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DEPARTMENT OF CLINICAL MICROBIOLOGY

PREVALENCE OF SALMONELLA AND OTHER GRAM NEGATIVE BACTERIAL INFECTIONS AMONG FEVER AND DIARRHOEAL PATIENTS VISITING HALF-ASSINI GOVERNMENT HOSPITAL

A THESIS SUBMITTED TO THE DEPARTMENT OF CLINICAL MICROBIOLOGY,

IN FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF

MASTER OF PHILOSOPHY IN CLINICAL MICROBIOLOGY

BY

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DECLARATION

Candidate's declaration

I affirm with certainty that this project work was carried out by me at Half-Assini Government Hospital, and it is the result of my own original research towards the award of Master of Philosophy in Clinical Microbiology, and that to the best of my knowledge, it contains no material previously published by another person or material which has been accepted for award of any other degree of the University, except where due acknowledgement has been made in the text.

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(Head of Department) Signature Date

DEDICATION

This thesis is dedicated in honour of my wife Philipine Esiawonam Deku. It is also to my children; Nelly Selikplim Deku and Kenny Seyram Deku.

ACKNOWLEDGEMENT

"For His grace is sufficient for me." I show my first appreciation to God. He who endowed me with all that l need and by His grace and mercy, l was able to come out with this work.

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LIST OF ABBREVIATIONS

µg	Microgram
CD4	Cluster of differentiation
CDC	Centres for Disease Control and Prevention
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
g	Gram
L	Litre
Ml	Millilitres
mm^3	Cubic millimetre
^OC	Degree Celsius
PFGE	Pulsed-Field Gel Electrophoresis
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
TBE	Tris Borate EDTA

ABSTRACT

Infections with Salmonella and other Gram negative bacteria are becoming more difficult to treat due to their resistance to commonly available antibiotics. Also, some district laboratories are not fully equipped to perform diagnostic tests to isolate and identify these pathogens. There have been reports of antimicrobial resistance as a result of production of Extended Spectrum Beta Lactamase (ESBL) in Accra and Kumasi but no such study has been done in Half-Assini. This study was therefore undertaken to determine the prevalence of *Salmonella* and other Gram negative organisms, their antimicrobial susceptibility pattern together with whether they were ESBL producers or not. Stool, blood and urine samples were collected non repetitively from patients with fever and diarrhoea and were cultured on their respective appropriate media. Growths that occurred were transferred onto Nutrient agar for biochemical and serological identification using the standard methods. Antimicrobial susceptibility of the isolates was done using disc diffusion Kirby Bauer method. The isolates were tested for ESBL production using double disc diffusion method. Pulsed-Field Gel Electrophoresis (PFGE) was done on *Salmonella* isolates. Prevalence of all the isolates was 10.9 %(42/384) consisting of 5 *Salmonella Choleraesuis* (from stool samples), 4 *Salmonella Typhi* (3 from stool samples and 1 from blood sample) and 27 *Escherichia coli* (23 from urine samples and 4 from blood samples) and 6 *Klebsiella pneumoniae* (2 from blood samples and 4 from urine samples). All the bacterial isolates were susceptible to ceftriaxone, cefuroxime and cefotaxime. All the *Salmonella* isolates were also susceptible to ciprofloxacin and amoxicillin/clavulanic acid. There was high level of susceptibility of the other isolates to gentamycin (81.0%), ciprofloxacin (88.1%) and amoxicillin/clavulanic acid (73.8%). None of the 42 isolates was susceptible to ampicillin and tetracycline. None of the isolates produced ESBL. Pulsed-field gel electrophoresis showed that the 5 *Salmonella Choleraesuis* were similar in terms of DNA band size and separation while the 4 *Salmonella Typhi* also had the same genetic fingerprint.

CHAPTER ONE

1.0 Introduction

Salmonella infection is a disease caused by a large group of bacteria of the genus *Salmonella* that affect man worldwide (Nesa *et al*., 2012). It belongs to a family of Gram negative bacteria called enterobacteriaceae. These bacteria share some biochemical and genetical characteristics (Baylis *et al.,* 2011). They are oxidase-negative and ferment glucose with acid production. They are aerobes and facultative anaerobes (Baylis *et al*., 2011). They are found in the intestinal tract of animals and man.

Some Gram-negative bacteria are responsible for causing food borne diseases, for example *Escherichia coli* and *Salmonella.* They also cause diseases in immunocompromised hosts, for example, *Escherichia coli, Salmonella* and *Klebsiella species* (Baylis *et al.,* 2011).

The diseases caused by *Salmonella* can be classified as non-typhoidal and typhoidal and paratyphoidal *Salmonella* infections (Baylis *et al.,* 2011). Non typhoidal salmonellosis can be defined as illnesses caused by all serotypes of *Salmonella* with the exception of *Salmonella Typhi, Salmonella Paratyphi A, Salmonella Paratyphi B* and *Salmonella Paratyphi C* (Smith *et al*., 2005). While *Salmonella Typhi* causes typhoid fever, *Salmonella Paratyphi A, Salmonella Paratyphi B* and *Salmonella Paratyphi C* cause paratyphoid fevers (Tennant et al., 2010). Both typhoid and paratyphoid fevers are termed enteric fevers (Crump et al., 2015). In severe cases, *Salmonella* infection may spread from the intestine to the blood stream. On the other hand, non typhoidal *Salmonella* causes gastroenteritis (Crump *et al.,* 2015).

Salmonella has a worldwide distribution. Even though the true prevalence is unknown, it has been estimated that 93.8 million episodes with 155, 000 fatalities occur each year

(Majowicz *et al.*, 2010). Data from the World Health Organization Global Food-borne Infections network revealed *Salmonella Typhimurium* and *Salmonella Enteritidis* account for about 80% of all human isolates (Vieira *et al*., 2009).

In Ghana, multi drug resistance development by Gram negative bacteria to commonly available antibiotics remains a major challenge for the health care system. The problem is compounded by the production of Extended-Spectrum Beta Lactamases (ESBLs). ESBLs are enzymes produced by certain bacteria types that are able to hydrolyze extended spectrum cephalosporins and are therefore effective against beta-lactam antibiotics (Ghafourian *et al.*, 2012). A study in Ghana showed that out of 405 bacterial isolates tested, 57.8% were ESBL producers (Feglo, 2011). In his study, 49.4% and 61.5% of the ESBL producing isolates were *Escherichia coli* and *Klebsiella species* respectively (Feglo, 2011).

In Half-Assini Government Hospital, culture systems for bacteriology started not long ago. Meanwhile, culture and sensitivity tests being conducted on bacteriological samples so far indicate high proportions of the isolates are detected to be resistant to many of the antibiotics tested including beta lactam antibiotics. Resistance to other antimicrobials like chloramphenicol, tetracycline and cotrimoxazole are also detectable (Personal observation).

1.1 Problem statement

Many bacterial pathogens have been reported to have developed resistance to the commonly prescribed antibiotics in Ghana. Most of these studies have been done in Kumasi and Accra. It is also not known the bacterial types causing invasive infections in these areas but no such study was conducted in Half-Assini to determine the true state of affairs. As a border town, Half -Assini receives patients across the

border from La Cote d' Ivoire. This study is expected to generate data that can be compared with what pertains in the two big cities (Accra and Kumasi).

1.2 Justification

Infections caused by *Salmonella* and other Gram-negative bacteria constitute heavy public health burden. They cause diseases like gastroenteritis, enteric fevers, pneumonia, urinary tract infections and meningitis in children leading to death (Crump et al., 2015). These enterobacteria can be transmitted by consuming food and water contaminated with human and animal faeces. Waste collection and disposal is a major problem facing residents of Half Assini, with no reliable source of water. Records from the Half- Assini Hospital show high fever and diarrhoea cases reporting to the hospital daily. Widal tests conducted on some of these patients are negative, necessitating the need for further investigations into the causes of fever and diarrhoea. This study was therefore undertaken to determine whether patients with fever and diarrhoea have Salmonella or other Gram negative bacteria as the aetiology. The study will also help in identifying whether Salmonella species causing the fever and diarrhoea show similar pattern distribution. The antimicrobial susceptibility pattern together with the beta lactamase production among the isolates would be determined.

1.3 Aim of the study

To determine the prevalence and antimicrobial susceptibility of *Salmonella* and other Gram-negative bacteria isolates, together with whether they are ESBL producers, in Half Assini Government- Hospital.

1.4 Objectives of the study

i. To culture blood and urine samples for *Salmonella* and other Gram-negative organisms.

ii. To culture stool samples for isolation of pathogenic enterobacteria. iii. To perform antimicrobial susceptibility testing on the isolates obtained.

iv. To determine if the isolates obtained produce Extended Spectrum Beta Lactamase (ESBL).

v. To fingerprint the Salmonella isolates obtained.

CHAPTER TWO

2.0 Literature review 2.1 The Salmonella organism

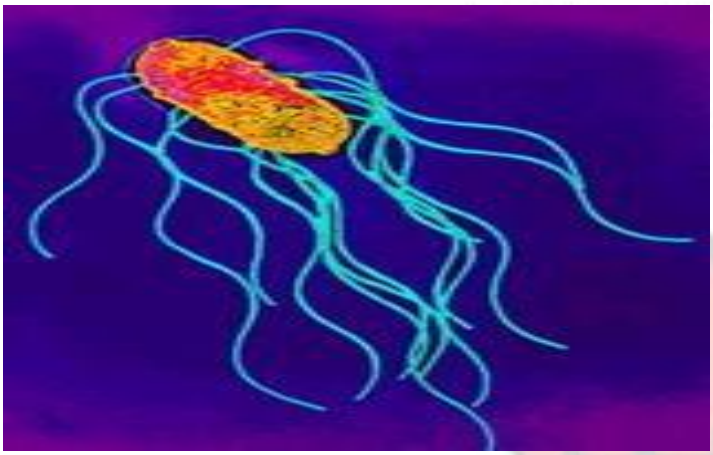

Fig. 2.1: Salmonella enterica

Source: www.textbook of bacteriology.net/themicrobialworld/Salmonella.htm l (accessed February 2, 2014 at 13: 40 GMT)

Salmonella species are Gram-negative facultative anaerobic rods. They belong to the family of enterobacteriaceae. They are non- sporing and possess peritrichous flagella (as shown in Figure 2.1) for motility except *Salmonella Gallinarum* which possesses no flagella (Agbaje et al., 2011). They can be found in the environment and can infect man (Agbaje *et al.*, 2011). *Salmonella* bacteria can survive for weeks in the environment. *Salmonella* can survive under harsh and dry conditions for a long period of time (Van Doren *et al.*, 2013). They are not destroyed by freezing but their death is accelerated by heat at 55^{o}C for ninety minutes or 60^{o}C for twelve minutes (Sorrells et al., 1970).

Other Gram-negative bacteria are aerobes or facultative anaerobes. Some are motile (for example, *Escherichia coli*) while others are non-motile (for example, *Shigella species*).

They are oxidase negative and ferment glucose with acid production (Baylis *et al.*, 2011). Whilst some are regarded as food borne pathogens (for example *Yersinia enterocolitica* and

Shigella species), others are regarded as opportunistic pathogens especially in clinical settings, for example *Klebsiella species, Citrobacter* and *Serratia species* (Baylis *et al.*, 2011).

2.2 Classification of Salmonella and other Gram negative organisms.

Gram-negative bacteria are broadly classified into two main groups based on their ability to ferment lactose, that is lactose-fermenting bacteria and non-lactose-fermenting bacteria (Agbaje et al., 2011). The genera of lactose-fermenting bacteria include *Escherichia, Klebsiella, Enterobacter* and *Citrobacter* while non lactose-fermenting genera are *Shigella*, *Proteus*, *Morganella*, *Salmonella* and many others (Agbaje et al., 2011).

