but were all susceptible to Cefuroxime, Gentamicin, and Ciprofloxacin. The Non-Typhoidal Salmonellae isolated were all found resistant to Ampicillin and 88.9% resistant to cotrimoxazole and 77.8% resistant to chloramphenicol. All the Salmonellae isolated by Groß *et al*., (2011) were found sensitive to Ciprofloxacin (Groß *et al*., 2011).

From Agogo in Ghana, 37 *S.* Typhi that were isolated showed 73% resistance to chloramphenicol, 71% resistance to trimethoprim/sulfamethoxazole, 70% to ampicillin/amoxicillin and 64% to tetracycline (Marks *et al*., 2010). The *S*. Typhi isolates that were resistant to Gentamicin was 46% and 24% to Amoxicillin/Clavulanic acid with none being resistant to ceftriaxone and ciprofloxacin (Marks *et al*., (2010).

High levels of multidrug resistance *Salmonellae* (44.2%; n=81/181) were reported between January 2010 and December 2013 at Korle-Bu Teaching Hospital in Ghana (Larbi *et al*., 2014), but showed low levels of resistance to ciprofloxacin (n = 1/127, 0.7%), Amikacin (n = 3/81, 3.7%) and Cefotaxime (n = 6/99, 6.1%) (Larbi *et al*., 2014).

More than half of the Non-Typhoidal *Salmonellae* (n=29; 57%) that were recovered from children who presented symptoms suggestive of severe malaria at the Komfo Anokye Teaching Hospital were found to be chloramphenicol-resistant (Evans *et al*.,

2004).

Non-Typhoidal *Salmonellae* recovered from blood cultures in Kumasi in 2005 showed higher sensitivity to amikacin (98.1%), ceftazidime (98.0%), ciprofloxacin (97.9%), ceftriaxone (95.8%), gentamicin (95.7%) and cefuroxime (82.4) (AwuaBoateng, 2007). *S.* Typhi isolated in that same study showed varied rate of sensitivity to cotrimoxazole (3.7%), ampicillin (8.3%), chloramphenicol (17.9%), amikacin (53.5%), Gentamicin (72.9%) and ceftriaxone (73.2%) (Awua-Boateng, 2007). The S. Typhi resistance rate to most drugs tested in that study was higher (Awua-Boateng, 2007).

Isolates that were obtained by Cajetan *et al*., (2013) from children in Federal Capital Territory Abuja, Nigeria were all susceptible to Nalidixic acid, Ciproflaxacin, and Ceftriaxone, whilst 44.4% were susceptible to Amoxycillin, Cephalexin and Cefuroxime and 22.2% were susceptible to Amoxycillin-clavulanic acid.

Antimicrobial patterns of 41 *Salmonellae* that were isolated from 300 children in Kano, Nigeria (Abdullahi, 2010) were varied. The *Salmonellae* susceptibility to Ciprofloxacin was 75.6% (n=31). That was followed by Co-trimoxazole with 61% (n=25), Chloramphenicol 43.9% (n=18), nalidixic acid 24.4% (n=10) and ampicillin 17.1% (n=7) (Abdullahi, 2010).

Akinnibosun and Nwafor (2015) isolated 4 *Salmonellae* from 50 children at University of Benin Teaching Hospital, Nigeria. All the *Salmonellae* isolated were identified resistant to Tetracycline and Gentamicin.

The sensitivity of Non-Typhoidal *Salmonella* that were obtained from cases in Kenya amounted to 23.4% (n=45) of all antimicrobials tested whilst the isolates from contacts were 31.3% (n=10) (Kariuki *et al*., 2006a). Few (5%) isolates showed resistance to only one antimicrobial agent tested. Two thirds of the Non-Typhoidal *Salmonella* that were obtained from cases showed resistance to at least one of the antibiotics tested. The isolates that were resistant to Tetracycline, Ampicillin and Cotrimoxazole where 66.7% whilst resistance to Gentamicin, Tetracycline and Ampicillin or Chloramphenicol was 15%. None of the isolates was found resistant to Ceftriaxone and Ciprofloxacin (Kariuki *et al*., 2006a).

Non-Typhoidal *Salmonellae* isolated from stool in Kenya recorded resistance to Ampicillin to be 49%, resistant to Co-trimoxazole as 48%, resistance to Chloramphenicol as 22% and Cefuroxime 28% (Kariuki *et al*., 2006b). The blood isolates in their study resistant to Ampicillin were found to be 54%, resistant to Cotrimoxazole to be 46%, resistant to Chloramphenicol to be 26% and Cefuroxime 30%. Out of 332 Non-Typhoidal *Salmonella* isolates, 147 (44.3%) were multidrug resistant. All the NTS were susceptible to Ceftriaxone and Ciprofloxacin (Kariuki *et al*., 2006b).

In Kenya, Kariuki and Onsare (2015) found that, 97% of *S*. Typhimurium exhibited resistance to at least one of the antimicrobials that was tested and 77% of *S*. Typhimurium was resistant to at least 3 antimicrobials. *Salmonella* Typhimurium resistant to ampicillin/chloramphenicol/Nalidixic acid/trimethoprim-sulfamethoxazole phenotype amounted to 20%. *Salmonella* Enteritidis isolates that showed resistance to not less than one of the antimicrobials that were tested were 92% and 30% were resistant to not less than 3 of the antimicrobials tested. Of all the

Salmonellae that were tested, 3% of *Salmonella* Typhimurium and 8% of the *Salmonella* Enteritidis were susceptible to all the antibiotics that they tested (Kariuki and Onsare, 2015).

In Gambia, out of 203 *Salmonellae* that were analyzed by Kwambana-Adams *et al*., (2015), 70% (142/203) were sensitive to the antibiotics tested. Multidrug resistance among isolates of *Salmonella* accounted for 4% (9/203) whilst1.4% (3/203) of which was *Salmonella* Enteritidis (Kwambana-Adams *et al*., 2015).

More than 80% of *Salmonella* isolates were reptorted in Ethiopia to be resistant to at least one of the antibiotics that were tested (Beyene, 2008). Greater proportions of the *Salmonella* isolates were resistant to amoxicillin (83.2%), ampicillin (82.3%), chloramphenicol (81.4%), trimethoprim-sulphamethoxazole (80.5%), ceftriaxone (78.8%), gentamicin (74.3%) and tetracycline (39.8%). High proportions of resistance were seen among serogroup C isolates where all the isolates (100%) were resistant to one or more drugs (Beyene, 2008).

A study in Ouagadougou, Burkina Faso (Bonkoungou *et al*., 2013) reported 28% of *Salmonellae* being resistant to Ampicillin and 20% being resistant to Chloramphenicol and Trimethoprim-Sulfamethoxazole. *Salmonellae* that showed resistance to Ciprofloxacin and Nalidixic acid stood at 4%.

The 24 *Salmonellae* that was recovered by Dembélé *et al*., (2014) in Burkina Faso were all found sensitive to ciprofloxacin, Nalidixic acid, Gentamicin, Cefotaxime,

Mecillinam and Imipenem. Resistance to Chloramphenicol, Ampicillin, Streptomycin, Trimethoprim and Sulfonamide were observed in 33% of the isolates. Multidrug resistance was seen in 33.3% of the isolates (Dembélé *et al*., 2014).

Boni-Cissé *et al*., (2012) identified 34.1% and 33.3% of Non-Typhoidal *Salmonellae* and *Salmonella* Typhi to be multidrug resistant, respectively. *Salmonella* isolates that were resistant to Amoxicillin were found to be 74.2% whilst 58.1% were resistant to amoxicillin-clavulanic acid, 14% were resistant to Ciprofloxacin and 8.1% were resistant to Cefotaxime. Boni-Cissé *et al*., (2012) found most of the isolates resistant to cotrimoxazole, chloramphenicol and tetracycline.

Wilkens *et al*. (1997) isolated 24 *Salmonellae* at Korle-Bu Teaching Hospital in Ghana and observed 57% of the *S.* Enteritidis isolates to be resistant to ampicillin, mezlocillin, trimethoprim-sulfamethoxazole and tetracycline. The other *Salmonellae* isolates showed high rate of susceptibility to the antibiotics that were tested. None were multidrug resistant *Salmonellae* (Wilkens *et al*., 1997). All the Non-Typhoidal *Salmonellae* that were isolated in 2010 at Tamale Teaching Hospital, Ghana were found to be sensitive to all the antibiotics tested in that study (Saba *et al*., 2013).

A study on systemic bacteraemia in children who presented with clinical pneumonia at Agogo Hospital in Ghana found that *Salmonellae* showed varied sensitivity of NTS isolates to Aminopenicillins (amoxicillin/ampicillin) (15%), Chloramphenicol (18%), Co-trimoxazole (23%), and Gentamicin 71%. The NTS that they isolated were all found susceptible to Ciprofloxacin and Ceftriaxone. Multidrug resistance (resistance

to chloramphenicol, amoxicillin and co-trimoxazole) was found in more than 70% (74/98) of the Non-Typhoidal *Salmonella* isolates (Schwarz *et al*., 2010).

A study covers eleven hospitals and nine regions in Ghana reported that all the NTS isolated were not resistant to Ciprofloxacin and Ceftriaxone. Multidrug resistance was seen in 77.0% (n=75) of the NTS isolates (Nielsen *et al*., 2012).

2.11 LABORATORY DIAGNOSIS OF *SALMONELLAE* INFECTIONS.

2.11.1. Diagnosis by culture.

In suspected *Salmonella* infection, culturing for the organism responsible for the infection is still the main efficient analytical method. The reference method to diagnose typhoid fever is to isolate *Salmonella* organism from the bone marrow (Wain and Hosoglu, 2008). This is reported by many authors as being more sensitive than blood culture. This is because, bone marrow harbor as much as ten times bacteria per volume than that found in blood (Wain and Hosoglu, 2008). Bacteria in the bone marrow can also be sheltered from the host immunoglobulins. On the other hand, culture using enough blood volume may elevate the sensitivity of blood culture to equal the culturing of bone marrow (Wain and Hosoglu, 2008). The media used to culture blood include thioglycollate broth, tryptone soy broth, brain heart infusion broth and others (Wain and Hosoglu, 2008). Stool culture may be positive when blood culture is negative and hence a vital addition for diagnosis of *Salmonella* (Wain and Hosoglu, 2008). Stool culture is also essential for checking carriers of *S*. Typhi after clear clinical cure (Wain and Hosoglu, 2008). *Salmonella* presence in samples may be in small amounts, injured or present with other micro-organisms. To diminish possibility of a wrong negative result, a large amount of faeces or food sample is to

be pre-enriched in a pre-enrichment non selective broth (Buffered Peptone Water) and then fortification in combination of two selective enrichment broth (Tetrathionate broth and Rappaport Vassiliadis soy peptone broth) (Wain and Hosoglu, 2008).

Afterwards, it is sub-cultured on to two selective but differential media such as Xylose Lysine Desoxycholate (XLD) agar and Brilliant Green agar (BGA) or on another selective-differential agar medium such as *Salmonella Shigella* (SS) Agar or Deoxycholate Citrate Agar (DCA) (WHO, 2003; Franco *et al*., 2011). Urine samples can be used to diagnose disseminated *Salmonella* infections (Itah and Uweh, 2005). Urine samples can be cultured for *Salmonellae* by inoculating onto McConkey agar, Salmonella-Shigella agar or Cystein-Lactose-Electrolyte Deficient (CLED) (Itah and Uweh, 2005). *Salmonella Shigella* (SS) agar has a stronger inhibitory power than Deoxycholate Citrate Agar (DCA) and both are selective and differential media used in culturing *Salmonellae* (Wain and Hosoglu, 2008). Specimens collected from other sites have been used in culturing for *Salmonella* but are not routinely used as diagnostic specimens. For instance, culture of the upper gastrointestinal tract using a duodenal string can be helpful but the procedure is poorly endured by young children (Wain and Hosoglu, 2008). Rose spots specimen can be used to grow *S*. Typhi, but the difficulty is that rose spots are not easily seen and present in only 4% of cases (Wain and Hosoglu, 2008). *S*. Typhi can be recovered from urine specimen but this is associated with urinary tract infection rather than typhoid fever (Wain and Hosoglu, 2008).

2.11.2 Diagnostic Characteristics of *Salmonella.*

Salmonella species are non-lactose fermenting bacteria and form colorless colonies on

Salmonella Shigella Agar (Imen *et al*., 2012). *Salmonella species* which produces H_2S show black-centered colonies. Colonies suspected to be *Salmonella* bacteria are identified using Gram staining technique (Imen *et al*., 2012). *Salmonella species* are short plump shaped Gram negative rods and non-spore-forming (Imen *et al*., 2012).

Biochemical tests are performed on both Gram-negative and oxidase-negative isolates to confirm the isolates (Imen *et al*., 2012). Colonies suspected to be *Salmonella* could be confirmed using set of Christensen Urea agar, lysine Iron Agar (LIA), Triple Sugar Iron agar, ONPG test (β-galactosidase), Indole test and Voges Proskauer test or the set of ornithine decarboxylase test, Christensen Urea agar, Triple Sugar Iron (TSI) agar, lysine Iron Agar (LIA) and mannitol (Imen *et al*., 2012). Alternatively, biochemical tests can be done by using API (Analytical Profile Index) 20E test kit (Imen *et al*., 2012; WHO, 2003). *Salmonella* species are urease negative, mannitol positive, betagalactosidase negative, Voges Proskauer negative, indole negative, ornithine decarboxylase positive and lysine decarboxylase positive (Imen *et al*., 2012).