The *Salmonella* organism can also be broadly classified into three groups based on host adaptability (Agbaje et al., 2011) and these are:

Group 1: *Salmonella* adapted to man. For example, *Salmonella Typhi.*

Group 2: *Salmonella* wholly or largely adapted to specific animal. For example, *Salmonella Dublin* in cattle, *Salmonella Gallinarum* in poultry, *Salmonella Abortusequi* in horses and *Salmonella Choleraesuis* in pigs.

Group 3: All other *Salmonella species* that are not host-adapted and produce infection in man. For example, *Salmonella Typhimurium* and *Salmonella Enteritidis* (Agbaje et al., 2011).

The *Salmonella* organism can also be separated into three species; *Salmonella enterica, Salmonella bongori and Salmonella substerranea* (H.-M. Chen et al., 2013). Salmonella enterica is further divided into six species based on host range specificity and these are

i. *Salmonella enterica sub species enterica ii.*

   Salmonella enterica sub species salamae

iii. *Salmonella enterica sub species arizonae*

iv. *Salmonella enterica sub species diarizonae*

v. *Salmonella enterica sub species houtenae* and vi. *Salmonella enterica sub species indica* (H.-M. Chen et al., 2013)

Strains that are pathogenic to humans are in group one, that is *Salmonella enterica sub species enterica* (Crum-Cianflone, 2008). This group is further categorized into typhoidal and non-typhoidal *Salmonella* (Abdullahi et al., 2012). While the typhoidal *Salmonella* causes typhoid fevers (Martins et al., 2011), the non-typhoidal *Salmonella* causes gastroenteritis (Majowicz et al., 2010) .

Salmonella nomenclature is based on the biotype, subspecies and serotype. For example, the *Salmonella* subspecies and serotype designation for typhimurium is *Salmonella enterica subspecies enterica serotype typhimurium*. This taxonomic designation was later changed to *Salmonella serovar Typhimurium*. The current and internationally accepted name is *Salmonella Typhimurium* (Velge et al., 2012).

2.3 Epidemiology and burden of *Salmonella* and other Gram negative bacterial infection

Salmonella is an important public health problem globally (Eguale et al., 2015). *Salmonella* has a worldwide distribution although serovar distribution may be different in different countries and regions (Agbaje et al., 2011). Certain serovars show high liking for causing bacteraemia. However these serovars differ from one country to the other (Gordon et al., 2008; Kiratisin, 2008). Predominant *Salmonella* species causing infection are *Salmonella Typhimurium* and *Salmonella Enteritidis* (Yamatogi et al., 2015).

Salmonella causes majority of bacterial gastroenteritis worldwide (Majowicz et al., 2010). 94.3 million cases of gastroenteritis are reported globally each year with a case fatality of

155,000 (Majowicz et al., 2010). Annual estimated infections caused by *Salmonella* in United States stands at 1.2 million with 450 deaths (Boore et al., 2015).

In Ontario, investigations into non typhoidal Salmonella revealed that 2%-8% of the infection was associated with bacteraemia and extra intestinal focal infections (eg. arthritis, meningitis, pneumonia) occur in 5%-10% of those with bacteraemia (Matheson et al., 2010).

A study in Malaysia by Dhanoa and Fatt on 55 non-typhoidal *Salmonella* bacteraemic patients projected *Salmonella Enteritidis* as having the highest blood invasive potential (Dhanoa & Fatt, 2009).

It is estimated that, among hospitalised patients in Africa, case fatality for invasive nontyphoidal Salmonella have been between 4.4% and 27% in children and between 22% and 47% in adults (Gordon et al., 2008).

A study in Kenya revealed that infection with non typhoidal *Salmonella* occurred in 5.5% of children with a case fatality of 21% to 36% (Oneko et al., 2015) . In Queen Elizabeth Central Hospital in Malawi, a study revealed that typhoid cases increased from fourteen per year between 1998 to 2010 to 843 in 2013 (Pitzer et al., 2015).

A study in Democratic Republic of Congo on suspected bacteraemic patients revealed that 1.4% was Salmonella Typhi and non typhoidal Salmonella accounted for 5.9% of the suspected cases(Kalonji et al., 2015). A retrospective study in Gambia showed that *Salmonella* bacteraemic case was 0.8% (106/13, 905) (Kwambana-Adams et al., 2015).

During 2003 and 2004 in South Africa, 1,138 cases of invasive non-typhoidal *Salmonella* were isolated and microbiologically confirmed out of which *Salmonella Typhimurium* and *Salmonella Enteritidis* accounted for 67% and 7% respectively.

In a study to determine the incidence of urinary tract infection in pregnant women in Nigeria, it came out that the incidence of *Escherichia coli* was 30% while that of *Klebsiella species* was 20.4% (Okonko et al., 2009).

In Ghana, it had been reported that the prevalence of enterobacteriaceae was 46.7% when research was conducted into the prevalence and associated risk factors of asymptomatic bacteriuria in ante-natal clients (Labi et al., 2015) whilst typhoid fever cases accounted for 3.2% of all infections recorded in three hospitals in Kumasi, Ghana (Saana et al., 2014).

2.4 Age distribution and burden of infection caused by Salmonella and other Gram negative bacteria

The infection with non-typhoidal *Salmonella* is mostly skewed towards the age extremes. A study conducted in Malawi showed that out of 2,517 children with non-typhoidal *Salmonella* bacteraemia, 85% were aged less than three years with estimated 19% to 35% of them being HIV positive (Gordon et al., 2008). In a study on non-typhoidal *Salmonella* infection in children relating age and infecting serotype, it has been reported that the predominant isolate was *Salmonella Typhimurium* isolated from people within the age bracket of two weeks to twenty years and that the chance of isolating *Salmonella* bacteria was high in the first three months of life (Asmar & Abdel-Haq, 2016).

In adults, there was a remarkable gender difference in the age at which the invasive disease acquisition occurs. In South Africa, invasive non-typhoidal *Salmonella* occurred in women at a younger age than men; a median age of 30 and 35 five years respectively (Feasey *et al.*,

2010). Similar results were recorded in Malawi with thirty three years for women and 37 for men with HIV as the major risk factor (Gordon et al., 2008).

A study in South Africa and Malawi showed a similar age distribution for the invasive disease in these countries; with a bimodal age distribution (Feasey *et al.*, 2010). A very low infection rate was also recorded in the first few months of life in Malawi (MacLennan et al., 2008) with 54% of the cases (2176/4044) seen in children below fifteen years while in South Africa, 32 % of the infection occurred under that same age (Feasey et al., 2010).

Shigellae and enterotoxigenic strains of *Escherichia coli* are important causes of diarrhoea associated morbidity and mortality in infants and children under five years
(Lamberti et al., 2014)

2.5 Pathogenesis of *Salmonella*

Salmonellae can cause a range of important diseases in humans (Achouri et al., 2015). To cause infection, Salmonella is first ingested and pass through the digestive system to reach the small intestine (Velge et al., 2012). For disease to occur in healthy humans, an infectious dose of 10^6 Salmonella cells must be ingested (Chen et al., 2013). This infectious dose may be reduced to 10^3 Salmonella cells in elderly with low gastric acidity and individuals who use antacids (Raffatellu et al., 2006). However, higher infectious dose is associated with higher attack rate and shorter interval to bacteraemia (Crump et al., 2015).

Salmonella species penetrate and multiply within the host cell, including macrophages and epithelial cells (Figueira & Holden, 2012). To do this, the bacteria pass through the lacteals of the small intestine and enter the lymph node. They then gain entrance to the blood stream resulting in septicaemia.

Salmonella is transported to various tissues by hijacking phagocytic cells; the body's defence system, thereby evading destruction by macrophages (Velge *et al*., 2012). The bacteria are also able to enter and invade the reticuloendothelial system, where they stay in the liver, spleen, bone marrow and lymph nodes(Raffatellu et al., 2006).

The Salmonella bacteria continue to replicate until adequate density is reached after which they break out into the bloodstream to invade other parts of the body (Afroza, 2003). The bacteria then re-enters the gastrointestinal tract and re-infect the Peyers patches and are shed in the stool(Afroza, 2003).

2.6 Clinical presentation of Salmonella and other Gram negative bacteria

Clinical presentation of non-typhoidal Salmonella infection is not specific, both in children and adults (Crump et al., 2015). Infection with Salmonella bacteria can be associated with fever, headache, diarrhoea, vomiting, splenomegaly and hepatomegaly, anorexia, malaise, dry cough and sore throat (Crump et al., 2015).

Other Gram negative bacteria can cause urinary tract infections, blood stream infections, pneumonia caused by *Klebsiella pneumoniae* and intra abdominal infections (Paterson, 2006).

Incubation period of Salmonella and other Gram negative bacteria is dependent on the host immune response and the infectious dose (Acheson & Hohmann, 2001). The illness usually lasts for 4-7 days and in immunocompetent host, treatment is not usually needed because the person's immune system will overcome the infection. In addition, immunocompromised patients, infants and the elderly are likely to have a severe illness (Acheson & Hohmann, 2001).

Complications such as endovascular infection, deep bone or visceral abscesses and septic metastases may develop and they are difficult to treat (Acheson & Hohmann, 2001). Arthritis and septicaemia can also be caused by *Salmonella* (Dhanoa and Fatt, 2009b) and urinary tract infection (Jones et al., 2008) can also be caused by *Salmonella* and other Gram negative bacteria.

2.7 Antimicrobial susceptibility Salmonella and other Gram negative bacteria

Multidrug resistance is described as resistance to three or more classes of antimicrobial agents as defined by the Clinical and Laboratory Standards Institute (CLSI) (Imanishi & Chai, 2013).

In United States, fluoroquinolones like ciprofloxacin and third generation cephalosporins (for example, ceftriaxone) are commonly used to treat severe *Salmonella* infections (Imanishi & Chai, 2013). According to the CDC, 38% of *Salmonella Enteritidis* and 21% of *Salmonella Typhimurium* were resistant to nalidixic acid. Again, 32% of *Salmonella Typhimurium* and 24% of *Salmonella Heidelburg* were resistant to ceftriaxone (Imanishi & Chai, 2013).

It has been reported that *Salmonella Typhi* and *Salmonella Paratyphi A* were all resistant to ampicillin. In the said study, *Salmonella Typhi* and *Salmonella Paratyphi A* showed 93.8% and 66.7% resistance to chloramphenicol. There was 100% susceptibility of the *Salmonella* bacteria isolates to ciprofloxacin (Abdullahi et al., 2012).

In Cote d'Ivoire, it was found that resistance of Salmonella isolates to amoxicillinclavulanic acid was 58.1%. Again, 8.1% and 14% of the isolates were resistant to cefotaxime and chloramphenicol (Boni-Cissé et al., 2012). In Central African Republic, it was reported that in a study to determine the antimicrobial resistance in *Salmonella* isolates, the isolates were

highly susceptible to ciprofloxacin (98%) and gentamycin (97%) (Mossoro-Kpinde et al., 2015). According to them, resistance of *Salmonella* bacteria to four or five antibiotics is also common in Africa(Mossoro-Kpinde et al., 2015).

Resistance of other Gram negative bacteria especially *Klebsiella species* and *Escherichia coli* is of major concern. In their studies, Laxminarayan and his colleagues reported that resistance of enterobacteriaceae to carbapenem rose from 0.0% in 2001 to 1.4% in 2010, with *Klebsiella species* showing the highest resistance (Laxminarayan et al., 2016). They revealed that 71% of *Klebsiella species* and 50% of *Escherichia coli* were resistant to gentamycin (Laxminarayan et al., 2016) . In the said study, nearly 100% of *Klebsiella species* were resistant to ampicillin(Laxminarayan et al., 2016).