2.11.3 Serotyping.

Serological analysis of *Salmonella* may be enough for investigations related to *Salmonella* serotypes which are uncommon and regularly the initial investigation in epidemiology. This serotyping differentiates bacteria on the basis of O (somatic) antigens that are found on the bacteria cell surface, the flagella (H1 and H2) antigen that correspond to phase 1 flagellum and phase 2 flagellum, respectively, and finally on the basis of capsular (Vi) antigens (Imen *et al*., 2012). The capsular antigen is found in a small number of serotypes of *Salmonella*. Some serovars that can be identified with capsular antigen are *S*. Paratyphi C*, S*. Typhi and *S*. *Dublin* (Imen *et al*., 2012).

Every *Salmonella* bacteria belong to a sero-group with unique surface 'O' antigen. In each specific group 'O' are diverse serotypes that are identified by the presence of both 'H' and 'O' antigens. The scheme for serotyping *Salmonellae* is illustrated in Figure 2.4

(Imen *et al*., 2012).

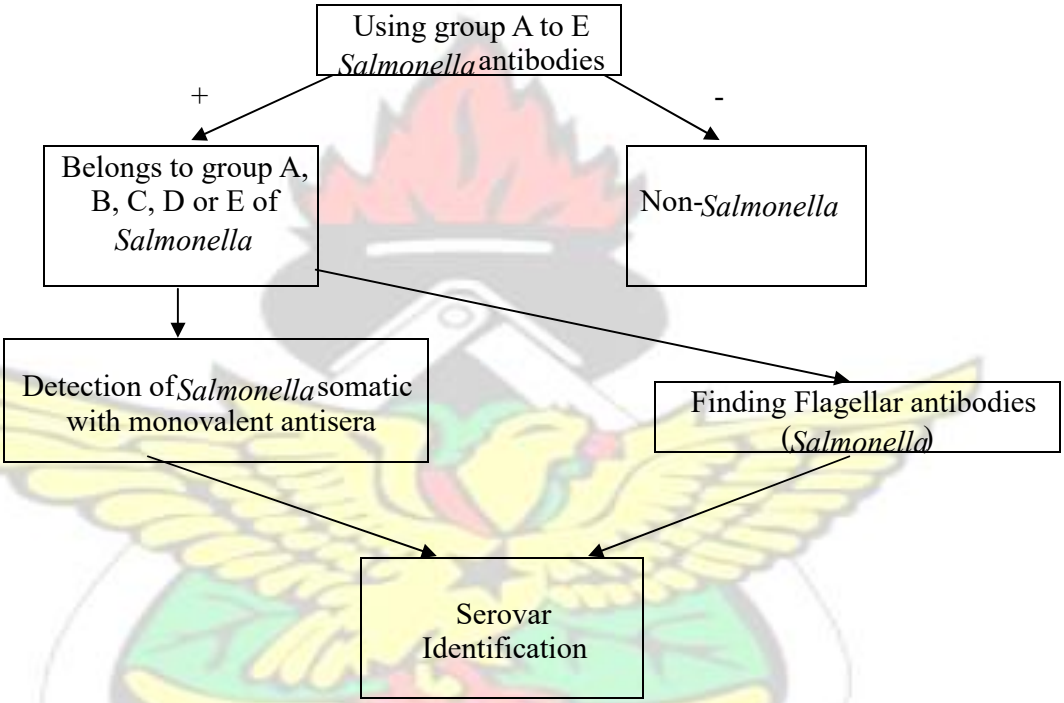

Figure 2.4: Scheme for serotyping *Salmonellae* (Imen *et al*., 2012).

2.11.4 Phage Typing.

Several serotypes of *Salmonella* differ in their vulnerability to be lysed by different bacteriophage (Imen *et al*., 2012). The reactivity of many *Salmonella* isolates to a panel or set of bacteriophages and the lytic pattern attained permits the assignment of that particular isolates to a specified phage type (Imen *et al*., 2012).

2.11.5 Molecular Methods.

The genetic make-up of microorganism provides information that is beneficial in identification and typing. Currently, the use of microorganism genetic make-up for typing are based on digestion with restriction endonuclease, amplification of nucleic acid or techniques of nucleotide sequencing (Imen *et al*., 2012; Silva *et al*., 2012).

2.11.5.1 Plasmid Profiling.

Most plasmids in *Salmonella* species harbor antimicrobial resistance and virulence properties (Moreno Switt *et al*., 2012; Imen *et al*., 2012). Inside the same serotype, plasmid content of the host makes known the differentiation based on the plasmid number and its molecular sizes (profile) obtained (Moreno Switt *et al*., 2012; Imen *et al*., 2012). The method of plasmid profile involves the separation of plasmids which are passed through agarose gel electrophoresis. Ethidium bromide is used to stain the agarose gel and viewed under UV light. Some of several limitations of plasmid analysis are that plasmids can rapidly be acquired or lost and various serotypes have single predominant plasmid that has become endemic (Moreno Switt *et al*., 2012; Imen *et al*.,
2012).

2.11.5.2. PFGE (Pulsed Field Gel Electrophoresis).

Considering molecular typing methods, PFGE was regard as the reference method (Imen *et al*., 2012). Restriction endonucleases are used to cut bacteria DNA and then separated with electrophoresis method that uses pulsed currents which changes

polarity at distinct intervals (Imen *et al*., 2012). DNA fragments that are larger than 12000 kb are separated by PFGE to give way to specific blueprints by this technique (Imen *et al*., 2012). The most commonly used enzymes to cut the bacteria DNA into fragments are NotI, Spel and Xbal (Imen *et al*., 2012). Multiple enzyme patterns comparisons can raise discriminatory ability of the method and make clear new subtypes (Imen *et al*., 2012). PFGE is very precise and results obtained are comparable both internally and externally and has been used to detect unsuspected cluster and track outbreaks of *Salmonella* occurring across nations. Improved Standardization of PFGE results among laboratories were enhanced by the use of the computerized gel-based data collection and analysis systems (Imen *et al*., 2012). This generates the capacity to quickly match up restricted DNA fragment patterns of isolates. Isolates around the world PFGE patterns are housed in large databanks and significantly enhance detection of *Salmonella* outbreaks. PulseNet is a molecular subtyping network and is involved in the development of standardized PFGE protocols and establishment of national database (Imen *et al*., 2012). To improve *Salmonella* species surveillance systems, common sources of contamination related strains can be identified by the use of combined typing techniques; for example PFGE and phenotypic methods (Imen *et al*., 2012). The following are some events that do not make PFGE successful: the genetically homogeneous serotypes such as those with certain distinct phage types and other serotypes are not discriminated by the use of numerous genotypic techniques and hence unable to make a distinction between outbreaks and non-outbreaks strains. Point mutation, deletion, integration, recombination or other genetic events can give dissimilarities in PFGE fragment patterns (Imen *et al*., 2012).

2.11.5.3 Ribotyping.

Some common serotypes and phage types of *Salmonella* are able to be subtyped using ribotype analysis (Imen *et al*., 2012). Ribotyping involves digestion of chromosomal DNA with endonuclease and separating it on agarose gels (Imen *et al*., 2012). The restriction digested DNA is then brought to a membrane and hybridized to a probe that identifies 16S and 23S rRNA. *Salmonella* has multiple copies of rRNA operon within its chromosome (Imen *et al*., 2012).

2.11.5.4 Multilocus Sequence Typing (MLST).

MLST scheme involves the recognition of about 400 to 500 bp sequences of internal nucleotide in several housekeeping genes (Larsen *et al*., 2012). A random integer number is assigned to unique alleles or sequences, and an allelic profile which comprised of distinct alleles combined at each locus that specifies the sequence type (ST) (Larsen *et al*., 2012). MLST begins with using primers specific for the MLST scheme loci in PCR amplification step (Larsen *et al*., 2012). Data is collected from all data bases by the Pub-MLST site and it is available on databases and softwares.

2.12 TREATMENT OF *SALMONELLA* INFECTIONS

Treatment of non-typhoidal *Salmonella* infection in the gastrointestinal tract (stool) is mainly aimed at replacement of fluid and electrolytes lost since it is a self-limiting disease (Allen and Poppe, 2002; Beyene, 2008). Susceptible people such as the young, elderly, and those compromised immunologically and individuals with invasive or severe infections may be given antimicrobials (Allen and Poppe, 2002; Beyene, 2008). Treatment of adult patient with local infection or life threatening *Salmonella*

bacteraemia should include trimethoprim sulfamethoxazole, third generation cephalosporin and fluoroquinolones whilst waiting for susceptibility patterns (Allen and Poppe, 2002; Beyene, 2008). Children with systemic salmonellosis are commonly treated with extended-spectrum cephalosporins such as Ceftriaxone (Allen and Poppe, 2002; Beyene, 2008). Fluoroquinolones usages are discouraged because of its adverse effect on collagen in children (Allen and Poppe, 2002; Beyene, 2008). Long standing carriage salmonellosis is effectively eradicated using amoxicillin and trimethoprimsulfamethoxazole (Allen and Poppe, 2002; Beyene, 2008).

2.13 PREVENTION AND CONTROL OF *SALMONELLA* INFECTIONS.

In salmonellosis, various animals such as pigs, cattle, and reptiles serve as reservoirs, and humans generally become infected by eating poorly cooked, contaminated food (Kariuki *et al*., 2006a). The main rational way to reduce the spread of *Salmonella* to humans through food chain is to routinely vaccinate farm animals using attenuated *Salmonellae* vaccine (Cheng-Hsun *et al*., 2004). This is hampered by the many serotypes of *Salmonella* (Cheng-Hsun *et al*., 2004). Prevention and control of salmonellosis also includes regular screening of food handlers, regular surveillance to identify sources of infection and managing infected persons with standard precautions (Cheng-Hsun *et al*., 2004). It is important to prevent contamination and spread of *Salmonella* in every step in food production and handling and maintain standards during food storage (Cheng-Hsun *et al*., 2004).

2.14 EPIDEMIOLOGY AND ANTIMICROBIAL PATTERN OF UROPATHOGENS.

A study by Nerurkar *et al*., (2012) reported 168 (60%) urinary tract infection from

280 patient's samples tested in western Mumbai, India, from January 2008 to December 2010. Females (57.7%; n=97) were found to be more infected than males (42.3%; n=71). Among the isolates, Gram negative bacilli accounted for 76.8% with *E.coli* (45.0%) been the most frequently isolated. That was followed by *Enterobacter* (17.8%), *Klebsiella species* (14.7%), *Citrobacter* (12.4%), *Proteus species* (7.0%) and

Pseudomonas (3.1%). Among the Gram-positive bacteria that were identified *Staphylococcus aureus* (92.3 %) was the commonest. The pathogens that were isolated showed varied sensitivity to the antibiotics tested. *E. coli* identified was found susceptible to Amikacin (82.2%), Ciprofloxacin (78.2%), Gentamicin (80.4%), Ampicillin (59%), Nitrofurantoin (57%), and Co-trimoxazole (23%) (Nerurkar *et al*., 2012).

Prevalence of bacteriuria was found to be 32.9% (n=9/280) among pregnant women at Vidarbha in India (Poonam and Bidwai, 2013). The bacteria pathogens isolated in that study were *Escherichia coli* 40 (43.5%), *Klebsiella species* 5 (5.4%), *Proteus vulgaris* 18 (19.6%), *Staphylococcus aureus* 22 (23.9%) and coagulase negative *Staphylococcus 7* (7.6%). *E. coli* were all sensitive to Amikacin and showed varied levels of resistance to ampicillin (87.5%), cotrimoxazole (75%), ceftazidime (62.5%), ciprofloxacin (62.5%), ceftriaxone (50%) and norfloxacin (50%). The levels of resistance were low to Augmentin (25%), Cefotaxime (37.5%), Gentamicin (25%) and Nitrofurantoin (12.5%) (Poonam and Bidwai, 2013).

A total of 109 uropathogens that were obtained from patients at Shaukat Khanum Cancer Hospital and Research Center, Lahore, Pakistan, were studied by Bashir *et al*., (2008) for their antibiotic susceptibility and frequencies. Among the bacteria

pathogens that were isolated *E. coli* represented 67.0% (n=73). That was followed by *Enterococci* with 8.3% (n=9), *Pseudomonas* with 7.4% (n=8), *Klebsiella* with 5.5% (n=6), *Enterobacter* 2.8% (n=3), *Proteus* 0.9% (n=1) and *Morganella* with 0.9% (n=1). *Escherichia coli* in that study recorded varied resistance to the following tested drugs, trimethoprim (86%), ampicillin (92%), cotrimoxazole (80%), gentamicin (49%), ciprofloxin (62%), nitrofuratoin (20%) and amikacin (4%). All the *E. coli* isolated were sensitive to Meropenem, Imipenem and Tazocin (Bashir *et al*., 2008).

In Imam Khomeini hospital Ahvaz, Iran, Amin *et al*., (2009) investigated 7056 patient urine samples for urinary tract infection and found 553 (8.7%) to be culturepositive. Females were found to be more infected with 376 (68%) than males (n=177; 32%). The Gram-negative bacilli that were isolated represented 94.4% (n=522).The isolates obtained in that study were *Escherichia coli* 326 (59%), *Klebsiella* 64 (11.6%), *Enterobacter* 54 (9.8%), *Proteus species* 16 (2.9%), *Citrobacter* 7 (1.3%), *Pseudomonas* 40 (7.2%), *Acinetobacter* 15 (2.7%), Coagulase positive *Staphylococci* 12 (2.2%), Coagulase negative *Staphylococci* 13 (2.3%) and α hemolytic *Streptococci* 6 (1.1%). The isolates recovered by Amin *et al*. (2009) showed broad variation in their sensitivity to the antimicrobials that were tested. The highest degree of susceptibility of the Gram negative isolates was recorded in Amikacin and ciprofloxacin. *E. coli* isolated were 90.5% susceptible to Gentamicin; resistance to ceftriaxone was 90.5%, 90.5% to amikacin, 89.6% to Cefotaxime, 85.3% to ciprofloxacin and 89.6% to tobramicin. The bacteria isolated by Amin *et al*., (2009) showed 92.4% resistance to ampicillin and 80.8% to cephalothin. In that study *Pseudomonas species* showed 77.5% sensitivity to Amikacin (Amin *et al*., 2009).