A study in Nigeria on diarrhoeagenic *Escherichia coli* showed that the isolates were resistant to ampicillin and tetracycline (over 90%) and ciprofloxacin (7.2%)

(Odetoyin et al., 2016). A gain, in Nigeria, a study showed that the resistance pattern of *Klebsiella pneumoniae* to beta lactam antibiotics, tetracycline and cotrimoxazole was 50% to 100% (Onanuga & Selekere, 2016).

A study in Tamale, Ghana to determine the antimicrobial susceptibility to *Escherichia coli* from drinking water in the metropolis showed high susceptibility of the isolates to ciprofloxacin (94.6%), gentamycin (91.1%) and ceftriaxone (89.3%) (Adzitey et al., 2015).

2.8 Epidemiology of bacteria expressing Extended Spectrum Beta Lactamase Extended spectrum beta lactamases are defined as enzymes produced by certain bacteria that have the ability to hydrolyse extended spectrum cephalosporins (Paterson & Bonomo, 2005). They are effective against beta-lactam containing antibiotics such as ceftriaxone, ceftazidime, cefotaxime and oxyimino-monobactam (Paterson & Bonomo, 2005). Enterobacteriaceae

producing ESBL have been implicated in numerous outbreaks of infection globally (Hijazi *et al.*, 2016). This enzyme is located on the plasmids of bacteria and it is transferrable among bacterial species (Rupp & Fey, 2003). It was first described in 1983 in Germany and England(Rupp & Fey, 2003). The prevalence of ESBL producing varies from one country to the other.

In a study to determine the prevalence of ESBL producing enterobacteriaceae in Germany,it was reported that 6.8% of the enterobacteriaceae were ESBL producers (Lübbert *et al.*, 2015). In Pakistan, it was reported that ESBL production in bacterial isolates was 0.7% (3/408) (Mahmood *et al*., 2012).

Luvsansharav and his colleagues analysed faecal samples in Thailand and observed that 30%-50% of these subjects were ESBL carriers (Luvsansharav *et al*., 2012). In a study in Iran undertaken between 2007 and 2008, 45% of *Klebsiella pneumoniae* from urinary tract infections were ESBL-producers (Ghafourian et al., 2012).

A study showed that regular consumption of meat and chicken were significantly associated with high carriage rate of ESBL-producing enterobacteriaceae. ESBL production in this study was 24.8% (Hijazi et al., 2016).

In Ethiopia, ESBL production was noted in *Escherichia coli* (28.6%) *and Klebsiella pneumoniae* (25.0%) (Mulisa et al., 2015). In Lagos Teaching Hospital in Nigeria, 38 isolates with ESBL characteristics were identified. Out of this 55.3% were *Escherichia coli*, 31.6% were *Klebsiella pneumoniae*, 7.9% were *Proteus species* while *Morganella morganii* and *Clostridium freundii* each accounted for 1.6% (Mulisa et al., 2015).

A study in an orphanage in Mali showed a very high prevalence where 63% of adults and

100% of the children were found to be carrying ESBL-producing Enterobacteriaceae (Tandé et al., 2009). This was however different in Akwatia, Ghana where a study showed that no Salmonella isolates produced ESBL(Dakorah, 2014). A study in Korle Bu Teaching Hospital in Ghana, however, reported that 49.3% of 300 Enterobacteria isolates were ESBL producers (Obeng-Nkrumah et al., 2013).

2.9 Transmission of Salmonella and other Gram negative bacteria

Transmission of Salmonella and *Escherichia coli* is predominantly through the consumption of contaminated foods and water with the faeces of man (Crump et al., 2015). Infection also can be contracted from sources like undercooked or incorrectly prepared poultry and infected eggs (Upadhyaya *et al*., 2015), reptiles such as turtles, snakes and lizards which carry the bacteria in their intestine (Lukac *et al.*, 2015) and contaminated fruits and vegetables (Raja *et al.*, 2015). Direct transmission from personto-person can also occur through certain types of sexual contact as in oral-anal contact (Quinn, 2015).

According to Acheson and Hohmann, Salmonella infection can be through contacts with infected pets, nosocomial transmission, waterborne transmission and contaminated drugs and solutions (Acheson & Hohmann, 2001).

Acquisition of Escherichia coli infection can be through person-to-person through direct contact or faecal-oral route or by contaminated food or water (Scott, 1990)

Other Gram-negative organisms like *Citrobacter,Enterobacter species, Serratia marcescens, Klebsiella species* and *Proteus species* can be transmitted by the health care givers (Bagattini *et al.*, 2006).

2.10 Risk factors for transmission of *Salmonella* and other Gram negative bacteria Risk factors for transmission of Salmonella can be categorised into:

i. Environmental risk factors and ii.

Host risk factors

2.10.1 Environmental risk factors for transmission of Salmonella

2.10. 1.1 Food and water as a risk factor for transmission of *Salmonella*

Transmission of *Salmonella* can be through oral-faecal route. The occurrence is high in the rainy season where food and water become contaminated due to flooding, suggesting that environmental risk factors are important in the spread of the infection (Kariuki et al., 2006; Milledge et al., 2005). Meat, egg, chickens and dairy products have been implicated as vehicles for transmission of the bacteria (Schlundt *et al.*, 2004). The bacteria have been isolated from goats, sheep, cattle and pigs; hence undercooking of these products can serve as a vehicle for transmission (Schlundt *et al.*, 2004).

2.10.1.2 Zoonosis as a risk factor for transmission of *Salmonella* and Gram negative bacteria

Infected animals like turtles and chicken put individuals at risk of infection (Gordon, 2008). Lizards and snakes can also serve as a vehicle for transmission (Lukac et al., 2015). Handling of chicken by children is a well established resk factor for acquisition of Salmonellae (CDC, 2007).

2.10.1.3 Nosocomial risk factors for transmission of *Salmonella* and other Gram negative bacteria

Nosocomial infections are described as diseases or infections that originate or are acquired in the hospital. Nosocomial infection of *Salmonella* and *Klebsiella species* can be severe in developing countries where most of the children are malnourished or have other host risk

factors (Morpeth *et al.*, 2009). Inability or improper hand washing by health care providers exposes their patients to the risk of infection (Morpeth *et al.*, 2009).

Failure to accurately sterilise reusable materials also predisposes the patients to infection.

2.10.2. Host risk factors for transmission of *Salmonella* and other Gram negative bacteria

2.10.2.1 Age as a risk factor for transmission of *Salmonella* and other Gram negative bacteria

Extremes of age, that is children less than three months and adults above fifty years of age are at risk of infection (Matheson et al., 2010). In African population, old age is a risk factor for *Salmonella* (Morpeth et al., 2009). This is because children's immunity is not fully developed while old age is associated with waned immunity.

2.11.2.2 Exposure to antimicrobial agents as a risk factor for transmission of *Salmonella*

Wrong use of antibiotics without prescription, not following prescribed instructions predisposes one to infection caused by *Salmonella* and other Gram negative bacteria (Morpeth *et al.*, 2009). Recent use of antibiotic is a risk factor for development of diarrhoea caused by *Salmonella* (Mabey et al., 1987). Endogenous bowel flora alteration as a result of surgery or antimicrobial therapy with loss of mucosal integrity is a predisposing factor (Morpeth *et al.*, 2009).

2.10.2.3 Achlorhydria as a risk factor for transmission of *Salmonella* and other Gram negative bacteria

Salmonella cannot survive at a gastric acidic content of less than 2.5 (Tennant et al., 2011). Gastric acid secretion has been shown to be reduced during acute enteric fever (Crump et al., 2015). It can therefore be said that gastric hypoacidity in infants or caused by medications is a predisposing factor for gastrointestinal infection, including the nontyphoidal Salmonella (Gendrel et al., 1994).

2.10.2.4 Malaria infestation as a risk factor for transmission of *Salmonella* and other Gram negative bacteria

Malarial disease predisposes an individual to infection with non typhoidal *Salmonella* (Graham *et al.,* 2000). Malaria has been suspected to increase the risk of non typhoidal Salmonella infection (Morpeth et al., 2009). Malaria can lead to haemolysis which impairs macrophage and neutrophil function. People with malaria are predisposed to Salmonella infection (Ao et al., 2015).The haemolysis leads to increased iron availability to the non-typhoidal *Salmonella*.

2.10.2.5 Malnutrition as a risk factor for transmission of *Salmonella* and other Gram negative bacteria

Malnutrition leads to reduction in the immune system. Children relatively grow healthier when they are well-fed. Malnourished people are at increased risk of infection with *Salmonella* and other Gram negative bacteria (Ao et al., 2015).

2.10.2.6 HIV infection as a risk factor for transmission of Salmonella and other Gram negative bacteria

HIV infection leads to suppression of immune system. HIV infected individuals have a higher chance of contracting *Salmonella* and other Gram negative bacteria (Ao et al., 2015). *Salmonella* bacteraemia is more common in HIV-infected individuals (Graham *et al.,* 2000).

2.11.0 Prevention of infection caused by *Salmonella* and other Gram negative bacteria.

Medical treatment is needed in severe cases so as to prevent environmental contamination with infected faecal materials. (Crump et al., 2015). Personal sanitation can help prevent the infection with Salmonella and other Gram negative bacteria including *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter* and *Enterobacter*. Using poultry as pets should

be discouraged (Behravesh et al., 2014). Thorough and proper hand washing with soap and copious amount of water prior to eating or before preparation of food, as well as after visiting the toilet, changing diapers, and after touching pets or other animals can help prevent infection with *Salmonella* and other Gram-negative bacteria (Ellingson et al., 2014) and other Gram negative bacteria (Wilson *et al.*, 2016).

2.12 Other Gram negative pathogens

2.12.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, non-sporing aerobic rod (Ryan and Ray 2004). It is found in the intestinal tract, soil, water, moist environment and sewage. *Pseudomonas aeruginosa* is primarily identified by its grape-like odour it produces on bacteriological medium. It can be grown under laboratory condition at 42^{o}C (Ryan & Ray, 2004).

It is an opportunistic pathogen and causes diseases in immunocompromised hosts. It can infect burns, wounds, urinary tract can also cause blood infections. It is diagnosed by its grape-like odour on bacteriological medium. It is oxidase and catalase positive (Srinivasan *et al*., 2003).

Pseudomonas is resistant to a wide range of antibiotics. However, aminoglycosides, monobactams and polymyxins can be used to treat infections caused by *Pseudomonas aeruginosa* (Hachem *et al*., 2007).

2.12.2 *Shigella species*

Shigella is a Gram-negative, facultative anaerobic rod. It is non-motile and non-spore forming organism. They are naturally found in the intestinal tract of man (Baylis *et al.*, 2011). *Shigella species* are classified into four serogroups based on their antigenic structure and biochemical reactions (Ansaruzzaman *et al*., 1995). The groups are:

Serogroup A: *Shigella dysenteriae*

Serogroup B: *Shigella flexneri*
Serogroup C: *Shigella boydii*

Serogroup D: *Shigella sonnei* .

Shigella can be transmitted by faecal-oral route. For infection to occur, less than 100 bacteria cells are required (Mounier *et al*., 1992). The *Shigella species* invade the epithelia of the small intestine (Mounier *et al*., 1992) and replicate; spreading to neighbouring epithelia cells resulting to tissue destruction (Suzuki & Sasakawa, 2001).
The most common clinical manifestations are diarrhoea, vomiting, fever and nausea.

Diagnosis is made by isolating the bacteria in fresh faecal sample cultures. They are nonmotile and non-lactose fermenters except *S. sonnei* (Ito *et al*., 1991). Infection with
Shigella can be preventing by frequent hand washing and cooking all foods before eating (Ram *et al*., 2008).

2.12.3 *Escherichia, Klebsiella, Citrobacter and Enterobacter*

The genera *Escherichia, Klebsiella, Citrobacter and Enterobacter* are collectively referred to as coliform bacteria. They are lactose-fermenters. They cause disease in immunocompromised hosts. *E. coli* is the most commonly isolated coliforms in the hospitals. They are all motile except Klebsiella. Choice of antibiotics is determined by the isolates circulating in a particular institution (Ryan & Ray, 2004).

2.13.4 *Vibrio*

The genus *Vibrio* is a Gram-negative bacteria comma shaped rods(Thompson *et a*l.,

2005). They are motile, aerobes and found in aquatic environment. It is associated with undercooked foods. Pathogenic *Vibrio species* include *V. cholera, V. parahaemolyticus and V. vulnificus.* Transmission is by contaminated water or food.

CHAPTER THREE

3.0 Materials and methods 3.1 The study area

Half Assini is the district capital for Jomoro District. The District is located in the SouthWestern part of the Western Region. It shares boundaries with Aowin-Suaman District to the North, the Gulf of Guinea to the South, the Ellembele District to the East and the Republic of La Cote D'Ivoire to the West. The District has a population of 150,107 and an annual growth rate of 3%.The indigenes are Nzemas. The District shares boundary with Republic of La Cote d' Ivoire. The major occupations of the people are farming, fishing and trading.

3.2 Ethical approval

Ethical approval was sought from a joint committee of Komfo Anokye Teaching Hospital and School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, before the commencement of the study.

3.3 Study patients

The study subjects were patients who visited the hospital with fever and diarrhoea irrespective of their country of origin, age, gender, socio economic status or religion. Informed consent was obtained from the patients after the essence and the procedure of the study was explained to them in a language they understood, sometimes with the assistance of an interpreter.

3.3.1 Inclusion criteria of the study patients

Any patient with fever and diarrhoea visiting Half Assini Government Hospital who consented to be part of the study had their samples included. Children's consent was obtained through their parents or guardians.

3.3.2 Exclusion criteria of the study patients

Patients taking antibiotics prior to the specimen collection were excluded from the study. This is because the antibiotics can have either bactericidal or bacteriostatic effect thereby preventing the growth of the bacteria on the artificial medium, resulting to false negative result. Patients who refused to be included in the study were not also enrolled even though they were having fever and diarrhoea.

3.4 Informed consent

The purpose of the study was explained to the patients. Samples were only collected after the patients understood the purpose of the investigation and consented in writing to partake in it. Patients who declined to participate were excluded from the study.

3.5 Determination of sample size for the study

A total of 384 non repetitive urine, stool and blood samples were collected for the study. In calculating this sample size, the formula $n = \frac{Z^2(P)(1-P)}{d^2}$ was used where

i. Z(1.96) was the standard score for the confidence level of 95%.

ii. P was the sample proportion or prevalence of the disease or bacterial isolates under investigation.

iii. d(5%) was the allowable error,=0.05 (Naing et al., 2006).

0.5 was used to represent the 'P' since the value was not known and could not be estimated (Lwanga & Lemeshow, 1991).

Therefore, sample size (n) = $\frac{1.96^2 \text{x } (0.5) \text{ x } (1-0.5)}{0.05^2} = 384$ samples

Therefore, a sample size of 384 was used for this study.

3.6 Sample type

Blood, urine and stool were collected at Half Assini Government Hospital Laboratory between the period of March 3, 2014 and October 31, 2015. The samples were collected from the patients using non probability consecutive sampling method where only those who visited the hospital with fever and diarrhoea during the study period were enrolled into the study.

3.7 Isolation and identification of Salmonella and other Gram-negative bacteria

3.7.1 Media used in the isolation and identification of Salmonella and other Gram- negative bacteria

To culture, isolate and identify the Salmonella and other Gram-negative bacteria from stool, blood and urine, various bacteriological media were used. These are Brain heart infusion broth, Selenite F broth, Blood agar, MacConkey agar, Salmonella-Shigella agar, Cystine Lactose Electrolyte Deficient (CLED) agar, Urea agar, Nutrient agar, Mueller Hinton agar, Triple Sugar Iron agar, Motility-Indole-Ornithine (MIO) agar, Citrate agar and Peptone water.

The preparation of these media is shown in Appendix 3.7.1A. The manufacturer's instructions were strictly followed in the preparation of the media. The prepared media were dispensed as in Appendix 3.7.1B.

3.8 Quality control of the prepared media

Quality control was performed to determine sterility and performance of various media employed in the isolation of the organisms, by performing sterility testing and performance testing.

3.8.1Sterility testing of the prepared media

This was done by incubating 3 of each batch of 30 plates aerobically at 37^{o}C for 2 days to determine if autoclaving was adequate. If more than two colonies were seen per plate, the whole batch was discarded.

3.8.2 Performance testing of the prepared media

This was done by inoculating the media with appropriate stock culture using standard inoculating procedure using control organism of *Escherichia coli* ATCC 25922 to determine if they grew well and produced the desired growth characteristics.

3.9 Samples collection for isolation of Salmonella and other Gram negative bacteria

3.9.1 Blood sample collection

After disinfecting the site of venepuncture using 70% alcohol, about 5ml of the blood was taken from the patients and aseptically dispensed into blood culture bottle containing 15ml of brain heart infusion broth.

3.9.1.1 Incubation of the blood culture bottle

The broths were incubated aerobically at 37^{o}C for 7 days, but inspected twice daily for signs of microbial growth such as haemolysis, turbidity, coagulation of the broth, a floccular deposit on top of the broth and a surface pellicle. The first subcultures were done on Blood agar and MacConkey agar after overnight of incubation of the broth. The

Blood agar and MacConkey agar plates were then incubated at 37^{o}C aerobically overnight. The final subcultures were done on the seventh day and incubated overnight.

After the overnight incubation, the plates were inspected for growth.

3.9.2 Stool sample collection for isolation of pathogenic enterobacteria

Stool samples were received in clean, leak proof containers with tight fitting lids. The collected stool samples were immediately inoculated into Selenite F broth. The broths were

then incubated overnight at 37ºC aerobically. The broths were sub cultured onto Salmonella-Shigella agar and incubated aerobically at 37ºC for 24 hours. After the overnight incubation, the plates were inspected for growth. Pale lactose non-fermenting colonies were looked for.

3.9.3 Urine sample collection

Clean-catch mid-stream urine samples were collected from the patients. The patients were advised to produce and collect urine sample as aseptically as possible. They were instructed to void the first parts of the stream and the remaining mid stream urine was collected into sterile containers given to them. The urine samples were then cultured immediately on Cystine lactose electrolyte deficient (CLED) agar using a calibrated loop of 1/500ml (0.002ml) and incubated aerobically at 37ºC overnight. After the overnight incubation, the plates were inspected for growth. Growths that occurred were counted to determine whether there was significant growth or not. Plates with 19 or less colonies (equivalent to 10^4 or less organisms) were considered insignificant and were not followed up. The test was repeated when the colony count was between 10^4 and 10^5. However, plates with colonies more than 10^5 were followed up and identified.

3.10 Identification of *Salmonella* and Gram negative bacteria

The following procedures were followed in isolating and identifying any bacterial growth on the media:

i. Colonial morphology ii. Gram reaction iii. Biochemical reactions iv. Serology (not used for the isolation of *Klebsiella pneumoniae* and *Escherichia coli*)

v. Analytical Profile Index (API), (protocol as shown in Appendix 3.10).

The following tests were also carried out:

i. Antimicrobial sensitivity test on the isolates using the Kirby-Bauer method.

ii. Double disc diffusion test to determine if the isolates were ESBL producers or not

iii. Pulsed-Field Gel Electrophoresis was carried out to determine genetic fingerprint of the isolates.

3.11 Colonial morphology for identification of Salmonella, *Escherichia coli* and *Klebsiella pneumoniae*

Identification of the growths on agar plates was performed using their growth characteristics on their various culture plates. While Salmonellae were non-lactose fermenters, Klebsiella and *E. coli* were fermenters of lactose. The growths were then picked and Gram staining was done on them. Salmonella, Klebsiella and *E. coli* were Gram negative rods. They were further identified using biochemical and sugar fermentation tests. The isolates so identified had their identification confirmed using the API.

3.12 Identification of the organism using biochemical reactions

3.12.1 Urease test

The test was done by inoculating the test organism in a bijou bottle containing 3ml of solidified urea agar and incubated aerobically at 37ºC for up to 12 hours.

Salmonella and *Escherichia coli* are non-urease producing organisms; hence there was no change in the original colour of the medium. *Klebsiella pneumoniae* is a urease producer, changing the colour of the medium from initial orange to pink. This test was used to separate *Klebsiella* from *Salmonella* and *Escherichia coli.*

3.12.2 Motility, indole and ornithine decarboxylase test for the identification of the isolates

a. Motility

The motility test was done by inoculating motility-indole-ornithine medium with the test organism using a sterile straight wire. The set-up was incubated aerobically at 37ºC overnight. Salmonella (except *Salmonella Gallinarum*) and *Escherichia coli* were motile indicating positivity by exhibiting cloudiness of the medium or growth spreading from the line of inoculation into the entire medium. Non-motile organisms, as was the case for *Klebsiella pneumoniae* grow only along the line of inoculation, leaving the surrounding medium clear, indicating a negative result. This test was used to differentiate *Klebsiella pneumoniae* from Salmonella and *Escherichia coli*.

b. Indole test

The test was done by inoculating the test organisms into motility-indole-ornithine medium and incubating aerobically at 37ºC overnight. After overnight incubation, three drops of Kovac´s reagent were added and shaken to mix. A pinkish surface layer indicated indole positivity while non-pinkish layer indicated that the organism was not indole producer. *Escherichia coli* were positive to the test while *Salmonella species* and *Klebsiella pneumoniae* were non-indole producers.

c. Ornithine decarboxylase production test

Salmonella species (except *Salmonella Typhi* and *Salmonella Gallinarum*) and *Escherichia coli* possess the enzyme ornithine decarboxylase that removes the carboxyl group from the ornithine to indicate a positive test. This was indicated by the turbid purple coloration of the medium. *Klebsiella pneumoniae* lacks this enzyme, so gives a negative result which shows as yellow coloration at the bottom of the medium. The test was done by inoculating the motility-indole-ornithine medium with the test organism and incubated aerobically at 37ºC overnight.

3.12.3 Triple sugar iron (TSI) agar

The test was done by stabbing the butt with the test organism and streaking the slant. The inoculated medium was incubated aerobically at 37^{o}C overnight with the cap loosely closed. The reaction suggestive of non-typhoidal *Salmonella species* was alkaline slant/acid butt, gas and Hydrogen sulphide production. A reaction suggestive of *Salmonella Typhi* was alkaline slant/acid butt with small amount of H_2S production. Reaction of both *Klebsiella pneumoniae* and *Escherichia coli* in TSI medium was slant/acid butt, gas production, with no Hydrogen sulphide produced.

3.12.4 Citrate utilisation test

Klebsiella pneumoniae was citrate positive. This was exhibited as a growth and a blue colouration of the medium. *Escherichia coli* was however negative to this test. This was used to separate *Klebsiella pneumoniae* from *Escherichia coli*. The test was done by inoculating the test organism in a bijou bottle containing the citrate agar and incubated aerobically overnight at 37^{o}C.

3.13 Serologic test for suspected *Salmonella* isolates

Two separate drops of normal saline were placed on a clean labeled glass slides. A small part of the suspected Salmonella colony from overnight nutrient agar culture plate was picked and thoroughly mixed with each drop of the normal saline to obtain a smooth suspension. Drop of antisera was added to one of the bacterial suspensions on the slide. To the other drop was added a normal saline to rule out auto agglutination. Both the antisera with the bacterial suspension and the normal saline with the bacterial suspension were mixed each with a sterile loop. The slides were gently tilted back and forth for 1 minute and agglutination was looked for.

When agglutination was observed for the bacterial suspension with group ´O´ polyvalent antiserum, it was repeated for the polyvalent 'H' antiserum. Then the set up was repeated for group specific antisera. The group specific *Salmonella* isolates were then confirmed using API.