A study by Beyene and Tsegaye (2011) in Ethiopia showed the prevalence of bacteriuria to be 9.2% out of 228 UTI cases analysed at Jimma University Specialized Hospital from April to June 2010. Among the pathogens that were isolated, *E. coli* occurred frequently with 33.3% (n=7). That was followed by *Klebsiella pneumoniae* which accounted for 19% (n=4), *S. saprophyticus* was 14.3% (n=3), *Citrobacter species* represented 9.5% (n=2), and *Enterobacter cloacae* with 9.5% (n=2). Each of *P. mirabilis Providencia stuart* and *S. aureus* represented 4.8% (n=1) of the total pathogens that were isolated. All the *E. coli* isolated in that study were resistant to ampicillin and amoxicillin and showed low resistance to Trimethoprim–sulphamethoxazole (28.6%),, Tetracycline (28.6%) and ciprofloxacin (14.3%) and were all sensitive to Choramphenicol, Nalidixic acid and Ceftriaxone. *Klebsiella species* displayed a similar resistance pattern to that of *E.coli*. *Citrobacter species* were all resistant to Ampicillin, Amoxicillin and Trimethoprim–sulphamethoxazole (Beyene and Tsegaye, 2011).

Agbagwa *et al*. (2015) reported the prevalence of UTI to be 48% (n=72) out of 150 urine samples that were examined between July, 2014 and December, 2014 in Port Harcourt, Nigeria. The occurrence of UTI was higher in females (77.4%) than males (24.6%). Among the organisms that were isolated, *Klebsiella species* had the highest frequency of 30.9%, followed by *E. coli* (22.6%), *S. aureus* (22.6%) and *Proteus species* (9.5%). Others were *Pseudomonas species* (5.9%), *Streptococcus* species (4.8%) and *Candida albicans* (3.6%) (Agbagwa *et al*., 2015).

A prevalence of 9.9% urinary tract infection was reported in 453 pregnant women from January 2010 to December, 2012 in Kumasi, Ghana by Addo (2014). Among the

bacteria organisms that were isolated, *Staphylococcus aureus* occurred most frequently and represented 40% (n=18). That was followed by *Escherichia coli* with 33.3% (n=15), *Streptococcus species* (11.1%; n=5), *Staphylococcus saprophyticus* (9%; n=4), *Proteus* species (4%; n=2) and *Klebsiella species* (2.2%; n=1). The isolates that were identified were all sensitive to Nitrofurantoin but showed varied sensitivity to Gentamicin (93.3%; n=42), ciprofloxacin (68.9%; n=31), nalidixic acid (60.0%; n=27), and Cefuroxime (53.3%; n=24), Ceftriaxone 22/45(48.9%), Augmentin (46.7%; n=21), erythromycin (28.9%; n=13), and ampicillin (24.4%; n=11) (Addo, 2014). No *Salmonellae* were isolated in that study (Addo, 2014).

Afriyie *et al*., 2015), analyzed 705 urine samples of patients at Ghana Police Hospital, Accra, Ghana from December 2013 to March 2014 and obtained 15.9% prevalence of urinary tract infection. Urinary tract infection was found to be higher in females (75%; n=84). *E. coli* was the isolate that occurred frequently and accounted for 46% (n=52), followed by Coliform which represented 41.1% (n=46). Other pathogens isolated in that study were *Pseudomonas species*, *Salmonellae*, *Candida species* and *Klebsiella species* (Afriyie *et al*., 2015). *Pseudomonas species, Salmonella species,* and *Klebsiella spp.* were all found sensitive to ciprofloxacin. *E. coli* susceptibility to ciprofloxacin was found to be 61.5% (Afriyie *et al*., 2015).

A prospective study by Turpin *et al*. (2007) had the prevalence of significant bacteriuria in 220 pregnant women who attended ante-natal clinic at Komfo Anokye Teaching Hospital (KATH), Kumasi, Ghana, between February and April 2003 to be 7.3%. The dominant bacteria were *E.coli* (37%) (Turpin *et al*., 2007). This was

followed by *Staphylococcus aureus* (31%). The others were *Klebsiella species* (6.3%), *Providencia species* (6.25%), other coliforms (6.3%), *Staphylococcus saprophyticus* (6.3%) and *Enterococcus faecalis* (6.3%). All the *E.coli* isolated was found sensitive to Nitrofurantoin, Gentamicin, Cefuroxime and Nalidixic acid. Sensitivity of *E. coli* to ampicillin and cotrimoxazole was found to be low (33.3%). The *Staphylococcus aureus* were all found sensitive to Nitrofurantoin and Ampicillin. Antibiotic susceptibility of *Staphylococcus aureus* was 80% to Cefuroxime, and 60% to Gentamicin and

cotrimoxazole (Turpin *et al*., 2007).

Obirikorang *et al*., (2012), reported prevalence of bacteriuria to be 9.5% (n=19) out of the 200 urine samples of asymptomatic pregnant women studied at Kwame Nkrumah University of Science and Technology Hospital, Kumasi, Ghana from April 2009 to July 2009. The most prevalent bacteria isolated in that study was *Escherichia coli* and accounted for 36.8% (n=7); that was followed by *Klebsiella* species (26.3%), *Staphylococcus aureus* (21.1%) and other coliforms (15.8%). Most of the *E. coli* that was isolated were found to be sensitive to Nitrofurantoin and Gentamicin (Obirikorang *et al*., 2012).

Chapter 3

MATERIALS AND METHODS

3.1 STUDY SITE.

The study area was Agona Swedru Municipal Hospital, located in Agona Swedru of Agona West District in the Central Region of Ghana. The district is located in longitudes 0º35' and 0º55'W and between latitudes 5º30' and 5º50'N. The hospital mainly serves the people of Agona West Municipality as well as people from AjumakoEnyan-Essiam Districts, Agona East District, Effutu District, Gomoa District, Asikuma, Odoben, Brakwa District etc and thereby making the catchment area of the hospital wider. The hospital is about 24km North of Winneba, 75km from Accra and 105km from Cape Coast off Winneba junction. Agona Swedru Municipal Hospital is easily accessible from Akim Oda in the Eastern Region through Akroso and from Accra through Kasoa-Bawjiase road. Population and Housing Census of 2010 estimated the population of Agona West Municipality to be 115,358 with females slightly dominating (Ghana Statistical Service, 2010). The source of water in the district is chlorinated pipe water. Although Agona Swedru is an urban area, there are still some challenges with waste collection and disposal. Some sections of the town have heaps of refuse at the collection sites. Agona Swedru Municipal Hospital has a bed compliment of 162.

3.2 ETHICAL APPROVAL.

Ethical approval for this study was obtained from the Committee on Human Research, Publications and Ethics, School of Medical Science, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. Approval was also obtained from

Municipal Hospital, Agona Swedru, Ghana. The aim and benefit of the study was made clear to partakers using questionnaire and informed consent was obtained by signature or thumb print by the participants or caregiver.

3.3 STUDY SUBJECTS.

3.3.1. Inclusion Criteria.

Patients of all ages who attended Agona Swedru Municipal Hospital with diarrhoea or gastroenteritis who consented to participate in the study were included.

3.3.2. Exclusion Criteria.

Persons or patients who had no diarrhoea were excluded from this study. Others who refused consent were excluded.

3.4. SAMPLE SIZE.

Sample size (Beyene, 2008) was estimated as follow:

$$n = \frac{Z^2 \rho q}{d^2}$$

Where:

n = the preferred sample size.

z = the normal standard deviation 1.96 p = Prevalence in the target population in the literature of study site {45% (0.45)} (Previous studies prevalence of salmonellosis at the study area was not available, the

45% prevalence was arbitrarily

taken) q = 1.0-p = 1.0-0.45 = 0.55 d

= degree of accuracy desired at 0 .05

$n = \frac{(1.96)^2 (0.45) (0.55)}{(0.05)^2}$

n = 380.3

Approximately = 380 samples

3.5. SAMPLE COLLECTION AND TRANSPORT.

In this study, diarrhoeic stool was defined as the passage of watery and or loose stool (Obi *et al*, 2007). The watery or loose stool samples were collected from the patients at Agona Swedru Municipal Hospital. The samples were processed at the hospital's laboratory department. Total of 380 patient's samples were collected and analyzed.

3.6 BACTERIAL ISOLATION.

Isolation of bacteria was done as described by Mir *et al*. (2010) and Obi *et al.* (2007) with some modifications as indicated in 3.6.1 and 3.6.2.

3.6.1. Blood Culture.

3.6.1. (A): Collection of Blood (Venepuncture) for Culture.

A suitable vein in patient's arm was located. Using a spiral motion, the skin area over the vein starting at the phlebotomy site was swabbed thoroughly using tincture of iodine followed by 70% ethanol (or ethanol-ether).

A tourniquet was applied on the upper arm and a vein located. Using 10ml sterile hypodermic syringe and needle, 10ml of blood was withdrawn from the patient (2ml

to 3mls of blood was withdrawn from children). The needle on the syringe was removed and replaced with another needle of the same size. The needle was then inserted into the rubber liner of the thioglycollate broth culture bottles and 5ml of blood was dispensed into each of the two culture bottles with each containing 50ml of thioglycollate broth to give a ratio of 1:10 (2ml to 3ml of blood withdrawn from children was dispensed into culture botlles containing 40ml of thioglycollate broth). Again, the top of the culture bottle was disinfected with tincture of iodine and the protective tape replaced. The inoculated culture bottles were mixed thoroughly. The bottles were labelled and placed in an incubator.

3.6.1. (B) Incubation of inoculated Thioglycollate broth.

The inoculated culture medium was incubated at 37^{o}C aerobically and sub-cultured every 48 hours for one week. The bottles were examined daily for visible signs of bacterial growth, turbidity in the blood layer, haemolysis, gas bubbles and clots.

3.6.1. (C) Sub-culturing inoculated Thioglycollate broth after incubation.

The inoculated thioglycollate broth was mixed gently and the cup of the bottle passed over Bunsen flame. The cup of the broth was gently opened and a sterile microbiological loop was used to take a loop full of the broth and inoculated on to Blood Agar Medium (BA), *Salmonella Shigella* (SS) Agar Medium and on MacConkey

Agar. The BA, SS Agar and MacConkey Agar were incubated aerobically at 37^{o}C for 24 hours.

3.6.2 Stool Culture

3.6.2. (A) *Collection and Transport of Stool for Culture.*

The patient was given a clean, dry, disinfectant-free bedpan to pass a stool specimen. About a spoonful of the stool sample particularly portions with mucus, blood or pus was transferred to a clean, dry, leak-proof, screw cap container. The specimen was then submitted to the laboratory for examination.

3.6.2. (B) *Macroscopic examination of stool.*

The stool specimen was examined for its colour, consistency (whether formed, semiformed, unformed or fluid / watery and whether it contained blood, mucus, pus. The result was recorded. Watery stool specimens were cultured for *Salmonella*.

3.6.2. (C) *Culturing Stool Specimen.*

A loop-full of the faeces was inoculated on *Salmonella Shigella* (SS) Agar (Liofilchem, Italy) and a loop-full also inoculated on Deoxycholate Citrate Agar (DCA). The inoculated SS Agar and DCA were incubated aerobically at 37ºC for 24 hours and then examined for colonial growths suggestive of *Salmonella* (*Salmonella* colonies appeared colourless, slightly raised, have entire smooth edges of diameter 2-3mm with or without black centers on SS Agar and DCA). In addition, 1ml of each stool sample was inoculated into 10ml of Selenite F Broth (SFB) and incubated aerobically at 37ºC for 24 hours with the cup slightly loosen. The inoculated Selenite F Broth (SFB) after incubation was mixed gently and a loopful of its content sub-cultured on Salmonella Shigella (SS) Agar and DCA. The inoculated SS Agar and DCA were then incubated aerobically at 37ºC for 24 hours. After the incubation, the inoculated *Salmonella Shigella* (SS) Agar and DCA were examined for colonies of

Salmonella. Each suspected colony of *Salmonella* (colourless, slightly raised, entire smooth edges colonies of diameter 2-3mm with or without black centers on SS Agar and DCA) was cultured onto nutrient agar to obtain pure culture. The growths suspected to be Salmonella were then tested and identified using morphological and biochemical tests.

3.6.3 Urine Culture.

3.6.3. (A) Collection and Transport of Urine for Culture.

A dry, leak-proof, wide-necked, sterile container was labeled with the patient identification number and given to the patient to collect mid-stream urine (clean-catch specimen). The patient was instructed to allow some urine to flow during urination and then open the sample container to catch some of the flowing urine. The patient was instructed to immediately close the specimen container and to return the specimen collected to the laboratory. The labeled specimen was processed immediately in the laboratory.

Each urine sample was inoculated on CLED (Cystine Lactose Electrolytes Deficient) agar, The end of calibrated wire loop that holds 0.002ml (2µL) of urine after sterilization over the flame of a Bunsen burner was dipped in the urine to just below the surface and remove vertically. The volume picked by the loop was inoculated on CLED agar. After overnight aerobic incubation at 37ºC, the cultured plate was examined for pure growth ≥ 10^5 CFU/ml (considered significant growth). Urine cultures that produced significant growth with pathogen other than *Salmonella* were identified using morphological and standard biochemical test and then recorded.

3.7 BACTERIAL IDENTIFICATION.

3.7.1 Gram Staining Technique (Morphological Test).

The dried heat fixed smear of the suspected colony of *Salmonella* was stained with crystal violet, lugol's iodine, decolourized with acetone alcohol and counterstained with neutral red. The back of the stained slide was wiped clean, blotted dry with filter paper and examined microscopically under oil immersion objective for Gram negative (red or pink) bacilli suggestive of *Salmonella*.

3.7.2 Biochemical Tests and Sugar Fermentation Tests.

Growths with colonial characteristics suspected to be *Salmonella* were subcultured on Nutrient Agar (Liofilchem, Italy) to obtained pure cultures for the biochemical and sugar fermentation tests. The test performed on the isolates included Indole Test, Oxidase Test, Urease Test, Motility Test, Triple Sugar Iron Agar and Lysine Iron Agar (Lysine Decarboxylation test). Each suspected *Salmonella* isolate from pure culture was tested for oxidase production and when found to be negative was inoculated in to a set of biochemical test (media); urea broth, Simmons' citrate agar, peptone broth, urea broth, Triple Sugar Iron Agar (Biomark Laboratories, India) and Lysine Iron Agar (Liofilchem, Italy). All the media were incubated overnight at 37°C aerobically. After overnight incubation, biochemical test reactions of each were read, recorded and interpreted as shown in Table 2.5. All isolates which were oxidase negative, urease negative, indole negative were suggestive of *Salmonella*. Tripple Sugar Iron Agar were examined for red slant and yellow butt that indicated glucose fermentation but non lactose fermentation, with or without blackened medium (H_2S production) and cracks or no cracks in the medium (gas production) were suggestive of *Salmonella*. The Lysine Iron Agar were examined for purple butt and purple slant (Lysine

decarboxylation) with or without blackening (H_2S production) of the slant apex were suggestive of *Salmonella*. The isolates were stored frozen in Nutrient Agar slope with a drop of glycerol added. The isolates were confirmed as *Salmonella* by API (Analytical Profile

Index) 20E identification test.

The bacterial isolates from positive blood cultures or isolates from urine cultures which growth were significant but were not suggestive of *Salmonellae* were also identified by their colonial characteristics, morphological test (Gram reaction), sugar fermentation test and biochemical test. Pure culture of each isolate was prepared on Nutrient Agar and was used to inoculate a set of biochemical test. The tests performed on the isolates that were tested to be Gram negative bacilli included Indole Test, Oxidase Test, Urease Test, Motility Test, Triple Iron Agar and Lysine Iron Agar (Lysine Decarboxylation test). All inoculated biochemical test media were incubated overnight at 37^{o}C, aerobically. After incubation, biochemical reactions for each test were read and interpreted.

3.8 SEROTYPING OF THE ISOLATES.

Two separate drops (40 µl) of saline were placed on clean grease free microscope glass slide. Sterilized bacteriological loop was used to pick portions of the colonies of the test organism and emulsified in each drop saline to obtain a homogenous dense suspension. One drop antiserum (polyvalent *Salmonella* "O" and "H") was added to one suspension. The other suspension was used as negative control. The slide was gently rocked for 1 min and observed for agglutination.

3.9 ANTIMICROBIAL SUSCEPTIBILITY TESTING.

The *Salmonella* isolates and the other pathogens obtained were tested for their susceptibility to the following antimicrobials: Ceftriaxone (30µg), Cefuroxime (30 µg), Ampicillin (10µg), Meropenem (10µg), Amoxicillin (10µg), Chloramphenicol (30µg),

Trimethoprim/sulfamethoxazole (1.25/23.75µg), Gentamicin (10µg), Amikacin (30µg),

Tetracycline (30µg), Nalidixic acid (30µg), Ciprofloxacin (5µg), Nitrofurantoin, Amoxicillin-Clavulanic Acid (30µg). The tests were performed using the Kirby-Bauer disc diffusion method.

This test was done by emulsifying five colonies of test organism in a tube that contained 2ml of Mueller Hinton broth (Liofilchem, Italy). The suspension turbidity was adjusted to 0.5 MacFarland standards and was used to seed a Mueller Hinton agar. Using sterile forceps, the antibiotic discs were placed onto the seeded Mueller Hinton Agar. The antibiotic discs were placed 24mm apart. The plate was incubated overnight at 37ºC aerobically. After incubation, the inhibition zones were measured in millimeters (mm) using ruler and interpreted either as sensitive (S), intermediate (I) or resistant (R) based on the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2010).

Escherichia coli (NTCT 10418) was used as a control strain.

3.10 TESTING THE ISOLATES FOR EXTENDED SPECTRUM BETALACTAMASE (ESBL) PRODUCTION.

The isolates were screened and then confirmed for ESBL production as described by Drieux *et al*. (2008) and HPA (2012) with some modifications as indicated in 3.10.1 and 3.10.2.

3.10.1 Screening the Isolates for ESBL Production.

Mueller Hinton Agar (Liofilchem, Italy) was seeded with the suspension of the test organism with the turbidity adjusted to 0.5 MacFarland standards. Sterile forceps was used to place a disc of ceftazidime (30µg), Cefpodoxime (10µg) and cefotaxime (30µg) on the seeded Mueller Hinton Agar. The plate was incubated overnight at 37ºC and the inhibition zones measured in millimeters (mm) using ruler and compass.

Any isolate which showed zone of inhibition less than 30mm to Cefotaxime, less than 20mm to Cefpodoxime or less than 27mm to Ceftazidime was suspected to be ESBL producer (HPA, 2012). ESBL producing *E. coli* and non ESBL producing *E. coli* were used as positive and negative controls respectively.

3.10.2 Phenotypic Confirmation of the Isolates for ESBL Production.

Isolates which were positive for ESBL production in the screening procedure were selected for confirmation. This was done by first using double synergy test followed by the combination disc method.

3.10.2.1 Double Disk Synergy Test

Suspension of the test organism was adjusted to 0.5 McFarland standards and was used to seed Mueller Hinton Agar. A disc of cefpodoxime (10µg) and amoxicillin-clavulanic acid (20µg/10µg) were positioned 20mm apart on the surface of the seeded

Mueller Hinton Agar using sterile forceps. The plate was incubated overnight at 37°C. A clearcut projection of the edge of the inhibition zone of cefpodoxime towards amoxicillinclavulanic acid (20µg/10µg) disc that resulted in a characteristic keyhole shape was observed as positive for ESBL production.

3.10.2.2 Combination Disc Method

This was done to confirm those isolates whose characteristic keyhole shape extension of the inhibition zone was not clear. Muller Hinton Agar was seeded with 0.5 McFarland turbidity suspension of the test organism. A disc of cefpodoxime (10µg) and a combined disc of cefpodoxime and clavulanic acid (10µg+10µg) were placed on the seeded Muller Hinton Agar. After overnight incubation at 37°C, the inhibition zone around each antibiotic disc was measured in millimeters and recorded. Any isolates whose difference between the zone diameters of cefpodoxime disc alone and combined disc of Cefpodoxime and Clavulanic acid was equal or more than 5mm were taken to be phenotypic confirmatory of ESBL production.

3.11 PULSED-FIELD GEL ELECTROPHORESIS (PFGE) ANALYSIS OF THE *SALMONELLA* ISOLATES.

The pulsed-field gel electrophoresis (PFGE) was done following protocol described by PulseNet USA. The *Salmonella* test strain was suspended in suspension buffer and the turbidity adjusted to 0.70 McFarland standards. Heated 1% Seakem Gold Agarose was mixed with the bacteria suspension in equal proportions. The mixture was poured into BIORAD plug mould to cast plugs. The bacteria cells in the agarose plugs were lysed using Lysis buffer and then washed six times using deionized water. The agarose

plugs were cut into 1-2mm thick slice. The bacteria DNA in the 1-2mm agarose plugs were restricted using 1x restriction enzyme buffer. The agarose slice was attached to a tooth comb and placed within a BIORAD agarose gel casting unit. Heated 1% Seakem Gold agarose was poured into the BIORAD agarose gel casting unit to cast gel. The gel was placed in electrophoresis chamber of a BIORAD CHEF-DR electrophoresis system. The electrophoresis run parameters were programmed as follows: initial switch time, 2.2 seconds; final switch time, 63.8 seconds; voltage, 6 volts; included angle, 120º, runtime, 19 hours. The electrophoresis run was started. After the run, ethidium bromide staining solution was used to stain the gel. The DNA fingerprint patterns were captured using the BIORAD gel documentation system. *Salmonella* Braenderup (H9812) was

used as control strain.

3.12 DATA ANALYSIS.

Data obtained were entered into Excel 2013 spread sheet and analyzed using SPSS 20. The data were presented in summary tables as frequencies and percentages. Others were presented as charts. Chi-square test was used to compare categorical values and paired t-test was used to compare continuous values. Significant values were set at a p-value of less than 0.05.

Chapter 4

RESULTS

4.1 STUDY SUBJECTS.

Three hundred and eighty (380) patients presenting with diarrhoea who visited the out patients department, accident and emergency department of Agona Swedru Municipal Hospital, and willingly participated in this study were investigated for *Salmonella* infection. The study was performed from April, 2014 to September, 2015 at Agona Swedru Municipal Hospital Laboratory Department. There were 131 (34.5%) males and 249 (65.5%) females in the study giving male to female ratio of 1:1.9. The age of the study subjects ranged from five (5) months to 85 years with mean age of 27.4 (± SD

15.6) years. The modal age class of the study subjects was 25-29 years, representing 21.8% (83/380). The age, sex and district distribution of study subjects with infection are shown in Table 4.1.

Table 4.1 Study population demographic characteristics stratified by *Salmonellae* and other pathogens infecting diarrhoeal patients at Swedru Municipal Hospital.

Bacteria Isolates	***Salmonellae* Isolated**			**Other Pathogens Isolated**					Total (n=46; 100%)	P-value
	S. Enterica n=9/13 **(69.2%)**	*S.* Choleraesuis n=3/13 **(23.1%)**	*S.* Paratyphi B n=1/13 **(7.7%)**	*E. coli* n=21/33 **(63.6%)**	*S. aureus* n=6/33 **(18.2%)**	*Citrobacter species* n=3/33 **(9.1%)**	*Proteus mirabilis* n=2/33 **(6.1%)**	*Pseudomonas aeruginosa* n=1/33 **(3.0%)**		
Parameters	F	F	F	f	F	f	f.	f.	f.(%)	
Age (Years)										**0.000**
0-4	1	0	0	2	2	0	0	0	5 (10.9)	
5-9	1	0	0	1	0	0	0	0	2 (4.3)	
10-14	0	0	0	1	1	0	0	0	2 (4.3)	
15-19	0	1	0	0	1	0	0	0	2 (4.3)	
20-24	3	0	0	3	0	1	0	0	7 (15.2)	
25-29	2	0	0	7	0	0	1	0	10 (21.7)	
30-34	1	1	0	2	1	0	0	1	6 (13.0)	
35-39	1	0	1	4	0	1	0	0	7 (15.2)	
40-44	0	0	0	0	0	0	0	0	0 (0)	
45-49	0	0	0	0	0	0	1	0	1 (2.2)	
50+	0	1	0	1	1	1	0	0	4 (8.7)	
Sex										**0.000**
Male (n=131)	1	0	1	7	1	0	1	0	11 (23.9)	
Female (n=249)	8	3	0	14	5	3	1	1	35 (76.1)	
Source										0.975
lood (n=378)	0	0	0	0	6	0	0	0	6 (13.0)	
Stool (n=380)	9	3	1	0	0	0	0	0	13 (28.3)	
Urine (n=375)	0	0	0	21	0	3	2	1	27 (58.7)	
District										**0.000**
Agona West	7	2	1	14	4	2	1	1	32 (69.6)	
Agona East	2	1	0	6	1	1	0	0	11 (23.9)	
Gomoa East	0	0	0	1	0	0	1	0	2 (4.3)	
Awutu Senya	0	0	0	0	1	0	0	0	1 (2.2)	

Others	0	0	0	0	0	0	0	0 (0)

Data were in frequencies (f), percentages (%) and were analyzed using Chi-square at 95% confidence intervals with p-value <0.05 considered significant. Note that S. Enterica, *Salmonella* Enterica; S. Choleraesuis, *Salmonella* Choleraesuis; S. Paratyphi B, *Salmonella* Paratyphi B; *S. aureus, Staphylococcus aureus*; *E. coli, Escherichia coli*. Other districts = Efutu, Gomoa West and Asikuma-Odobeng-Brakwa

4.2 *SALMONELLAE* AND OTHER PATHOGENS ISOLATED.

4.2.1 *Salmonellae* Isolated.

From a total of three hundred and eighty (380) patients tested, 13 were positive for *Salmonellae*. This gave the prevalence of *Salmonellae* in the study area to be 3.4%. All the *Salmonellae* were isolated from stool (n=380) samples of the study subjects. Urine and blood samples were culture negative for *Salmonellae* (see Table 4.2). Serotypes of the *Salmonellae* isolated are shown in Table 4.1. *Salmonella* Enterica was the most occurring *Salmonellae* isolates with 69.2%. (n=9/13), followed by *Salmonella* Choleraesuis with 23.1% (3/13) and *Salmonella* Paratyphi B with 7.7% (n=1/13). Most of the *Salmonellae* isolates were non-typhoidal (92.3%). The difference between the proportion of study subjects whose culture were negative for *Salmonellae* and the study subjects whose culture were positive for *Salmonella species* was significant with a pvalue of 0.000. *Salmonella* infection (salmonellosis) in females (11/249; 4.4%) was higher than males (2/131; 1.5%). Higher proportions of *Salmonella* isolates (23.1%; n=3) were recovered from patients between the age group of 20 and 24 years. The study subject's demographic characteristics stratified by *Salmonellae* isolates are shown in

Table 4.1.

Table 4.2: Prevalence of *Salmonella* infection stratified by samples and sex.