3.14 Antimicrobial susceptibility testing

To perform this test, three isolated colonies were touched using a sterile wire and emulsified in 4 mls of sterile peptone water. The turbidity was matched with 0.5 McFarland. Using a sterile swab, a Mueller Hinton agar was inoculated by dipping the swab into the inoculum and streaking the swab all over the surface of the medium. With the lid closed, 5 minutes was allowed before the antibiotic disc was placed on the Mueller Hinton agar using sterile forceps for the following antibiotics: Ampicillin (10µg),

Tetracycline (10µg), Cotrimoxazole (25µg), Gentamycin 10µg), Cefuroxime (30µg), Chloramphenicol (10µg), Ceftriaxone (30µg), Cefotaxime (30µg) amoxicillin/clavulanic acid [(20µg+10µg)] and ciprofloxacin. The plates were then incubated aerobically at 37^{o}C overnight.

Using a ruler, the diameter of the zone of inhibition was measured and compared to a set of standard chart to determine if the organisms were susceptible, resistant or intermediately susceptible depending on the diameter of the zone of inhibition produced by the organism.

3.15. Detection of Extended Spectrum Beta Lactamase using double discs diffusion method

Three isolated colonies were inoculated into 1% peptone water and the turbidity was adjusted to 0.5 McFarland. The test organism was streaked onto the Mueller Hinton agar. With the lid closed, 5 minutes was allowed before the discs [(cefotaxime (30µg) and amoxicillin/clavulanic acid (20µg+10µg)] were placed onto the inoculated Mueller Hinton, 25mm apart centre to centre. The plate was then incubated aerobically at 37^{o}C overnight.

3.15.1 Interpretation of the double discs diffusion test

After the overnight incubation, if the diameter of the zone of inhibition is extended towards the clavulanic acid disc, then the organism was identified as an ESBL producer, hence ESBL positive.

3.16 Pulsed-Field Gel Electrophoresis (PFGE) of the Salmonella isolates

The test was performed by making a suspension of Salmonella isolates from a fresh overnight culture, not more than 18 hours old, in cell suspension buffer to a turbidity of 0.70 using Dade Behring Microscan turbidity meter. One percent molten SeaKem Gold agarose was added to the cell suspension and allowed to solidify as a plug. The plug was immersed in lysis buffer containing Xbal restriction enzyme. The plug was lysed using lysis buffer to expose the genomic DNA. Restriction enzyme Xbal was used to cut the DNA into fragments at specific restriction sites. Slice was prepared and attached to tooth comb and placed in the electrophoresis chamber of BIORAD CHEF-DR electrophoresis system for electrophoresis. After the electrophoresis, the agarose gel was stained using Ethidium bromide and the image of the DNA finger print was captured using BIORAD gel documentation system. Salmonella Braenderup [H9812] was used as a control. PFGE was performed according to protocol developed Pulse Net international(Ribot et al., 2001).

3.17 Data analysis

Data generated in the study were presented in summary tables and analysed statistically. The statistical analysis was performed using Microsoft excel and Statistical Package for the Social Sciences (SPSS) version 18. Most variables were categorical or continuous.

Continuous variables were presented as mean ± standard deviation and the categorical data were presented as proportions. The continuous data were compared to each other using chi-square tests. To assess the level of significance of an assumed hypothesis, Pearson chi square test ($P<0.05$) was employed.

CHAPTER FOUR

4.0 Results

4.1: Age, gender and religion of the study patients

A total of 384 patients of all ages, gender, religious beliefs and occupational status were screened for non typhoidal Salmonella and other Gram negative bacteria. Out of the 384 patients who visited the hospital with fever and diarrhoea and chosen for the study, 140 representing 36.5% were under five years. Two hundred and forty nine (64.8%) were females compared to 135 (35.1%) males (as shown in Table 4.1). As low as 26 (6.8%) subjects were Moslem, with 358 (93.2%) being Christians (Table 4.1).

Table 4.1: Demographic characteristics of the study patients stratified by the bacterial isolates.

Demographic characteristics		Bacterial isolates				P value		
		Salmonella isolates (n=9)	E. coli (n=27)	K. pneumoniae (n=6)	Total (n=42)	P value [x]	P value [y]	P value [z]
Mean age (years)		31.1±26.3	30±23.6	22±18.0	29.1±22.9	0.16	0.59	0.14
Gender	M (n=135)	2 (22.2%)	11(40.7%)	1(16.7%)	14(33.3%)	0.32	0.79	0.27
	F (n=249)	7(77.8%)	16(59.6%)	5(83.3%)	28(66.7%)			
Sample type	Blood (n=384)	1(11.1%)	4(14.8%)	2(33.3%)	7(16.7%)	0.17	0.79	0.16
	Stool (n=384)	8(88.9%)	0(0%)	0(0%)	8(19.0%)			
	Urine (n=280)	0(0%)	23(85.2%)	4(66.7%)	27(64.3%)			
Country	Ghana (n=368)	9(100%)	26(96.3%)	6(100%)	41(97.6%)	0.56	N/A	0.63
	La Cote d' Ivoire (n=16)	0(0%)	1(3.7%)	0(0%)	1(2.4%)			
Religion	Christian (n=358)	8(88.9%)	26(96.3%)	6(100%)	40(95.4%)	0.40	0.40	0.63
	Moslem (n=26)	1(11.1%)	1(3.7%)	0(0%)	2(4.8%)			
Educational status	Tertiary (n=26)	1(20%)	0(0.0%)	1(16.7%)	2(4.7%)	0.19	0.11	**0.04**
	SHS (n=43)	0(0%)	7(25.9%)	0(0.0%)	7(16.7%)			

	JHS (n=33)	1(20%)	1(3.7%)	1(16.7%)	3(7.1%)			
	Primary (n=73)	1(0%)	2(7.4%)	3(50%)	6(14.3%)			
	Others (n=9)	0(0%)	0(0.0%)	0(0.0%)	0(0.0%)			
	None (n=200)	6(60%)	17(63.0%)	1(16.7%)	24(57.1%)			

Key: P= Used to determine level of significance, P value[x] = Salmonella isolates vrs *E. coli*, P value[y] = Salmonella isolates vrs *K. pneumoniae*, P value[z] = *E. coli vrs K. pneumoniae*, Salmonella isolates= Salmonella Choleraesuis and Salmonella Typhi, N/A= Not applicable, : M= Male, F=Female, n=total number, SHS=Senior High School, JHS= Junior High School, , Others=Crèche, Day care, Nursery or Kindergarten, None= No formal education

4.2 Bacterial isolates and the samples from which they were isolated

There were twenty seven (27) *Escherichia coli* isolated; 4 (14.8%) were obtained from blood and 23(85.2%) were isolated from urine. No *Salmonella* pathogen was isolated from urine. All the *Salmonella Choleraesuis* (5) were isolated from stool samples. One *Salmonella Typhi* out of the four isolates was isolated from blood and the rest were from stool. Whilst two (2) *Klebsiella pneumoniae* were isolated from blood samples, 4 were from urine samples. The total number of isolates obtained from blood sample was 7 (16.67%) consisting of 4 *E. coli*, 2 *Klebsiella pneumoniae* and 1 *Salmonella Typhi*. Five Salmonella Choleraesuis and 3 Salmonella Typhi were also obtained from stool. Twenty seven isolates comprising of 23 *Escherichia coli* and 4 *Klebsiella pneumoniae* were isolated from urine sample as shown in Table 4.2. There were no significant differences observed among the isolates from various sample types when Salmonella isolates and *Escherichia coli* were compared (P =0.17) and when Salmonella isolates and *Klebsiella pneumoniae* were compared (P=0.79). There was also no significant difference (P=0.16) when *Escherichia coli* and *Klebsiella pneumoniae* were compared as shown in Table 4.1.

Table 4.2: Proportions of positive bacteria isolated

Bacteria isolates	Frequency	Percentage

Salmonella Choleraesuis	5	11.9
Salmonella Typhi	4	9.5
Escherichia coli	27	64.3
Klebsiella pneumoniae	6	14.2

Table 4.3: Bacterial isolates and the samples from which they were isolated

Sample type	*S. Choleraesuis*	*S. Typhi*	*Escherichia coli*	*Klebsiella pneumoniae*
Blood(7/384)	0	1	4	2
Urine(27/280)	0	0	23	4
Stool(8/384)	5	3	0	0

4.3 Prevalence of Salmonella and other Gram negative bacteria

The bacterial isolates were isolated from 42 patients representing 10.94%. Out of the 42 bacterial isolates, 5(11.9%) were *Salmonella Choleraesuis*, 4 (9.5%) were *Salmonella Typhi.* There were 27(64.3%) *Escherichia coli* and 6(14.3%) *Klebsiella pneumoniae* isolates (Table 4.2).

4.4 Bacterial isolates and gender of the study patients

All the *Salmonella Choleraesuis* isolates were from females. *Salmonella Typhi* isolates were evenly distributed among the males and females. Twenty seven (27) patients were infected with *Escherichia coli* out of which sixteen (16) (60.0%) were females and eleven (11) (40.7%), being males (as shown in table 4.1 above). There was no significant difference between the male and female patients infected with Salmonella isolates and

Escherichia coli (P=0.32). No significant difference was also recorded when Salmonella isolates and *Klebsiella pneumoniae* were compared (P=0.79). So also was there no significant difference when *Escherichia coli* and *Klebsiella pneumoniae* were compared (P=0.27) as shown in Table 4.1.

4.5 Bacterial isolates and country of origin of the study patients

Table 4.1 shows that three hundred and sixty eight (368) of the subjects, representing 95.8% came from Ghana. Those subjects who came from La Cote d' Ivoire were 4.2%. All the *Salmonella* isolates were isolated from Ghanaian subjects. There was however no significant difference in the country of origin of the patients when *Salmonella* isolates and *Escherichia coli* were compared (P=0.56) and also when *Escherichia coli* and *Klebsiella pneumoniae* were compared (P=0.63).

4.6 Bacterial isolates and the religion of the study patients

Patients for this study came from two main religions denominations: Christianity and Moslem. No patient from other religion was recorded. There was no *Salmonella Choleraesuis* and *Klebsiella pneumoniae* isolates obtained from the Moslems (Table 4.1). Whilst Christians accounted for 93.2% of the patients visiting the Half Assini Government Hospital, those with Islamic faith were 6.8%. There was significant difference in the religion of the study patients infected with the bacterial pathogens.

4.7 Bacterial isolates and educational status of the study patients

About 52% of the patients who took part in this study had no formal education; 6.8% (26) of the patients were educated up to tertiary level. Twenty four (57.1%) of the isolates were isolated from those patients with no formal education, with none isolated from the educational group named 'others'. There was no significant difference in the educational

status of the patients infected with bacterial isolates when *Salmonella* isolates and *Escherichia coli* were compared (P=0.19) and also when *Salmonella* isolates and *Klebsiella pneumoniae* were compared (P=0.11). Significant difference was however observed when *Escherichia coli* and *Klebsiella pneumoniae* were compared (p=0.04).

4.8 Antimicrobial susceptibility and ESBL test results

Antimicrobial susceptibility test results indicated that all the forty two (42) isolates tested were susceptible to ceftriaxone, cefotaxime and cefuroxime. The *Salmonella Typhi* isolates were all susceptible to amoxicillin/clavulanic acid and gentamycin. High, but variable proportions of the isolates were susceptibility to gentamycin (81.0%), ciprofloxacin (88.1%) and amoxicillin/clavulanic acid (73.8%). None of the 42 isolates was susceptible to ampicillin and tetracycline. Varied level of resistance of the isolates to the antimicrobial agents was also observed against chloramphenicol (97.6%) and cotrimoxazole (85.7%). None of the 42 bacterial isolates produced ESBL. The details of these results are presented in table 4.4.

Table 4.4: Antimicrobial susceptibility test results of the isolates obtained at Half Assini.

Antibiotics	Bacterial isolates/number sensitive				
	Salmonella Choleraesuis(n=5)	*Salmonella Typhi* (n=4)	*Escherichia coli (n*=27)	*K. pneumoniae* (n=6)	Total susceptible
Ceftriaxone	5 (100%)	4 (100%)	27(100%)	6 (100%)	42 (100%)
Cefotaxime	5 (100%)	4 (100%)	27 (100%)	6 (100%)	42 (100%)
Cefuroxime	5 (100%)	4 (100%)	27 (100%)	6 (100%)	42 (100%)
Ciprofloxacin	5 (100%)	4 (100%)	24 (88.9%)	5 (83.3%)	37 (88.1%)
Gentamycin	4 (80%)	4 (100%)	21 (77.8%)	5 (83.3%)	34 (81.0%)
Amoxicillin/clavulanic acid	5 (100%)	4 (100%)	17 (63.0%)	5 (83.33%)	31 (73.8%)
Cotrimoxazole	0 (0%)	1 (25.0%)	2 (7.4%)	3 (50.0%)	6 (14.3%)
Chloramphenicol	0 (0%)	1 (25.0%)	0(0.0%)	0 (0.0%)	1 (2.4%)
Ampicillin	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0(0.0%)
Tetracycline	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0(0.0%)

4.9 Characterization of Salmonella isolates by Pulsed –Field Gel Electrophoresis The Salmonella isolates were characterised by Pulsed –Field Gel Electrophoresis (PFGE) by detecting variations or otherwise among them at the level of their Deoxyribonucleic Acid (DNA) as shown in Figure 4.1. All the *Salmonella Choleraesuis* showed similar pattern of DNA separation. The *Salmonella Typhi* isolates also showed similar separation of bands when subjected to electrical field.

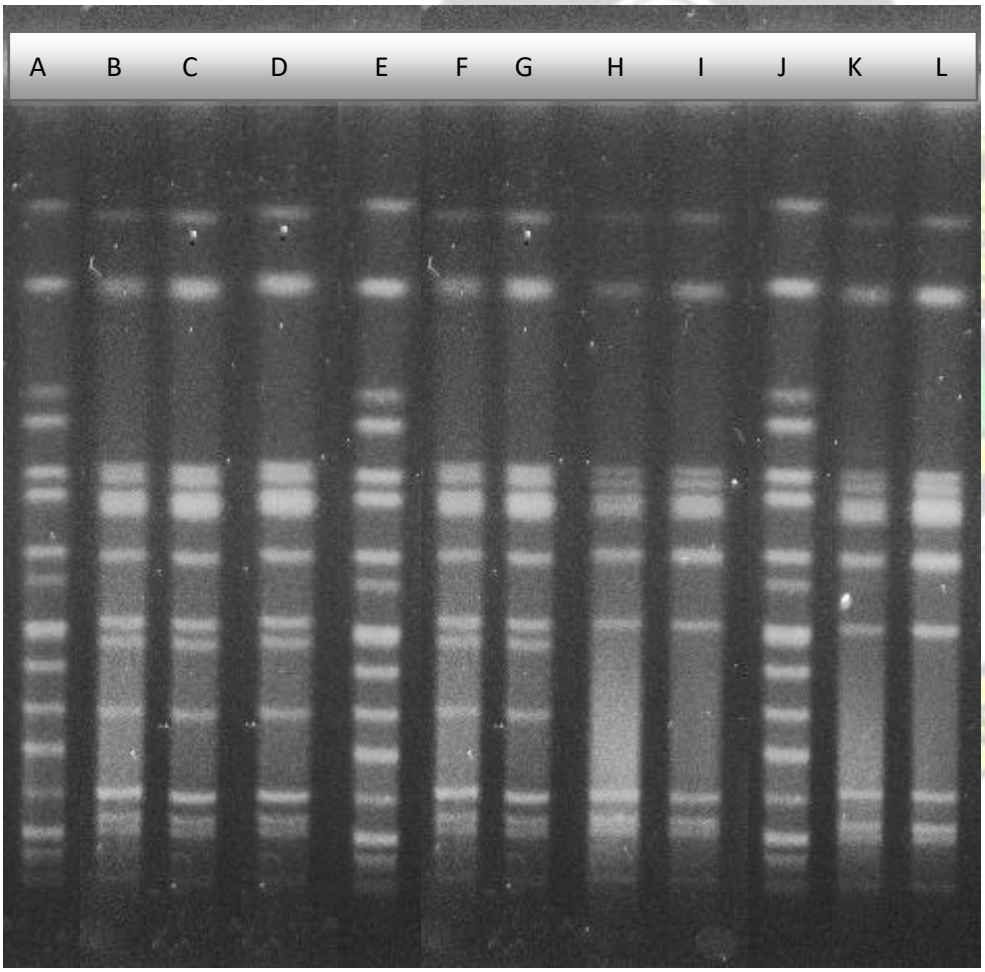

Figure 4.1 PFGE images of Salmonella Choleraesuis and Salmonella Typhi.

A, E and J were the controls used [*Salmonella Braenderup* (H9812)].

B, C, D, F and G were the test organisms (*Salmonella Choleraesuis*).

H, I, K and L were the test organisms (*Salmonella Typhi*).

CHAPTER FIVE

DISCUSSION

5.1 The bacterial isolates obtained in relation to the age of patients

There were 9 *Salmonella* isolates obtained from subjects who took part in this study. They comprised of 5 *Salmonella Choleraesuis* forming 55.6% and 4 Salmonella Typhi forming 44.6% of the *Salmonellae*. The isolation of only *Salmonella Choleraesuis* did not agree with result of a study in Brazil where *Salmonella Typhimurium* and *Salmonella Enteritidis* were the predominant serovars isolated (Yamatogi et al., 2015). This is probably due to the ecological factors which affect serovar distribution (Agbaje et al., 2011). These ecological factors include the environmental cleanliness, availability of safe water source and the presence of animal reservoir for transmission.

The *Salmonella Choleraesuis* isolates were mostly obtained from subjects at the age extremes. In this study, 40% of the non-typhoidal *Salmonellae* were isolated from patients under 3 years. Another 40 %(2/5) were also isolated in subjects above 70 years. This is in conformity with a study in Kenya where children less than 3 years were at a higher risk of nontyphoidal *Salmonella* infection (Brent et al., 2006). It is similar to a study carried out in Malawi where 85% of the non-typhoidal *Salmonellae* were obtained from children less than 3 years (Gordon, 2008). This could be attributed to the transmission of the *Salmonella* bacteria being through faecal-oral route, as children play in the environment, they pick the pathogen from the contaminated ground into their month (Rivera-Chávez & Bäumler, 2015) .

Old age has been reported as a risk factor for acquisition of non-typhoidal *Salmonella* in African population (Morpeth et al., 2009). This is so because the elderly have waned (reduced) immunity. Matheson and his co researchers also ascertained this in their study where they stated that adults above 50 years were at a risk of infection (Matheson et al., 2010). This is consistent with the results obtained in this study where 40% of the nontyphoidal Salmonella were isolated in subjects above 70 years.

The age of the patients infected with other Gram negative organisms was evenly distributed, that is the organisms show no age preference. This finding supports a report in Kumasi, Ghana where *Klebsiella pneumoniae* and *Escherichia coli* showed no significant preference for age *(*Feglo, 2011). This is so because the isolates were opportunistic pathogens and they can only infect and cause diseases when the opportunity is created. This current report was not in agreement with the findings by Preethishrees and others where both *Escherichia coli* and *Klebsiella species* were mostly isolated in patients between sixty one years to seventy years (Preethishree et al., 2016). This could probably be due to differences in the age of the patients recruited into the study.

5.2 The bacterial isolates obtained in relation to the gender of patients

More females were infected with the bacterial isolates (66.7%) as compared to the male patients (33.3%).This may be due to the fact that women mostly perform domestic duties like washing and bathing of the children as well as farming activities, hence they are more prone to the infection as compared to their male counterparts who mostly engage in sea fishing. In the present study, female patients constituted majority of the patients hence the higher possibility of isolating bacteria from them.

There was no significant difference in the gender of the patients infected with the bacterial isolates. This may be due to the fact the lactose-fermenting isolates are opportunistic pathogens and can equally infect both genders if the opportunity is created.

5.3 The bacterial isolates obtained in relation to the sample from which they were isolated

All the *Salmonella Choleraesuis* were isolated from stool sample, with none from the blood and urine. The *Salmonella Choleraesuis* is largely adapted to pigs (Agbaje et al., 2011). Isolation of these bacteria in man may mean faecal contamination of the environment by pigs or consumption of undercooked pork (Agbaje et al., 2011).

In this current study, the predominant isolates were *Escherichia coli* and were obtained from urine samples. A similar pattern was also seen in South Africa where *Escherichia coli* bacteria were responsible for causing 75% to 95% of urinary tract infections in women (Van Schoor, 2016). The high level of isolation of *Escherichia coli* from urine sample was because unlike *Klebsiella pneumoniae* and *Salmonella* isolates, *Escherichia coli* are able to adapt to bladder environment, causing urinary tract infections (S. L. Chen et al., 2013).

5.4 Antimicrobial susceptibility of the bacterial isolates

All the 42 isolates were susceptible to ceftriaxone, cefotaxime and cefuroxime. In Half Assini Government Hospital where the facility for culture and sensitivity is available, clinicians most of the time rely on the laboratory result before prescribing antibiotics. This could have accounted for high level of susceptibility of the bacterial isolates to these antibiotics. This agrees with a study in Tamale, Ghana where high susceptibility was reported for ceftriaxone (Adzitey et al., 2015).

All the 9 Salmonella isolates were susceptible to ciprofloxacin. This is in conformity with what was reported in Akwatia, Ghana where there were high level of susceptibility of the

Salmonella isolates to ciprofloxacin (Dakorah, 2014). This could be as a result of high cost of ciprofloxacin making it inaccessible to most of the patients in Half Assini. It however did not support a study by Imanishi and Chai in United States of America where susceptibility was 68% for *Salmonella Typhimurium* and 76% for *Salmonella Heidelberg* (Imanishi & Chai, 2013).

All the isolates tested against ampicillin and tetracyclines were resistant in this study. These antibiotics have been abused over the years. They are not expensive and can easily be bought over the counter.

Bacterial isolates were resistant to chloramphenicol (97.1%) and cotrimoxazole (85.7%). This is in conformity with a study by Odetoyin and his researchers in Nigeria where high level of resistance by *Escherichia coli* was reported against ampicillin and tetracycline (Odetoyin et al., 2016). These antibiotics have been used indiscriminately in humans and poultry. They were put into poultry feeds and used as growth promoters, accounting for high level of resistance recorded. (Odetoyin et al., 2016).

Susceptibility or resistance to antimicrobial agent can be influenced by many factors among which can be abuse of antimicrobial agents and inaccurate dosage by over-the counter medicine sellers and patients not following complete course of treatment. These have led to different susceptibility patterns around the globe.

5.5 The bacterial isolates and extended spectrum beta lactamase production All the 42 isolates were non ESBL producers. This is consistent with a study conducted by in La Cote d' Ivoire in 2012 where no Salmonella isolates produced ESBL (Boni-Cissé et al., 2012). On the contrary, a study in Korle Bu Teaching Hospital reported high ESBL production (47%) among 300 enterobacteria (Obeng-Nkrumah *et al.*, 2013). The antibiotic usage in Korle Bu will be higher than in Half Assini. The teaching hospital is situated in

the Accra, Ghana where most patients can easily afford expensive antibiotics, even without prescriptions. On the contrary, Half Assini is rural, so many isolates might not have encountered many of the antibiotics in circulation.