Parameters	Number Tested	*Salmonellae* Positive Culture	Prevalence/Rate of Infection (%)
Samples			
Stool	380	13	3.4
Urine	375	0	0
Blood	378	0	0

Sex			
Female	249	11	4.4
Male	131	2	1.5

4.2.2 Other Pathogens Isolated.

During the investigation of patients' samples for *Salmonellae,* other bacteria pathogens were encountered and were recorded. There were 33 other bacteria pathogens isolated from blood and urine samples. Of these, 27 were isolated from urine samples and 6 were isolated from blood samples (see Table 4.1). The most common bacteria pathogen isolated in urine was *Escherichia coli* constituting 77.8% (n=21/27) of the isolates. The prevalence of other isolates was *Citrobacter species* 11.1% (n=3/27), *Proteus mirabilis* 7.4% (n=2/27) and *Pseudomonas aeruginosa* 3.7% (1/27). All the bacteria recovered from urine were Gram negative bacilli. Out of the 27 (100%) urine bacteria isolated from this study, 33.3% (n=9) were isolated from males and 66.7% (n=18) were isolated from females. *Staphylococcus aureus* (n=6) was the only pathogen isolated from blood. Mixed infections were not seen among any of the patients whose samples were culture positive. The pathogens isolated in this study and their associated age, sex, sample type and district distributions are shown in Table 4.1.

4.3 CLINICAL COMPLAINTS FROM PATIENTS.

Patients with *Salmonellae* culture positive who had duration of diarrhoea lasted for not more than 3 days before they visited the hospital represented 3.7% (n=12/322) whilst those who complained of abdominal pains were 3.0% (n=8/266). The other

clinical complaints obtained from *Salmonella* culture positive patients are shown in Table 4.3.

Table 4.3 Clinical complaints from *Salmonellae* culture positive and negative patients.

Clinical Complaints	***Salmonellae* positive culture (n=13) (%)**	***Salmonellae* Negative culture (n=367) (%)**	**Total (%)**	**p-value**
Abdominal Pains	8 (3.0)	258 (97.0)	266 (70.0)	**0.000**
Fever	6 (2.8)0	209 (97.2)	215 (56.6)	**0.001**
Nausea	3 (1.6)	181 (98.4)	184 (48.4)	0.544
Vomiting	6 (5.1)	111 (94.9)	117 (30.8)	**0.037**
Blood in Stool	0 (0,0)	22 (100)	22 (5.8)	**0.000**
Diarrhoea Duration				**0.000**
0-3 days	12 (3.7)	310 (96.3)	322 (84.7)	
4-7 days	1 (2.0)	48 (98.0)	49 (12.9)	
≥8 days	0 (0)	9 (100)	9 (2.4)	

4.4 ANTIMICROBIAL SENSITIVITY TEST RESULTS.

4.4.1 Antimicrobial Sensitivity of *Salmonellae* Isolates.

The *Salmonellae* isolated were tested against twelve (12) antimicrobial agents. The *Salmonellae* isolated were all resistant (13/13; 100%) to Cefuroxime, but there were varied resistance proportions to the other antimicrobials. The proportion of resistance was higher for Cotrimoxazole (69.2%; n=9/13) than for Tetracycline (23.1%; n=3/13), Amoxicillin (15.4%; n=2/13) and Ampicillin (15.4%; n=2/13). The detailed results are presented in Table 4.4. None of the *Salmonella* isolates were multidrug resistant (MDR, defined as resistance to Amoxicillin, Cotrimoxazole and Chloramphenicol), but resistant to Cefuroxime, Ampicillin, Cotrimoxazole and Amoxicillin (4 antimicrobials) were seen in 33.3% of *Salmonella* Choleraesuis isolates. Two thirds (66.7%) of *Salmonella* Choleraesuis were resistant to Cefuroxime, Tetracycline and

Cotrimoxazole.

Table 4.4 Antimicrobial Resistance Pattern of *Salmonellae* and other pathogens isolated from diarrhoea patients at Swedru Municipal Hospital.

	Isolates								
Antibiotics	*S*. Enterica n=9 (%)	*S*. Choleraesuis n=3 (%)	*S*. Paratyphi B n=1 (%)	*E. coli* n=21 (%)	*S. aureus* n=6 (%)	*C. species* n=3 (%)	*P. mirabilis* n=2 (%)	*P. aeruginosa* n=1 (%)	Total = 46 (%)
Cefuroxime	9 (100)	3 (100)	1 (100)	15 (71.4)	5 (83.3)	3 (100)	1 (50)	1 (100)	38 (82.6)
Cotrimoxazole	6 (66.7)	3 (100)	0 (0)	18 (85.7)	5 (83.3)	3 (100)	2 (100)	1 (100)	38(82.6)
Tetracycline	0 (0)	3 (100)	0 (0)	16 (76.2)	3 (50)	3 (100)	2 (100)	1 (100)	28 (60.9)
Ampicillin	0 (0)	1 (33.3)	1 (100)	18 (85.7)	6 (100)	3 (100)	1 (50)	1 (100)	31(67.4)
Amoxicillin	0 (0)	1 (33.3)	1 (100)	18 (85.7)	6 (100)	3 (100)	1 (50)	1 (100)	31(67.4)
Amikacin	0 (0)	0 (0)	0 (0)	1 (4.8)	1 (16.7)	0 (0)	0 (0)	0 (0)	2 (4.3)
Gentamicin	0 (0)	0 (0)	0 (0)	8 (38.1)	2 (33.3)	2 (66.7)	1 (50)	0 (0)	13 (28.3)
Ciprofloxacin	0 (0)	0 (0)	0 (0)	18 (85.7)	1 (16.7)	3 (100)	0 (0)	1 (100)	23 (50)
Ceftriaxone	0 (0)	0 (0)	0 (0)	12 (57.1)	3 (50)	3 (100)	0 (0)	1 (100)	19 (41.3)
Amoxicillin-Clavulanate	0 (0)	0 (0)	0 (0)	14 (66.7)	5 (83.3)	3 (100)	1 (50)	1 (100)	24 (52.2)
Chloramphenicol	0 (0)	0 (0)	0 (0)	14 (66.7)	3 (50)	3 (100)	1 (50)	1 (100)	22 (47.8)
Meropenem	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nitrofurantoin	NT	NT	NT	8 (38.1)	NT	3 (100)	2 (100)	1 (100)	14 (51.9)
Nalidixic Acid	NT	NT	NT	20 (95.2)	NT	3 (100)	0 (0)	1 (100)	24 (88.9)
ESBL (% POS.)	0	0	0	12 (57.1)	NT	2 (66.7)	0 (0)	NT	14 (35.9)

Data were in frequencies and percentages (%). Note that S. Enterica = *Salmonella* Enterica, S. Choleraesuis = *Salmonella* Choleraesuis, S. Paratyphi B = *Salmonella* Paratyphi B, *E. coli* = *Escherichia coli, S. aureus* = *Staphylococcus aureus*, *C. species* = *Citrobacter species*, *P. aeruginosa* = *Pseudomonas aeruginosa, P. mirabilis* = *Proteus mirabilis.* NT = Not Tested.

4.4.2 Antimicrobial Sensitivity of Other Pathogens Isolated.

The bacterial pathogens were tested against fourteen (14) antimicrobial agents. The results are shown in Table 4.4. The bacterial pathogens isolated were all sensitive to Meropenem but showed varied resistance levels to most tested antimicrobials.

Escherichia coli isolates recorded high proportions of resistance to Nalidixic acid (95.2%; n=20/21), Ciprofloxacin (85.7%; n=18/21), Amoxicillin/Ampicillin (85.7%; n=18/21), Cotrimoxazole (85.7%; n=18/21), Chloramphenicol (57.1%; n=12/21) and to most tested antimicrobials (see Table 4.4). Low proportion of resistance was observed against Gentamicin (38.1%; n=8/21), Nitrofurantoin (38.1%; n=8/21) and Amikacin (4.8%; n=1/21). Most of the *Escherichia coli* isolated (85.7%) were multidrug resistant (MDR, defined as resistant to 3 or more antibiotics). The *Citrobacter species* isolated showed high proportion of resistance to Gentamicin (66.7%; n=2/3) but were all resistant to the remaining tested antimicrobials except Amikacin and Meropenem (see Table 4.4). All the *Proteus mirabilis* were resistant to Tetracycline, Cotrimoxazole and

Nitrofurantoin and half of them showed resistance to Amoxicillin, AmoxicillinClavulanic Acid, Chloramphenicol, Gentamicin but all were sensitive to Cefuroxime, Ceftriaxone, Meropenem, Amikacin, Ciprofloxacin and Nalidixic Acid (see Table 4.4).

The *Pseudomonas aeruginosa* isolated was sensitive to Meropenem, Amikacin and Gentamicin but was resistant to the remaining tested drugs. The *Staphylococcus aureus* isolates were all resistant to Amoxicillin but resistance to the remaining tested antimicrobials were of varied proportions (see Table 4.4).

4.5 EXTENDED SPECTRUM β-LACTAMASE (ESBL) TEST RESULTS.

None of the *Salmonellae* isolated were ESBL producers. Most of the *Escherichia coli* (`57.1%; n=12/21) isolates and *Citrobacter species* (66.7%; n=2/3) were ESBL producers. *Proteus mirabilis* isolates were all ESBL non-producers (see Table 4.4). The majority (92.9%; n=13/14) of ESBL producers were resistant to Ciprofloxacin.

4.6 PFGE RESULTS OF *SALMONELLA* ISOLATES

The DNA fingerprint image of isolates is presented in Figure 4.1. Lanes 1 and 5 are *Salmonella* Branderup (H9812) used as a standard strain. Isolates with the same number and size of DNA fingerprints were considered similar strains. Lanes 2, 3 and 4 were the 3 *Salmonella* Choleraesuis isolates (see Figure 4.1). PFGE results showed that of the three (3) *Salmonella* Choleraesuis isolates, two (Lanes 2 and 3) had the same number and size of DNA fragments and hence are similar strains. The number and size of DNA fingerprint image of *Salmonella* Choleraesuis in Lane 4 is different from those of Lanes 2 and 3.

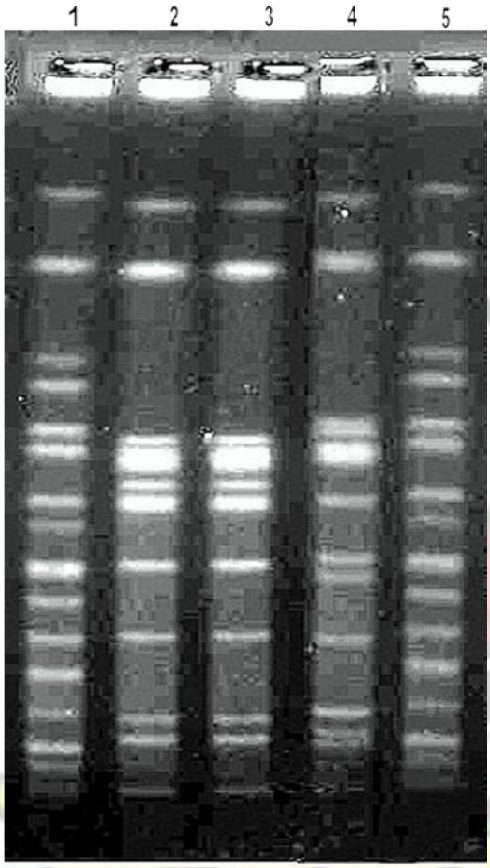

Figure 4.1: Pulsed-field gel electrophoresis (PFGE) patterns of *Xba*I-digested genomic DNA of *Salmonella* Choleraesuis strains obtained from stool samples of patients who presented with diarrhoea at Agona Swedru municipal Hospital.

Lanes: 1 and 5 = *Salmonella* Branderup (H9812) control strain.

Lanes: 2, 3 and 4 = *Salmonella* Choleraesuis isolates.

Chapter 5

DISCUSSION

The current study was done to determine the *Salmonella species* among diarrhoeal patients attending Agona Swedru Municipal Hospital.

5.1 PREVALENCE OF *SALMONELLAE*.

The current study recorded 3.4 % prevalence of *Salmonella species* among patients who presented with diarrhoea at Agona Swedru Municipal Hospital. There was a significant difference between the proportion of study subjects whose culture were negative for

Salmonella species and those with culture positive for *Salmonella species* (p-value = 0.000). The prevalence obtained in this study (3.4%) is higher than 2.3% recorded in a study conducted in children in Abuja (Cajetan *et al*., 2013) and 1.3% in Dhaka, Bangladesh (Leung *et al*., 2013). Saba *et al*., (2013) recorded 3.9% prevalence of *Salmonella species* in the Northern Region of Ghana, which is similar to the 3.4% obtained in this study. The 3.9% recorded in the Northern Region was attributed to lack of potable drinking water for the residents in the Northern Region of Ghana (Saba *et al*., 2013). Agona Swedru has adequate supply of potable drinking water. It is therefore unclear as to the cause of the prevalence similar to what was obtained in the Northern Region. Agona Swedru is an urban town and *Salmonellae* infection may be acquired through eating street foods that may have been contaminated by *Salmonellae*. This may have accounted for the similarity in prevalence of this study and that of Saba *et al*., (2013). Feglo *et al*., (2004) reported a lower prevalence of 2.3% among food handlers in Kumasi. Unlike this study which was conducted in diarrhoeal patients,

their study (Feglo *et al*., 2004) was conducted among respondents who were not ill but at work.

The difference in study subjects and geographical location may have contributed to the

difference in prevalence of *Salmonellae*. In contrast to the findings in this study, Beyene (2008) reported a higher prevalence (5.3%) of Salmonellosis in Ethiopia and Dembélé *et al*., (2014) also had 6% prevalence of *Salmonellae* in Burkina Faso. Another study

(Abdullahi, 2010) in Kano State, Nigeria also recorded a higher prevalence of 13.7%.

The majority of *Salmonella* isolates in this study were Non Typhoidal (NTS). Previous studies (Wilkens *et al*., 1997; Beyene, 2008; Cajetan *et al*., 2013; Saba *et al*., 2013) showed high prevalence of Non Typhoidal *Salmonellae* which correlated with the findings in this study.The increased prevalence of Non Typhoidal *Salmonella* may be attributed to the instances of multiple animal reservoir hosts of NTS which make containment more difficult as transmission is mainly through taking in contaminated animal food products (Kariuki *et al*., 2006a). Also, the many serotypes of *Salmonella* make it difficult to control the spread of salmonellosis through vaccination of farm animals (Cheng-Hsun *et al*., 2004) and may have contributed to the increased prevalence of NTS in this study.