In Half Assini Hospital where this present study was carried out, strict adherence to safe and hygienic practices, including hand safety is the norm. This is to prevent the case where health care givers become vehicle for transmission. The hospital has the facility for culture and sensitivity, guiding the clinicians on the choice of antibiotics; hence the case where clinicians prescribed antibiotics without laboratory investigations is not part of the hospital culture.

CHAPTER 6

6.0 Conclusion and recommendation

6.1 Conclusion

Samples from 384 patients were cultured. A total of 42 isolates were obtained. Seven (16.7%) of the isolates were obtained from blood and 8(19.0%) were also obtained from stool. The total number of bacteria isolated from urine was 27 being 64.3% of the total isolates. The prevalence of *Salmonella* isolates was 2.3% and that of *Klebsiella pneumoniae* was 1.6%. *Escherichia coli* prevalence was at 7.0%.

This study revealed that non-typhoidal *Salmonella* (*Salmonella Choleraesuis*) affect more females than males and they mostly affect females at their age extremes. The *Salmonella Choleraesuis* isolated were not blood invasive.

All the bacterial isolated were susceptible to ceftriaxone, cefotaxime and cefuroxime. All the *Salmonella* isolates were also susceptible to ciprofloxacin. High but varied proportions of the isolates were also susceptible to gentamycin (88.10%) and amoxicillin/clavulanic acid (80.95%). All the isolates were resistant to tetracycline and ampicillin. Most of the isolates were also resistant to chloramphenicol (97.62%) and cotrimoxazole (85.72%).

None of the isolates produced ESBL.

From PFGE result, all the 5 *Salmonella Choleraesuis* exhibited similar separation of DNA. The 4 Salmonella Typhi also exhibited related based on DNA band size and separation.

6.3 Recommendations

i. Ceftriaxone, cefotaxime and cefuroxime are recommended for treatment of infections caused by *Salmonella Choleraesuis, Salmonella Typhi, Escherichia coli* and *Klebsiella pneumoniae*.

ii. More health education should be done on the spread and prevention of Salmonella and other Gram negative bacteria as well as on the judicious use of antibiotics. iii. Equipment for culture and sensitivity should be provided to district hospitals in the country. This will help reduce the case where clinicians prescribe antibiotics without testing.

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APPENDICES

Appendix 3.7.1A: Composition and preparation of culture media

Composition of brain heart infusion broth (LAB M LIMITED, UNITED KINGDOM)

i. Brain-Heart Infusion solids(Porcine) 17.5g

ii. Tryptose 10.0g iii. Glucose 2.0g

iv. Sodium chloride 5.0g v. Disodium Hydrogen Phosphate 2.5g

vi. Distilled water 1L

Preparation of brain heart infusion broth

37g of the powder was dissolved in 1L of distilled water. The suspension was soaked for 10 minutes, swirled to mix and warmed gently to dissolve the powder completely. The mixture was dispensed into culture bottles and sterilise by autoclaving at 121^{o}C at 15 pounds pressure for 15 minutes.

Composition of Selenite F Broth (Rapid Labs, United Kingdom)

i. Peptone 5.0g ii. Lactose 4.0g

iii. Sodium phosphate 10.0g iv. Sodium Selenite 4g

v. Distilled water 1L

Preparation of Selenite F broth

4g of Sodium Selenite and 19g of powder was added to 1L of distilled water.

Mixture was warmed until the powder dissolved.

Mixture was distributed into bottles and sterilised for 10 minutes in boiling water.

Composition of blood agar base (ACCUMIX, INDIA)

i. Beef Heart 500g ii. Sodium chloride 5.0g iii. Tryptose 10.0g

iv. Agar 15.0g

Preparation of blood agar base

40.0g of the powder was suspended in 1L of distilled water. Suspension was thoroughly mixed.

Mixture was brought to boil with frequent agitation to dissolve the powder completely.

Mixture was sterilised by autoclaving for 15minutes at 15 pounds pressure at 121^{o}C.

Blood agar was prepared from this as follows:

The base was cooled to 45^{o}C and 5%v/v sterile blood was added. The blood and the base were thoroughly mixed well. Plates were poured into sterilised petri plates.

Composition of MacConkey agar (ACCUMIX, INDIA)

i. Peptone 20.0g
ii. Lactose 10.0g
iii. Neutral red 0.04g
iv. Sodium taurochlorate 20.0
v. Agar 20.0g

Preparation of MacConkey agar

55.0g of the powder was suspended in 1000ml distilled water and thoroughly mixed. Suspension was brought to boil with frequent agitation to dissolve the powder completely

Mixture was sterilised by autoclaving at 121^{o}C at 15 pounds pressure for 15 minutes.

Composition of Salmonella-Shigella agar (RAPID LABS, UNITED KINGDOM)

i. Peptones 4.0g ii.
Beef extract 4.og iii.
Lactose 10.0g
iv. Brilliant green 0.33mg
v. Bile salts 8.0g vi.
$Na_2S_2O_3$ 8.0g
vii. Sodium citrate 8.0g
viii. Ferric citrate 1.0g ix.
Neutral red 0.025g
x. Agar 11.50g

xi.	Distilled water	1L

Preparation of Salmonella Shigella agar

54.5g of the powder was added to 1L of distilled water.

The suspension was swirl to mix and brought to boil.
The mixture was allowed to cool to 47^{o}C, mixed well and the plates were poured.

Composition of Cystine Lactose Electrolyte Deficient (CLED) agar

i.	Tryptone	4.0g ii.
	Peptone	4.0g iii.
	Lactose	10.0g
iv.	Beef extract	3.0g
v.	L- cystine	0.128g
vi.	Bromothymol blue	0.02g
vii.	Agar	15.0g
viii.	Distilled water	1L

Preparation of cystine lactose electrolyte deficient (CLED) agar

36.15g of the powder was suspended in 1L of distilled water. The suspension was thoroughly mixed and brought to boil with frequent agitation to dissolve the powder completely. Sterilisation was done by autoclaving at 121^{o}C for 15 minutes at 15 pounds pressure.

Composition of urea agar base (BIOMARK, INDIA)

i.	Peptic digest of animal tissue	1.0g
ii.	Dextrose	1.0g

iii. Sodium chloride 5.0g iv.

Disodium phosphate 1.20g v.

Monopotassium phosphate 0.80g

vi. Phenol red 0.012g vii.

Agar 15.0g

viii. Distilled water 1L

Preparation of urea agar base

24g of the powder was suspended in 950 ml of distilled water.

Suspension was boiled to dissolve the medium completely.

Sterilisation was done by autoclaving at 10 pounds pressure at 115^{o}C for 20 minutes.

Mixture was cooled to 50^{o}C and 50ml of 40% urea solution was added and mixed thoroughly.

Mixture was dispensed into sterilised tubes and allowed to set in a slant position.

Preparation of urea solution

40g of urea powder was dissolved into 100ml of sterile distilled water and gently mixed.

Composition of nutrient agar (ACCUMIX, INDIA)

i. Peptone 5.0g

ii. Sodium chloride 5.0g

iii. Beef extract 1.5g

iv. Yeast extract 1.5g

v. Agar 15.0g

Preparation of nutrient agar

28g of the powder was suspended in 1L of distilled water.

The suspension was thoroughly mixed.

Suspension was boiled with frequent agitation to dissolve the powder completely. Sterilisation was done by autoclaving the mixture at a pressure of 15 pounds pressure for 15 minutes at 121ºC.

Mixture was allowed to cool to 45ºC and the plates were poured into sterilised petri plates.

Composition of Mueller Hinton agar

i. Casein acid hydrolysate 17.5g ii. Beef heart infusion 2.0g

iii. Starch 1.5g iv. Agar 17.0g

Preparation of Mueller Hinton agar

38g of the powder was suspended in 1L of distilled water. The suspension was thoroughly mixed.

Suspension was boiled with frequent agitation to dissolve the powder completely. Sterilisation was done by autoclaving the mixture at a pressure of 15 pounds pressure for 15 minutes at 121ºC. Mixture was allowed to cool to 45ºC and the plates were poured into sterilised petri plates.

Composition of triple sugar iron agar (BIOMARK, INDIA)

i. Peptic digest of animal tissue 10.0g ii. Casein enzymic hydrolysate 10.0g

iii. Yeast extract 3.0g iv. Beef extract 3.0g

v. Lactose 10.0g vi. Sucrose 10.0g vii. Dextrose 1.0g

viii.	Sodium chloride	5.0g ix.
	Ferrous sulphate	0.20g x.
	Sodium thiosulphate	0.30g xi.
	Phenol red	0.024g
xii.	Agar	12.0g
xiii.	Distilled water	1L

Preparation of triple sugar iron agar

65g of the powder was suspended into 1L of distilled water.

Suspension was brought to boil, mixed well and distributed into tubes.

Sterilisation was done by autoclaving the mixture at a pressure of 15 pounds pressure for 15 minutes at121^{o}C.

The medium was allowed to set in a slope form.

Composition of motility- indole -ornithine medium (BD, USA)

Pancreatic Digest of Casein	9.5g
Pancreatic Digest of Gelatin	10.0g
Yeast Extract	3.0g
Dextrose	1.5g
L-Ornithine Monohydrochloride	5.0g
Bromocresol Purple	0.02g
Agar	2.0g
Distilled water	1L

Preparation of motility- indole- ornithine medium

31g of the powder was suspended in 1L of distilled water.

The suspension was mixed well.

The suspension was brought to boil with frequent agitation for complete dissolution of the powder

The mixture was dispensed in screw-capped tubes and sterilised in an autoclave at 121^{o}C for 15 minutes at 15 pounds pressure. The prepared medium was stored at 4^{o}C.

Composition of Simmon's citrate agar (BIOMARK, INDIA)

i. Magnesium sulphate 0.20g
ii. Ammonium dihydrogen phosphate 1.0g iii.
Dipotassium phosphate 1.0g iv.
Sodium citrate 2.0g v.
Sodium chloride 5.0g vi.
Bromothymol blue 0.08g
vii. Agar 15.0g
viii. Distilled water 1L

Preparation of Simmon's citrate agar

24.28g of the powder was suspended in 1L of distilled water. Medium was boiled for complete dissolution of the powder. Medium was sterilised at 1bar pressure for 15 minutes at 121^{o}C at 15 pounds pressure.

Composition of peptone water (ACCUMIX, Belgium)

i. Peptone 10.0g
ii. Sodium chloride 5.0g iii.
Distilled water 1L

Preparation of peptone water

15g of the powder was suspended in 1L of distilled water. The suspension was mixed thoroughly

The suspension was warmed slightly with frequent agitation to dissolve the powder completely.

The mixture was dispensed into tubes and sterilised at 1bar pressure for 15 minutes at 121^{o}C 15 pounds pressure.

Appendix 3.7.1B: Dispensing of the prepared media

i. Sterile petri dishes were laid out on a level surface.

ii. Media were mixed by rotation of the flask and bottle gently. iii. The necks of the flasks and bottles were flame sterilised. iv. 15ml of the medium was poured into the petri dish and allowed to set.

v. Prepared media were stored in a refrigerator at a temperature of 4^{o}C.

APPENDIX 3.10 IDENTIFICATION OF ISOLATES USING ANALYTICAL PROFILE INDEX

API ID 32E is a standardised, miniaturized microtube system consisting of 32 biochemical tests for identification for Enterobacteriaceae and other non- fastidious Gram negative rods. The 32 microtubes contain dehydrated substrates.

The 32 biochemical tests were:

- ODC- Ornithine decarboxylase
- ADH- Arginine dihydrolase
- LDC- Lysine decarboxylase
- URE- Urease
- LARL- L-Arabitol (Acidification)
- GAT- Galacturonate (Acidification)
- 5KG- 5- Ketoglutarate (Acidification)
- LIP- Lipase

- RP- Phenol red(Acidification)
- βGLU- β-Glucosidase
- MAN- Mannitol (Acidification)
- MAL- Maltose (Acidification)
- ADO- Adonitol (Acidification)
- PLE- Palatinose (Acidification)
- βGUR- β-Glucuronidase (Acidification)
- MNT- Malonate
- IND- Indole production
- βNAG- N-acetyl-β-glucosaminidase
- βGAL- β-Galactosidase
- GLU- Glucose(Acidification)
- SAC- Saccharose (Acidification)
- LARA- L-Arabinose (Acidification)
- DARL- D-Arabitol (Acidification)
- αGLU- α-Glucosidase
- αGAL- α-Galactosidase
- TRE- Trehalose (Acidification)
- RHA- Rhamnose (Acidification)
- INO- Inositol (Acidification)
- CEL- Cellobiose (Acidification)
- SOR- Sorbitol (Acidification)
- αMAL- α-Maltosidase
- AspA- L-Aspartic acid arylamidase

In performing the test, a single well isolated colony was emulsified in an ampoule containing 3ml sterile normal saline until the turbidity was equivalent to

0.5McFarland. Each of the 32 microtubes was filled with 55µl of the suspension with automatic pipette.

The following tests were covered with by overlaying 2 drops of mineral oil so as to obtain anaerobic conditions: ODC, ADH, LDC, URE, LARL, GAT and 5KG. They were then incubated at37^0C for 24 hours aerobically.

After the 24 hour incubation period, were read using the mini API instrument. One drop of James reagent was added to the 'IND' well (to reveal the indole reaction) before it was read. Positive test results were scored as a seven digit number (profile). The identity of the bacteria is then derived from the database with relevant identification software.

Appendix 3.12.1 Principle of urease test

The basis of this test is to test the ability of the organism to produce urease which splits urea into ammonium and carbon dioxide. The alkaline product is detected by phenol red which is pink in alkaline medium and yellow in acid medium, thus a positive reaction will appear as pink-red.

Appendix 3.12.2. Principle of the motility, indole, ornithine test.

Motile organisms cause turbidity in the medium as they diffuse from the stab line. Organisms initially ferment dextrose forming acidic environment which cause the bromo cresol purple which is a pH indicator to change from purple to yellow. If the organisms possess ornithine decarboxylase, the ornithine will be decarboxylated to putrescine which increases the PH making it alkaline, resulting to a colour change from the yellow to purple. Indole production is detected by the ability of the organisms to break down tryptophan to indole by the action of tryptophanase.

Appendix 3.12.3 Principle of the triple sugar iron agar test

Triple sugar iron agar contains dextrose, sucrose and lactose with phenol red and ferrous ammonium sulphide as indicators.If the organism ferments only dextrose, an acidic environment is created in both the slant and the butt, creating a yellow colour in the butt. On the slant however, aerobic fermentation or respiration takes place and not enough acid is produced to lower the Ph, so the colour remains red.

If sucrose or lactose is fermented, enough acid is produced resulting in the phenol red indicator turning yellow in both the slant and the butt. Gas production may result from the fermentation and can be detected as bubbles trapped in the agar or splitting of pushing the agar upwards.

Hydrogen sulphide production is detected by the ferrous ammonium sulphate incorporated into the medium. This reacts with Hydrogen sulphide forming ferrous sulphide; an insoluble black precipitate.

Appendix 3.12.4 Principle of the citrate test

The principle of this test is to test the ability of the organism to utilise citrate as the only carbon source for metabolism. If this occurs, an alkaline environment is created which changes the initial green colour to blue.

Appendix 3.13.0 Principle of the serological test

The principle of this test is the clumping or agglutination of the suspected Salmonella isolates with their specific antibodies in the antiserum. Because the antibodies were known, the antigen can be detected since antibodies react with their homologous antigens.

Appendix 3.14 Preparation of 0.5 McFarland

i. 1% v/v solution of sulphuric acid was prepared by adding 1 ml of the concentrated acid to 99ml of water.

ii. 1%w/v of barium chloride was prepared by dissolving 0.5g of barium chloride in 50ml distilled water. iii. 99.4ml of sulphuric acid solution was added to 0.6ml of barium chloride solution and mixed.

Appendix 3.15.0 Principle of Extended Spectrum Beta Lactamase detection

ESBL are beta lactamases that confers resistance to penicillins, first generation, second generation and third generation cephalosporins by hydrolysing these antibiotics. In the presence of clavulanic acid however, the beta lactamase effect is inhibited.

3.16.0 Principle behind Pulsed-Field Gel Electrophoresis

Pulsed-Field Gel Electrophoresis allows for the separation of extremely large DNA fragments when subject to an electric field that constantly changes direction. This is done by digesting genomic DNA using restriction enzyme, resulting to several DNA fragments. These fragments are then subjected to an electric field, producing a DNA fingerprint pattern.

3.16.1 Procedure for Pulsed-Field Gel Electrophoresis

i. The sample for this procedure was a fresh overnight culture.

ii. 800µl of cell suspension buffer(appendix 3.16.1 R1) was pipetted into 5ml tubes (1 tube for each bacterial strain, 1 tube for Salmonella Braenderup [H9812] control strain and 1 tube to serve as a blank for turbidity measurement).

iii. A sterile cotton wool swab was used to pick some bacterial colonies from the agar plate and suspended into the suspension buffer.

iv. Turbidity of the cell suspension was measured using Dade Behring Microscan turbidity meter to read 0.70. The tubes were kept on an ice after the reading to prevent bacterial multiplication thereby increasing the turbidity.

v. 200 µl of the cell suspension was transferred into a 1.5ml tube. 20 µl of 20mg/ml proteinase K (appendix 3.16.1 R2). The tube was gently tapped with fingers to mix the suspension. The tube was incubated at 37^{o}c for 5 minutes using a thermomixer.

vi. 280 µl of a 1% SeaKem Gold agarose: 1%SDS mixture (appendix 3.16.1 R3) (held at 55^{o}C). The molten agarose was gently mixed with the bacterial sample. 300 µl of the mixture was transferred into a BIORAD plug mould. 10 minutes allowed for the agarose block to solidify.

vii. The plug mould was opened and the agarose plug was transferred to a 50ml screw-cap tube using a small spatula, which was cleaned with 70% alcohol between each step.

viii. 5ml of lysis buffer (appendix 3.16.1 R4) was added and incubated at 55^{o}C for120 minutes with gentle shaking using a shaking water bath at a speed of 70rpm.

ix. The blue screw cap of the 50ml tube was replaced with a green strainer. The lysis buffer was poured out. 15ml of preheated deionised water at 50^{o}C was added to wash the block for 15 minutes at 50^{o}C in a shaking water bath at a speed of 70rpm. The water was poured out and this wash step was repeated.

x. The water was poured out and 15ml of preheated (50^{o}C) TE buffer (appendix 3.16.1 R5) was added. Washing was done for 15 minutes at 50^{o}C in a shaking water bath at a sped of 70rpm. The wash step was repeated another 5 times.

xi. 100 µl of 1x restriction enzyme buffer (appendix 3.16.1 R6) in a 1.5ml tube.

xii. The agarose plug was placed onto a glass slide and 2mm thick slice of the plug was cut using scalpel blade. The blade was clean with 70% alcohol between each cut.

xiii. The agarose slice was placed into the 1x restriction enzyme buffer and incubated for 15 minutes at 37^{o}C.

xiv. The 1x buffer was pipetted out and 150 µl of fresh 1x restriction enzyme buffer containing 50 units of restriction enzyme (appendix 3.16.1 R7) was added. Incubation was done for 4 hours at 37^{o}C.

xv. The buffer/enzyme mix was pipetted out and 200 µl of 0.5x TBE (appendix 3.16.1 R8) was added. Incubation was done for 10 minutes at room temperature.

xvi. The agarose slice was removed from the tube and attached to a comb. The comb was placed within a BIORAD agarose gel casting unit.

xvii. A 150ml solution of 1% SeaKem Gold agarose (appendix 3.16.1 R9) was prepared, allowed to cool to 50^{o}C, and poured into the agarose gel casting unit. 25 minutes was allowed for the gel to solidify.

xviii. Electrophoresis running buffer (0.5xTBE) (appendix 3.16.1 R8) was prepared and the electrophoresis chamber of a BIORAD CHEF-DR was filled with the buffer. The cooling unit was set to a run temperature of 14^{o}C and the running buffer was allowed to chill at chill for 30 minutes before the electrophoresis was started. The temperature was checked with a thermometer to ensure that it was really at 14^{o}C.

xix. The gel was removed from the casting unit and placed onto its resting position within the electrophoresis chamber of the BIORAD CHEF-DR III electrophoresis system.

xx. The electrophoresis run parameters was set as follows:

a. Initial switch time 2.2 seconds
b. Final switch time 63.8 seconds
c. Included angle 120^0
d. Voltage 6 volts
e. Run time 19 hours

xxi Electrophoresis run was started.

xxii After the run, the agarose gel was removed from the electrophoresis chamber and placed into a plastic container. 250ml of Ethidium bromide staining solution (appendix 3.16.1 R8) was added and incubated at room temperature for 30 minutes with gentle shaking.

xxiii The staining solution was removed from the container, and 400ml of deionised water was added and destained with gentle shaking at room temperature for 30 minutes. The water was changed after 15 minutes of distaining.

xxiv An image of the DNA fingerprint was captured using BIORAB gel documentation system

Appendix 3.16.1 Constituents of reagents used for Pulsed-Field Gel Electrophoresis

Appendix 3.16.1 R1 :Cell suspension buffer (100mM Tris: 100mM EDTA, pH 8)

10ml of 1M Tris, pH 8

20ml of 0.5M EDTA, pH 8

70ml deionised water

Appendix 3.16.1 R2: Proteinase-K (20mg/ml)

20mg proteinase-K

1mlTE buffer (3.20.1 R5)

Appendix 3.16.1 R3: 1% SeaKem Gold agarose:1% SDS mixture

0.25g SeaKem Gold agarose

22.5ml TE buffer (3.20.1 R5)

The suspension was dissolve by boiling in a microwave oven and allowed to cool to 50^{o}C. 2.5 ml of 10% SDS (3.20.1 R11) was added and mixed thoroughly at 55^{o}C.

Appendix 3.16.1 R4: Lysis buffer

0.5M EDTA, pH 8

1% N-laurosylsarcosine sodium salt (sarcosyl) [freshly added]

0.1 mg/ml proteinase-K (freshly added)

Appendix 3.16.1 R5: Tris-EDTA(TE) buffer (10Mm Tris: 1Mm EDTA, pH 8)

10ml of 1 M Tris, pH 8

2ml of 0.5M EDTA, pH 8

988ml of deionised water

Appendix 3.16.1 R6: 1x restriction enzyme buffer

Deionised water	90µl
10x buffer	10µl

Appendix 3.16.1 R7: 1x restriction enzyme buffer with restriction enzyme

10x buffer	15 µl
Enzyme (10U/ µl)	5 µl
Deionised water	130 µl

Appendix 3.16.1 R8: 0.5x TBE buffer

50ml of 10x TBE (3.20.1 R12)

950ml deionised water

Appendix 3.16.1 R9: 1% SeaKem Gold agarose

1.5g SeaKem Gold agarose

150ml of 0.5x TBE buffer (3.20.1 R8)

The suspension was dissolved in a microwave oven until the powder dissolved.

Appendix 3.16.1 R10: Ethidium bromide staining solution

250ml of 0.5x TBE (3.20.1 R8)

25µl of 10mg/ml Ethidium bromide

Appendix 3.16.1 R11: 10% SDS

10g Sodium dodecyl sulphate

100ml deionised water

Appendix 3.16.1 R12: 10x TBE buffer

108g Tris

55g Boric acid

7.5g EDTA powder

1000ml deionised water.