Salmonella Enterica was the dominant serovar isolated among the *Salmonellae* in this study. This correlated with a study in Akwatia, Ghana which reported *Salmonella* Enterica as the most predominant serovar (Puopelle, 2014). The findings in this study differ from other reported studies in Europe (de Jong and Ekdahl, 2006; O'Brien, 2013) and in Africa (Kariuki *et al*., 2006a; Kariuki *et al*., 2006b; Boni-Cissé *et al*., 2012; Bonkoungou *et al*., 2013; Kariuki and Onsare, 2015; Kwambana-Adams *et al*.,

2015) which had *Salmonella* Enteritidis and *Salmonella* Typhimurium as the most frequently isolated serovar.

5.2 STUDY SUBJECTS AND CLINICAL COMPLAINTS.

This study showed that *Salmonella* infection was higher in females (11/249; 4.4%) than in males (2/131; 1.5%). Higher *Salmonella* infection in females has been reported in Akwatia, Ghana (Puopelle, 2014) and is consistent with the findings in this study. On the contrary, in Abuja, Nigeria, Cajetan *et al*. (2013) reported relatively higher *Salmonella* infection in males than in females.

This study identified no invasive non typhoidal *Salmonella* infection and no bloodstream infection of *Salmonellae.* This is consistent with reports that non Typhoidal *Salmonellae* are associated with diarrheal disease which is self-limiting and seldom cause invasive infections (Kwambana-Adams *et al*., 2015). Previous studies by Schwarz *et al*. (2010) and Newman *et al*. (2011) in Ghana and other studies in Africa (Kariuki *et al*., 2006a; Beyene 2008; Abdulahi 2010) reported invasive *Salmonellae* infection. Though it is unclear why their finding contrasts that of this study, it may also be due to the differences in geographical locations and differences in study subjects. In spite of this, *Salmonella* Choleraesuis isolated in this study is a prominent cause of invasive infections (Cheng-Hsun *et al*., 2004) and considering the fact that 5% of patients with non Typhoidal *Salmonella* gastroenteritis will develop potentially fatal bacteraemia (Beyene, 2008) therefore, patients in this study with salmonellosis are more likely to develop bacteraemia.

Infections of *Salmonellae* were generally across all age groups in this study but a higher prevalence was seen in patients between the age group of 20 and 24 (see Table 4.1). *Salmonellae* infections are reported to occur across all age groups (Kwambana-Adams *et al*., 2015). Children between 6 and 15 years of age in Ghana were reported to be more infected with *Salmonellae* than the other age groups (Puopelle, 2014). Also, previous studies (Abdullahi, 2010; Kwambana-Adams *et al*., 2015) reported high prevalence of *Salmonellae* infection in children which is not consistent with the findings in this study.

5.3 ANTIMICROBIAL SENSITIVITY OF *SALMONELLAE*.

Gastroenteritis caused by non Typhoidal *Salmonella species* is self-limiting and hence treatment with antibiotics is not required (Silva *et al*., 2012). Antimicrobial treatment in NTS gastroenteritis does not facilitate prognosis of the disease but rather may prolong faecal discharge of the bacteria, promote risk of relapse and production of antimicrobial resistant strains (Silva *et al*., 2012). Extra intestinal disease caused by NTS therefore requires antimicrobial treatment (Silva *et al*., 2012).

In this study, the *Salmonellae* isolated showed high resistance to Cotrimoxazole (69.2%; n=9) but low resistance to Aminopenicillins (15.4%; n=2) and were all susceptible to Chloramphenicol. This indicates that, among the first line of drugs previously used for the treatment of *Salmonellae* infections, Chloramphenicol is the only reliable drug in our study area. Studies in Ghana (Schwarz *et al*., 2010) and in Ethiopia (Beyene, 2008) observed resistance among *Salmonellae* more than 70% for aminopenicillins, chloramphenicol and co-trimoxazole. Interestingly, a study in the

Northern part of Ghana (Saba *et al*., 2013) reported all the non-typhoidal *Salmonellae* isolated were susceptible to all antimicrobial agents used in that study. This gives an indication of to variations in *Salmonellae* susceptibility to antimicrobials in Ghana.

In *Salmonella,* multidrug resistance (MDR) is defined as resistance to amoxicillin, chloramphenicol and co-trimoxazole (Schwarz *et al*., 2010). All the *Salmonella* isolates in this study were susceptible to Chloramphenicol and therefore none was multidrug resistant (MDR). This is reassuring because MDR *Salmonellae* may be associated with increased morbidity and a major public health concern (Kwambana-Adams *et al*., 2015). This finding conforms to Wilkens *et al*., (1997) and Saba *et al*., (2013) studies in Ghana which found none of the *Salmonellae* that they isolated to be multidrug resistance (MDR). Other studies (Schwarz *et al*., 2010; Nielsen *et al*., 2012) in Ghana reported over 70% Multidrug resistant *Salmonellae* which are not in agreement with
the findings in this study.

The overall susceptibility of *Salmonellae* to antimicrobials tested in this study was high.

The *Salmonellae* were all sensitive to Ceftriaxone, Amoxicillin-Clavulanic Acid, Meropenem, Amikacin, Gentamicin, Chloramphenicol and Ciprofloxacin. This does not threaten existing regimens: Ceftriaxone (Third generation cephalosporins) and Ciprofloxacin (fluoroquinolones) which are suggested for the treatment of *Salmonella* infections and leaving adequate antimicrobial treatment options in the study area. This notwithstanding, there should be continuous surveillance and monitoring of *Salmonella* susceptibility to antimicrobials in the study area.

The easy availability and acquisition of drugs over the counter without prescription leads to antibiotic misuse by the public and physicians poor prescribing habits without adequate laboratory testing are among the factors leading to the emergence of resistance strains (Newman *et al*., 2011). Ceftriaxone is administered intravenously or intramuscularly and hence its administration requires well-trained personnel. Also the fact that Ceftriaxone is about three to ten times more costly than amoxicillin (Schwarz *et al*. 2010) may discourage its misuse by the residents in the study location. Possibly, these may have contributed to the sensitivity of *Salmonella species* to Ceftriaxone in this present study.

5.4 *SALMONELLAE* ISOLATED AND ESBL PRODUCTION.

Bacteria producing extended spectrum β –lactamase (ESBL) enzymes are able to hydrolyze and inactivate large range of β-lactams which includes third generation cephalosporins, penicillins and monobactams (Beyene, 2008). ESBL producers show less susceptibility to the quinolones and are usually multidrug resistance (MDR) (Abdul *et al*., 2013). In the present study, none of the *Salmonellae* isolated were ESBL producers. This may have contributed to the complete susceptibility of *Salmonellae* to β-lactam antibiotics and ciprofloxacin tested in this study. The finding in this study correlates with that of Puopelle (2014) study in Ghana and Boni-Cissé *et al.* (2012) study in Cote d'Ivoire that found none of their *Salmonella* isolated as ESBL producers. The finding in this study contrast earlier reported case of ESBL producer strains of *Salmonellae* circulating in Bangladesh (Dilruba *et al.*, 2014).

5.5 PFGE ANALYSIS OF *SALMONELLA* ISOLATED.

The PFGE results shows that, of the three (3) *Salmonella* Choleraesuis isolates, two (2) had the same number and size of DNA fragments and hence are closely related (clonal). Thus, two PFGE types of *S. Choleraesuis* were identified as circulating in our study

area.

5.6 OTHER PATHOGENS

5.6.1 Prevalence

Urinary tract infection is mainly caused by bacteria and is one of the common reasons for seeking medical attention in the community (Sharma and Bidwai U, 2013). In this study, the prevalence of bacteria pathogens isolated from urine (bacteriuria) was 7.2%. This prevalence correlates with findings from another study in Ghana which reported 7.3% (Turpin *et al*., 2007). Obirikorang *et al*. (2012) reported 9.5% prevalence for asymptomatic bacteriuria among pregnant women in Kumasi, Ghana. A higher percentage of 56.5% has also been reported in Cape Coast, Ghana (Boye *et al*., 2012). These indicate variations in prevalence of bacteriuria (Urinary Tract Infection) and may be related to the kind of study subjects, geographical site, environment, community, social habits, educational level and personal hygiene (Addo, 2014).

The present study recorded *Escherichia coli* (77.8%; n=21/27) as the most common bacterial pathogen causing bacteriuria. This agrees with those of other reported studies in Ghana (Turpin *et al*., 2007; Obirikorang *et al*., 2012) and in Iran (Amin *et al*., 2009). This affirms reports that *E. coli* is the major pathogen responsible for causing urinary

tract infection (Vasudevan, 2014). In Ghana, Addo (2014) reported *Staphylococcus aureus* as the commonest cause of asymptomatic bacteriuria followed by *Escherichia coli*. Our study recorded *Citrobacter species* (11.1%; n=3/27) as the second highest bacteria pathogen isolated. *Proteus mirabilis* (7.4%; n=2/27) was the third bacteria pathogen recovered from urine and it is the only *Proteus species* isolated in this study. This may be attributed to the fact that among the *Proteus species, Proteus mirabilis* has a different pathogenicity which gives it the higher tendency for colonizing the urinary tract (Feglo *et al*., 2010). *Pseudomonas aeruginosa* (1/27 (3.7%) was the lowest pathogen isolated. All the urine pathogens isolated in this study were Gram negative

bacilli.

Two thirds of urinary tract infections were found in females. This is consistent with the finding that women are frequently susceptible to urinary tract infections due to their anatomy and reproductive physiology (Vasudevan, 2014). Male urethra is longer (8 inches) than women (1.5-2 inches) (Vasudevan, 2014). The shorter urethra in women enhances the scope for pathogen to invade the bladder and contribute to higher incidence of urinary tract infection in females. Afriyie *et al*. (2015) study in Ghana and other studies in the world showed higher incidence of urinary tract infection in females

(Vasudevan, 2014).

5.6.2 Other Pathogens Antimicrobial Sensitivity.

Antimicrobial resistance pattern of bacteria isolates causing urinary tract infection varies in different regions (Sharma and Bidwai, 2013). The improved antibacterial activity and pharmacological properties of fluoroquinolones enhanced its high clinical cure rate as most common uropathogens show low resistance (Afriyie *et al*., 2015). These make fluoroquinolones the preferred antibiotics for the treatment of urinary tract infection (Afriyie *et al*., 2015). In this study, uropathogens exhibited high resistance (81.5%; n=22/27) to ciprofloxacin (fluoroquinolones). *Escherichia coli* (77.8%), being the most common uropathogen isolated in this study showed high resistance (85.7%) to ciprofloxacin. A study in Ghana (Afriyie *et al*., 2015) and in India (Abdul *et al*., 2013) reported resistance of uropathogens to ciprofloxacin (fluoroquinolones) lower than that observed in this study. The high rate of resistance may be due to its misuse as fluoroquinolones can easily be obtained over the counter without prescription at the study area (Personal observation).

All the uropathogens showed high resistance to the other antimicrobial agents: Nalidixic

Acid (88.9%), Cotrimoxazole (88.9%), Ampicillin (85.2%), Amoxicillin (85.2%), Tetracycline (81.5%), Cefuroxime (74.1%), Amoxicillin-Clavulanic Acid (70.4%), Chloramphenicol (70.4%), Ceftriaxone (59.3%), Nitrofurantoin (51.9%), and Gentamicin (40.7%) and low resistance to Amikacin (3.7%). This high prevalence of uropathogens resistance to antimicrobial agents tested in this study is of concern. The high resistance may result in increased morbidity, increased cost of treating urinary tract infections, extended length of hospital stay and increased mortality. In some instances, the bacteria strains that have acquired antimicrobial resistance genes enhanced transmissibility and spread the resistance genes (Silva *et al*., 2012).

In this study nearly 40% of the isolates that belonged to the Enterobacteracae were ESBL producers. More than 90% of these ESBL producers were resistant to Ciprofloxacin and all were resistant to Nalidixic acid. This finding exceeds that of one to two-thirds of Enterobacteriaceae producing extended spectrum β Lactamase enzymes being resistant to fluoroquinolones (Abdul *et al*., 2013). A higher proportion (79.9%) of ESBL producers were reported resistant to ciprofloxacin in India (Abdul *et al*., 2013). ESBL producers are usually reported to be multidrug resistant (Abdul *et al*., 2013). This is consistent with the finding in this study where 88.5% of the uropathogens belonging to the Enterobacteriaceae were multidrug resistant. This may have contributed to the high resistance of uropathogens to β–Lactam antibiotics and fluoroquinolones tested in this study as ESBL hydrolyzes and inactivate β–Lactam antibiotics.

In the present study, there was high prevalence of resistant uropathogens to the commonly prescribed antimicrobials for the treatment of urinary tract infection. The reliable antimicrobial agents that may be used for the treatment of urinary tract infection from this study were Amikacin and Meropenem (Carbapenems).

5.7 LIMITATIONS.

The PFGE on the *Salmonella* isolates was performed at the Pasteur Institute, Abidjan, and Lá Côte d'Ivoire, where they lack the BioNumeric software for the PFGE fingerprint analysis.

Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Prevalence of *Salmonella* among patients who presented with diarrhoea at Agona Swedru Municipal Hospital was 3.4% and prevalence of non-Typhoidal *Salmonellae* was 3.2%. All the *Salmonellae* were isolated from patients stool samples. Three *Salmonella* serotypes were identified with *Salmonella* Enterica dominating with 69.2%

(n=9/13), followed by *Salmonella* Choleraesuis with 23.1% (n=3/13) and *Salmonella* Paratyphi B 7.7% (n=1/13). Three *Salmonella* Choleraesuis were isolated and found to be of two different gene types by the PFGE fingerprint. There were low proportions of *Salmonella* isolates resistant to the antimicrobial agents tested. None of the *Salmonellae* was multidrug resistant (MDR). The *Salmonellae* isolated in this study were susceptible to Ceftriaxone, Amoxicillin-Clavulanic Acid, Meropenem, Amikacin, Gentamicin, Chloramphenicol and Ciprofloxacin and none of the *Salmonella* isolates produced ESBL. The most common pathogen isolated from urine was *Escherichia coli* (63.6%; n=21/33), followed by *Citrobacter species* (9.1%; n=3/33), *Proteus mirabilis* (6.1%; n=2/33) and *Pseudomonas aeruginosa* (3.0%; n=1/33). *Staphylococcus aureus* (18.2%; n=6/33) was the only pathogen isolated from blood. The pathogens isolated other than *Salmonella* had high proportions resistant to the antibiotics tested. More than one third of the isolated pathogens belonged to the family Enterobacteriaceae and was ESBL

producers.

6.2 RECOMMENDATIONS

- It is recommended that surveillance and monitoring of *Salmonella* susceptibility to antimicrobials in the study area be continued to detect any change in trend of *Salmonella* isolates susceptibility to antimicrobials and to detect when *Salmonella* begin to produce ESBL.

- The public health reference laboratories in Ghana should be equipped by Ghana Ministry of Health to serotype *Salmonellae* and to identify *Salmonellae*. Also, a national surveillance should be instituted on Salmonellosis in Ghana to ascertain the national burden.

- Ghana Ministry of Health policy on rational antibiotics usage should be enforced with intensive public education to help stop practices that creates and spread antibiotic resistance strains.

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APPENDIX I: QUESTIONNAIRE

Investigation of salmonellosis in patients with diarrhoea at Agona Swedru Municipal Hospital

Interviewer ID:_____ Interview Date: ___/____/_____
DD /MM/ YYYY

A. PATIENT IDENTIFICATION

Patient's Name: _____ Participant's ID: _____

Age: _____ Sex: _____ Telephone number: _____

Address:

_____

District/Municipality: [1] Awutu Senya [2] Effutu [3] Agona East [4] Agona West [5]

Other (specify) _____

Caretaker's Name (if child) _____

_ B. CLINICAL DATA

Do you have any of the following symptoms?

Diarrhoea …….☐ Yes ☐ No

(Diarrhoea refers to three or more loose stools in 24 hour period)

If yes specify date of onset (DD/MM/YYYY) _____/____/_____,

Duration_____

Blood in stool……☐ Yes ☐ No

Nausea……...☐ Yes ☐ No Vomiting……☐ Yes ☐ No

Abdominal/Stomach pain …….….☐ Yes ☐ No

Fever……...☐ Yes ☐ No

Other ……..☐ Yes ☐ No

Specify: _____

Have you been on antibiotics within the last month for any reason…….. ☐ Yes ☐ No

If yes, name of antibiotics _____, duration _____

Have you been told that you have any underlining condition or disease that will affect or lower your immune system, e.g. Pregnancy, Diabetes etc? ☐ Yes, Specify: __ ☐ No

Clinical Diagnosis_____

C. LABORATORY DATA

Type of specimen(s) obtained: Blood ☐ Stool ☐ Urine ☐

Consistency of stool

Watery…… ☐ Yes ☐ No

Mucoid ……..☐ Yes ☐ No Bloody……..

.☐ Yes ☐ No

Mixed (blood and mucus)…...☐ Yes ☐ No

Loose……..☐ Yes ☐ No

Other, specify _____-

Microscopic examination

Stool:

Culture and identification

Stool_____

Blood_____

Urine_____

__ **Phenotypic characterization** Serogroup:

Serotype/serovar: _____

Antibiotics susceptibility result_____

Antimicrobials	**Inhibition Zone (mm)**	**Sensitive (S)**	**Intermediate (I)**	**Resistant (R)**
Ceftriaxone (µg),				
Cefuroxime(30 µg)				
Ampicillin (10 µg),				
Amoxicillin				
Chloramphenicol (30 µg),				
Trimethoprim/sulfamethoxazole (1.25/23.75 µg),				
Gentamicin (10 µg),				
Amikacin (30 µg),				
Tetracycline (30 µg),				
Nalidixic acid (30 µg),				
Ciprofloxacin (5 µg),				
Imipenem (10 µg)				

ESBL Testing Results:

Molecular Characterization of *Salmonella* isolates

PFGE to determine genetic relatedness:

PCR for ESBL genotyping:
Comment:

APPENDIX II: PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

This leaflet must be given to all prospective participants to enable them know enough about the research before deciding to or not to participate

Title of Research: Characterization of Non-Typhoidal Salmonellae Isolates Obtained from Patients Attending Agona Swedru Municipal Hospital.

Name(s) and affiliation(s) of researcher(s): This study is being conducted by Dr. Patrick K. Feglo of SMS, KNUST and Mr. Michael Nkansah of Agona Swedru Municipal Hospital.

Background (Please explain simply and briefly what the study is about): NonTyphoidal Salmonella (NTS) serotypes are zoonotic and have emerged as a significant and most common food-borne pathogen throughout the world. Typhoid fever cases are stable with low numbers in developed countries, but Non-Typhoidal Salmonellosis cases have increased worldwide and hence an important public health problem worldwide. Recorded cases of gastroenteritis have increased in patients attending Agona Swedru Municipal Hospital. These increases could be the cause of Non-Typhoidal Salmonella (NTS) and hence required further investigation. There are also reports of invasive (NTS) with reduced susceptibility to the currently used antibiotics for the treatment of salmonellosis and hence putting infected persons at risk of not being treated. This study will identify the relatedness of Salmonella species including invasive NTS circulating in our environment and their resistant to antimicrobials.

Purpose(s) of research: The purpose of this research is to isolate and determine the phenotypic and genotypic characteristics of salmonella species affecting diarrhoeic patients in Agona Swedru Municipal Hospital.

Procedure of the research, what shall be required of each participant and approximate total number of participants that would be involved in the research: Informed consent will be sought from subjects who would agree to take part in this study. Blood, urine and stool samples will be taken from each participants (diarrhoeic patient) attending Agona Swedru Municipal Hospital at the out-patient's department. Each sample will be cultured for Salmonella species. Each of the Salmonella isolates will be serotype using polyvalent and monovalent antisera by slide agglutination technique. Antimicrobial susceptibility test will be determined by the Kirby-Bauer disc diffusion technique. Extended Spectrum Beta-Lactamase producing Salmonella

species will be determined by Double Disk Synergy Test (DDST). Genetic relatedness and clones of the isolates will be determined by PFGE (Pulse Field Gel Electrophoresis) and PCR (Polymerase Chain Reaction). The results of this research will be expressed statistically and subjected to analysis using SPSS or STATA or Excel. A total of 200 participants are expected to be recruited in this research.

Risk(s): There will be the slight temporal pain at the site of venepuncture during blood sample taking; In addition, participants would be directed to produce stool and urine samples.

Benefit(s): The goal of this research is to determine Salmonella species associated with gastroenteritis and provide clinicians with appropriate guidance for prevention, control and antibiotic treatment of Salmonellosis in the study vicinity.

Confidentiality: All information and samples collected in this study will be given code numbers hence no name will be recorded. Data collected cannot be linked to you in anyway. Information will however be available to the investigators and concern group. However, as part of our responsibility to conduct this research properly, we may allow officials from ethics committees to have access to your records

Voluntariness: Taking part in this study should be out of your own free will. You are not under obligation to take part in this study. Research is entirely voluntary.

Alternatives to participation: If you choose not to participate, this will not affect your treatment in this hospital in any way.

Withdrawal from the research: You may choose to withdraw from the research at anytime without having to explain yourself. You may also choose not to answer any question you find uncomfortable or private.

Consequence of Withdrawal: There will be no consequence, loss of benefit or care to you if you choose to withdraw from the study. Please note however, that some of the information that may have been obtained from you without identifiers (name etc), before you chose to withdraw, may have been modified or used in analysis reports and publications. These cannot be removed anymore. We do promise to make good faith effort to comply with your wishes as much as practicable.

Costs/Compensation: There will be no compensation given for your time or inconvenience.

Contacts: If you have any question concerning this study, please do not hesitate to contact Mr. Nkansah Michael on 0244 755803.

Further, if you have any concern about the conduct of this study, your welfare or your rights as a research participant, you may contact:

The Office of the Chairman
Committee on Human Research and Publication Ethics
Kumasi
Tel: 03220 63248 or 020 5453785

CONSENT FORM

Statement of person obtaining informed consent:

I have fully explained this research to _____
and have given sufficient information about the study, including that on procedures, risks and benefits, to enable the prospective participant make an informed decision to or not to participate.

DATE: _____ NAME: _____

Statement of person giving consent:

I have read the information on this study/research or have had it translated into a language I understand. I have also talked it over with the interviewer to my satisfaction.

I understand that my participation is voluntary (not compulsory).

I know enough about the purpose, methods, risks and benefits of the research study to decide that I want to take part in it.

I understand that I may freely stop being part of this study at any time without having to explain myself.

I have received a copy of this information leaflet and consent form to keep for myself.

NAME:_____

DATE: _____ SIGNATURE/THUMB PRINT: _____

Statement of person witnessing consent (Process for Non-Literate Participants):

I _____ (Name of Witness) certify that information given to

_____ (Name of Participant), in the local language, is a true reflection of what l have read from the study Participant Information Leaflet, attached.

WITNESS' SIGNATURE (maintain if participant is non-literate): _____

MOTHER'S SIGNATURE (maintain if participant is under 18 years): _____

MOTHER'S NAME: _____

FATHER'S SIGNATURE (maintain if participant is under 18 years): _____

FATHER'S NAME: _____

APPENDIX III; ETHICAL APPROVAL

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICAL SCIENCES / KOMFO ANOKYE TEACHING HOSPITAL
COMMITTEE ON HUMAN RESEARCH, PUBLICATION AND ETHICS

Our Ref: CHRPE/AP/118/14

16th April, 2014.

Dr. Patrick Kwame Feglo
Department of Clinical
Microbioogy
School of Medical Sciences
KNUST-KUMASI.

Dear Sir,

LETTER OF APPROVAL

Protocol Title ***"Molecular Characterization of Non-Typhoidal Salmonellae Isolates Obtained from Patients Attending Agona Swedru Municipal Hospital."***

Proposed Site: ***Laboratory Department, Agona Swedru Municipal Hospital, Agona Swedru, Central Region, Ghana.***

Sponsor: ***Principal Investigator.***

Your submission to the Committee on Human Research, Publications and Ethics on the above named protocol refers.

The Committee reviewed the following documents:

- A notification letter of 21st June, 2013 from the Swedru Municipal Hospital (study site) indicating approval for the conduct of the study in the Hospital.
- A completed CHRPE Application Form.
- Participant Information Leaflet and Consent Form.
- Research Proposal.
- Questionnaire.

The Committee has considered the ethical merit of your submission and approved the protocol. The approval is for a fixed period of one year, renewable annually thereafter. The Committee may however, suspend or withdraw ethical approval at anytime if your study is found to contravene the approved protocol.

Data gathered for the study should be used for the approved purposes only. Permission should be sought from the Committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee should be notified of the actual start date of the project and would expect a report on your study, annually or at the close of the project, whichever one comes first. It should also be informed of any publication arising from the study.

Thank you Sir, for your application.

Yours faithfully,

Osomfuor Prof. Sir J. W. Acheampong MD, FWACP
Chairman

Room 7 Block J, School of Medical Sciences, KNUST, University Post Office, Kumasi, Ghana
Phone: +233 3220 63248 Mobile: +233 20 5453785 Email: chrpe.knust.kath@gmail.com / chrpe@knust.edu.gh

APPENDIX IV: CULTURE MEDIA PREPARATION

SELENITE BROTH (LIOFILCHEM)

The typical formula of dehydrated base of the Selenite Broth (g/L) is; Tryptone 5.0, Lactose 4.0, Sodium Selenite 4.0, Sodium Phosphate 10.0. pH 7.0 +/- 0.2 at 25ºC.

23.0g of the dehydrated base of Selenite Broth was suspended in one litre of distilled water in flat bottomed flask. The mixture was then heated to boiling until complete dissolution. The preparation was not autoclave.

LYSINE IRON AGAR (LIOFILCHEM, ITALY)

The typical formula of dehydrated base of the Lysine Iron Agar (g/L) is; Peptospecial 5.0, Glucose 1.0, L-lysine Hydrochloride 10.0, Ferric Ammonium Citrate 0.5, Yeast Extract 3.0, Sodium Thisulphate 0.04, Brom Cresol Purple 0.02, Agar 14.05.

Preparation - Suspend 34.1 g in one liter of distilled water. Heat until completely dissolved. Autoclave at 121ºC for 15 minutes.

TRIPLE SUGAR IRON AGAR (BIOMARK LABORATORIES, INDIA)

Formular of dehydrated base of Triple Sugar Iron in g/L is; Peptic digest of animal tissue 10.0, Casein enzymic hydrolysate 10, Yeast extract 3.0, Beef extract 3.0, Lactose 10, Sucrose 10, Dextrose 1.0, Sodium Chloride 5.0, Ferrous sulphate 0.20, Sodium thiosulphate 0.30, Phenol red 0.024, Agar 12.0. Final pH at 25ºC is 7.4 +/- 0.2.

Preparation - Suspend 65 g in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes. Sterilized by autoclaving at 15 lbs pressure (121 ºC) for 15 minutes. Allow the medium to set in sloped form with a butt about one inch long.

UREA BROTH (LIOFILCHEM, ITALY)

Suspend 39.0 g in one litre of distilled water. Mix to completely dissolved. Sterilized by filtration. Formular (g/L): Yeast Extract 0.1, Monopotassium phosphate 9.1, Disodium phosphate 9.5, Urea 20.0, Phenol Red 0.01. PH 6.8 +/- 0.1 at 25ºC

CYSTEINE LACTOSE ELECTROLYTE DEFICIENT AGAR (CLED) AGAR (LIOFILCHEM, ITALY)

Formular of dehydrated base of CLED (g/L) is; Beef Extract 3.0, Peptone 4.0, Tryptone 4.0, L-Cystine 0.128, Lactose 10.0, Brom Thymol Blue 0.02, Agar 15.0.

36.1 g of dehydrated base of CLED was suspended in one litre of distilled water in a flat bottomed flask. The preparation was heated until completely dissolved. The dissolved mixture was autoclaved at 121ºC for 15 minutes.

MÜELLER-HINTON AGAR (LIOFILCHEM)

Formular of dehydrated base of the Müeller-Hinton Agar (g/l) is; Meat Extract 2.0, Casamino Acids, Technical 17.5, Starch 1.5, Agar 15.0. pH 7.3 +/- 0.1 at 25 °C.

36.0 g of dehydrated base of Müeller-Hinton Agar was suspended into 1000 ml of distilled water in a flat bottomed flask. The preparation was heated until completely dissolved. The dissolved mixture was autoclaved at 121ºC for 15 minutes. The

autoclaved medium was immediately transferred into water bath at a temperature of 50 °C and allowed to cool to 50 °C. The freshly prepared medium was poured into flat-bottomed glass petri dishes on a level, horizontal surface to give a uniform depth of 4 mm. The agar medium was allowed to cool to room temperature. The plate was sealed or wrapped in rubber bag and stored in a refrigerator at 2 – 8 °C when not used the same day of preparation. The stored prepared culture medium was used within seven days after preparation. A representative sample of each batch of plates was incubated 35°C for at least 24 hours to test for sterility.

MÜELLER-HINTON BROTH (LIOFILCHEM)

Formular of dehydrated base of the Müeller-Hinton Agar (g/l) is; Meat Extract 2.0, Casamino Acids, Technical 17.5, Starch 1.5. pH 7.3 +/- 0.1 at 25 °C.

Preparation – Suspend 21.0 g of dehydrated base of Müeller-Hinton Broth into 1000 ml of distilled water in a flat bottomed flask. Heated until completely dissolved and pippette 2ml into bijou bottles and autoclaved at 121ºC for 15 minutes.

MACCONKEY AGAR (LIOFILCHEM, ITALY)

Composition of dehydrated base of the MacConkey Agar (g/l) is; Pancreatic digest of gelatin 17.00g, Peptones (meat and casein) 3.0g, Lactose monohydrate 10.0g, Bile salts 1.5g, Sodium chloride 5.00g, Neutral red 0.03g, Agar 15.00g. pH 7.1+/- 0.2 at 25ºC.

Preparation – Suspend 51.5g of dehydrated base in 1L distilled water and heat to dissolve the medium completely. Sterilize by autoclaving at 121ºC for 15 minutes. Cool to 50ºC in a water bath and pour into sterile Petri plates.

NUTRIENT AGAR (OXOID, ENGLAND)

Typical formular of dehydrated base of Nutrient Agar is (g/L); Peptone 5.078g, Beef Extract 3.0g, Sodium chloride 8.0g, Agar No. 2 12.0. pH 7.3 +/- 0.2 at 25ºC.

Preparation - Weigh 28g of dehydrated base and dissolve in 1L of de-ionised water. Allow to soak for 10 minutes, swirl to mix and sterilize by autoclaving for 15 minutes at 121^0C. Cool to 50^0C, mix well and pour into sterile Petri plates.

***SALMONELLA SHIGELLA* AGAR (LIOFILCHEM, ITALY)**

Typical formula of dehydrated base of *Salmonella Shigella* Agar is; Meat extract5.0g, Yeast extract 5.0g, Peptone 5.5g, Lactose 10.0g, Sodium citrate 1.0g, Sodium thiosulphate 8.5g, Ferric Ammonium Citrate 1.5g, Brilliant green 0.00033g, Neutral red 0.025g, Agar 14.0g. pH 7.0 +/- 0.2 at 25oC.

Preparation - Suspend 52.0g of dehydrated base of *Salmonella Shigella* Agar into 1000mls of distilled water. Heat to boil until completely dissolved. Do not autoclave.

BLOOD AGAR BASE (**LIOFILCHEM, ITALY)**

Typical formula in g/l is; Beef Extract 10.0, Tryptose 10.0, Sodium Chloride 5.0, Agar 15.0. pH 7.3 ± 0.2 at 25ºC.

Preparation: Suspend 40.0g in 1000 ml of distilled water. Heat until completely absorbed. Autoclave at 121ºC for 15min and cool to 50oC. Aseptically add 50ml of defibrinated sheep blood. Mix gently and pour into sterile petri plates.

APPENDIX V: BIOCHEMICAL TEST

Indole Test

The indole test is useful in detecting bacteria which produces tryptophanase and therefore can hydrolyze an amino acid tryptophan with the production of indole. To perform indole test, loopful of suspected colony of *Salmonella* was inoculated into a sterile peptone broth and then incubated overnight at 37ºC aerobically. After, Pasteur pipette was used to add few drops of Kovac's reagent to the inoculated culture broth. Appearance of a red layer on top of the culture broth was indicative of positive indole test whereas yellow layer indicated a negative test. *Salmonellae* are indole test negative.

Citrate Utilization Test (CUT).

Citrate utilization test (CUT) was done to determine the ability of bacteria to grow on citrate as the sole carbon source. The test was perforemed by stabbing Citrate agar in a test tube with suspected colony of the test organism using sterile straight loop. After overnight aerobic incubation at 37ºC, a change in colour of the agar from green to blue signified citrate utilization (positive test) and no change in colour implied a negative test. *Salmonellae* are not able to utilized citrate (Citrate untilization test negative).

Oxidase Test

This test detects bacteria which produce the enzyme cytochrome oxidase. The test for oxidase test was performed by adding 2-3 drops of oxidase reagent to a filter paper placed in clean petri dish. The edge of a glass slide was used to pick a colony from the pure culture of the test organism and smeared on the filter paper with the oxidase reagent. The appearance of blue-purple colour within 10 seconds indicated positive oxidase test whereas no appearance of blue-purple colour indicated negative oxidase test. *Salmonellae* do not produce cytochrome oxidase and hence are oxidase negative.

Urease Test

Urease test was used to detect bacteria that produce urease enzyme and therefore can cleave urea to produce carbon dioxide and ammonia. To test for urease production, a colony of the test organism was inoculated into urea broth and then incubated overnight, aerobically at 37ºC. Pink colour change in the medium indicated a positive urease test. *Salmonellae* are urease negative.

Triple Sugar Iron Agar (TSI)

Straight wire loop was used to pick a colony of the test organism to stab the butt and streak the slant of Tripple Sugar Iron Agar in a tube and then incubated overnight at 37ºC aerobically. After, Triple Sugar Iron Agar (TSI) was examined for the following reaction: a red or yellow slant, red or yellow butt, hydrogen sulphide (H_2S) production

(blackening in the medium) and gas production (cracks in the medium). Red slant and yellow butt indicated glucose fermentation but non lactose fermentation. Yellow slant and yellow butt indicated lactose fermentation. *Salmonella species* show red slant, yellow butt, blackening in medium (H_2S production) or not, cracks (gas production) or no cracks (no gas production) in medium.

Lysine Iron Agar (LIA)

This medium was used to differentiate bacteria based on their ability to deaminate or decarboxylate lysine and produce abundant of hydrogen sulfide. Sterilized straight microbiological loop was used to stab the butt and streaked the slant of Lysine Iron Agar with suspected colony of *Salmonella species.* The cup of the tube was loosely opened or capped to ensure aerobic conditions and incubated at 37°C for 18 – 48 hours. It was examined at 18 – 24 hours and 40 - 48 hours for growth and colour changes in tube butt and slant and blackening at the apex of the slant. Purple (alkaline) butt and purple slant indicated positive lysine decarboxylation reaction. Yellow (acid) butt and purple (alkaline) slant indicated negative reaction. A red slant indicated lysine deamination reaction. Medium blackened at the apex of the slant indicated positive hydrogen sulfide reaction. *Salmonella species* decarboxylate lysine hence produced purple butt and purple slant with blackened at the apex of the slant (hydrogen sulfide production).

APPENDIX VI: PFGE PROTOCOL

Preparation of Cell Suspension

The test strains and *Salmonella Braenderup* (H9812) control strain were cultured on a 5% blood agar plate and incubated overnight at 37°C. 800 µl of cell suspension buffer was aliquot into 5 ml tubes (1 tube for each bacterial test strain, 1 tube for the *Salmonella Braenderup* (H9812) control strain and 1 tube which served as a blank for turbidity measurements). Cotton swab was used to collect some bacterial culture from the agar plate and re-suspend into the suspension buffer. The turbidity of the cell suspension was measured using the Dade Behring Microscan turbidity meter. The cell concentration was adjusted to a turbidity reading of 0.70 McFarland. The tubes were kept on ice after each reading.

Casting Plugs

200 µl of the cell suspension was transferred to a 1.5 ml tube. 20 µl of 20 mg/ml proteinase-K was added and then mixed by gently tapping the tube with the fingers. The mixture was incubated at 37°C for 5 min. 280 µl of a 1% Seakem Gold agarose: 1% SDS mixture (held at 55°C) was added. The agarose was mixed with bacterial sample by gently pipetting mixture up and down. The mixture was dispensed immediately into a BIORAD plug mould, two plugs of each sample was made. The plugs were allowed to solidify at room temperature for 15 min.

Lysis of Cells in Agarose Plugs

Each plug mould was opened and agarose block transferred to a 50 ml screw-cap tube

(the agarose blocks were handled with a small spatula which was cleaned with 70% alcohol between each step). 5 ml of lysis buffer was added and incubate at 55ºC for 2 hours with gentle shaking in a shaking incubator at a speed of 200 rpm.

Washing of Agarose Plugs after Cell Lysis

The plugs were held in tubes with spatula and lysis buffer was carefully poured off. 15 ml of preheated (50ºC) deionized water was added and washed for 15 min at 50ºC in a shaking incubator at a speed of 200 rpm. The water was poured out and the wash step repeated. The water was poured out and15 ml of preheated (50ºC) TE buffer was added and washed for 15 min at 50ºC in a shaking incubator at a speed of 200 rpm. The washed step was repeated for another 5 times. The agarose plug was transferred to a 10 ml tube and 3 ml of fresh TE buffer added.

Restriction Digestion of DNA in Agarose Plugs.

The agarose plug was placed onto a glass slide and 1-2 mm thick slice was cut of the plug. The #22 scalpel blade that was used to cut was cleaned with 70% alcohol between each cut. The agarose slice was placed in a 1.5 ml tube containing 100 µl of 1× restriction enzyme buffer and then incubated for 15 min at 37ºC. The 1× buffer was pipetted out and 150 µl of fresh 1× restriction enzyme buffer containing 50 units of restriction enzyme was added. The set up was incubated for 4 hours at 37ºC. The buffer/enzyme mix was pipetted out and 200 µl of 0.5× TBE (Tris-Borate EDTA Buffer) added. Then incubated for 5-10 minutes at room temperature.

Casting Agarose Gel and Running the Electrophoresis.

The agarose slice was removed from the tube and attached to a tooth comb using a spatula. The comb was placed within a BIORAD agarose gel casting unit. 150 ml solution of 1% SeaKem Gold agarose was prepared and cooled to 50ºC. The 1% SeaKem Gold agarose was poured into the agarose gel casting unit and allowed 25min for the gel to solidify. The electrophoresis chamber of a BIORAD CHEF-DR electrophoresis system was filled with electrophoresis running buffer (0.5× TBE). The cooling unit was set to a run temperature of 14ºC and the running buffer was left to chill for at least 20 min before electrophoresis was commenced. The gel was removed from the casting unit and placed onto its resting position within the electrophoresis chamber of the BIORAD CHEF-DR electrophoresis system. The BIORAD CHEF-DR III electrophoresis system run parameters were program as follows: initial switch time, 2.2 sec; final switch time, 63.8 sec; voltage, 6 volts; included angle, 120º, runtime, 19 hours. The electrophoresis run was started.

Staining and Documentation of PFGE Agarose Gel.

Upon completion of electrophoresis, the agarose gel electrophoresis chamber was removed and place into a plastic container. 250 ml of ethidium bromide staining solution was added and incubated with gentle shaking at room temperature for 20-30 min. The staining solution was removed from the container, 400 ml deionized water was added to destain with gentle shaking at room temperature for 30 min. The water was changed after 15 min of destaining. The DNA fingerprint patterns were captured using the BIORAD gel documentation system.

Reagents Preparation for PFGE

TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0)

10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure water (Clinical Laboratory Reagent Water (CLRW))

1% SeaKem Gold agarose in TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0

a. Weigh 0.50 g (or 0.25 g) SeaKem Gold (SKG) agarose into 250 ml screw-cap flask.
b. Add 50.0 ml (or 25.0 ml) TE Buffer; swirl gently to disperse agarose.
c. Loosen or remove cap and cover loosely with clear film, and microwave for 30sec; mix gently and repeat for 10-sec intervals until agarose is completely dissolved.
d. Recap flask and return to 55- 60ºC water bath and equilibrate the agarose in the water bath for 15 minutes or until ready to use.

Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0

10 ml of 1 M Tris, pH 8.0
20 ml of 0.5 M EDTA, pH 8.0
Dilute to 100 ml with sterile Ultrapure water (CLRW)

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl)

25 ml of 1 M Tris, pH 8.0
50 ml of 0.5 M EDTA, pH 8.0
50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)
Dilute to 500 ml with sterile Ultrapure water (CLRW)