UNIVERSITY OF NAIROBI

DETECTION OF *SALMONELLA SPECIES* IN KENYA USING 16S rRNA PCR COUPLED TO HIGH RESOLUTION MELTING POINT ASSAY (HRMA).

BY

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Reg No: H56/81336/2012

A thesis submitted in partial fulfillment of the requirements for the award of a Master of Science degree in Biochemistry.

NOVEMBER 2016

DECLARATION

This research thesis is my original work and has not been presented for a degree award in any other University.

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ACKNOWLEDGMENT

I give thanks to God Almighty, for he endowed me with wisdom, strength and perseverance while undertaking my research project and continues to do so in my life. I wish to express my deepest sense of gratitude to my research supervisors, Dr.Juma, Dr.Kamau, Dr.Atunga and Prof Kinyanjui all from the department of biochemistry, University of Nairobi, for your great ideas, invaluable suggestions, constructive criticism, guidance and support throughout my research study. Special Thanks to Dr.Allan, from KEMRI for your invaluable suggestions and assistance.

To all my colleagues in the laboratory, classmates and technical staff in Biochemistry department, Institute of Primates Research and KEMRI I thank you for your kind cooperation, suggestions and encouragement. Many thanks to the staff in Ndumbuini slaughter house, Dagoretti slaughter house and the sales men and women in Gikomba, Machakos, Kariakor and Burma chicken markets for your assistance during sample collection.

To my three colleagues Diana, Pauline and Shiku this journey wouldn't be the same without your suggestions, encouragement and support. Thank you. To my family and friends thank you for your moral support, and for urging me on when the journey got tough.

DEDICATION

To, My Parents Mr. and Mrs. Kimathi thank you for your financial and moral support, May God bless you.

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ABSTRACT

Salmonella species have been identified worldwide as a common cause of bacteremia among the immunocompromised individuals and infants where in severe cases it has proved fatal. Although various tests including culture and serological methods have been traditionally used for *Salmonella spp.* detection they have been reported to be less sensitive and time consuming. In Kenya, the serological based widal test is the most widely used assay for *Salmonella* detection. Although the assay is rapid, its utility has been limited by its low sensitivity. A more specific DNA based assays, though quite promising in speed, specificity and sensitivity, have also not been able to distinguish among different *Salmonella species*. Thus, a more rapid and specific assay is required for *Salmonella* identification. High-resolution melting point assay (HRMA) is a new, rapid nucleic based diagnostic test that has been recently developed to detect specific DNA sequence variations and has thus been applied in the specific identification and differentiation of many closely related bacterial species such as *Salmonella*. This study was hence aimed at developing and evaluating HRMA as a rapid, precise and more sensitive technique for the identification of *Salmonella* serovars circulating among domestic animals in Kenya.

Fecal materials of pigs and cattle and cloacal swabs from poultry as well as the egg contents were each used for the isolation of *Salmonella* serovars by first being grown in enrichment media and subsequently isolated on XLD a selective media. The isolated *Salmonella species* were further characterized biochemically using API 20E strips based on the different colour changes upon specific substrate utilization. To identify specific serovars in the samples, DNA was then extracted from the identified *Salmonella spp*. and then subsequently subjected to HRMA assay using the V1, V3 and V6 primers specific for the 16S rRNA region. The control DNA sample was serially diluted tenfold and used to evaluate the limit of detection of the HRMA. The HRMA

specificity was also evaluated by subjecting an artificially prepared poly microbial sample consisting *S.* Enteritidis, *S.* Gallinarum, *S.* Typhi, and *S.* Braenderup DNA as the positive controls.

The results indicated that the egg and pig fecal materials had the highest prevalence of *Salmonella* at 9.21% and 8.24 % respectively. On the other hand chicken and cattle samples had the least prevalence at 4.1% and 4% respectively as determined by the culture method. However, there was a sizeable decline in the prevalence of *Salmonella* in the sample when biochemically characterized by API 20E strips. Using this method, only 5.99% of the egg samples were contaminated with *Salmonella*, 3.85% were positive in the pig's fecal material, 3.66% in chicken swabs while, 2.67% of the sampled cows fecal materials were contaminated with *Salmonella.* This clearly indicates that biochemical detection of *Salmonella* by API 20E strips was more sensitive than culture method. HRM assay generated unique melt profiles for each *Salmonella* serovar controls including *S.* Typhi, *S.* Typhimurium, *S.* Enteritidis, *S.* Gallinarum, *S.* Braenderup, *S.* Dublin, and *S.* Choleraesuis. Based on this assay the main circulating serovars identified in Nairobi and the surrounding environment were *S.* Typhimurium, *S.* Enteritidis, *S.* Braenderup, and *S.* Choleraesuis. The limit of detection of *Salmonella* DNA in the samples was as low 0.031ng/µl indicating that not only was the technique specific but it's also more sensitive.

In conclusion the HRM assay developed in this study is rapid, sensitive and specific but is also capable of differentiating among different *Salmonella* serovars. This assay is a valuable and promising diagnostic tool, which could enhance the early detection, identification and prevalence determination for an effective control strategy of *Salmonella species*. This could be important in reducing misdiagnosis of *Salmonella*, which has currently led to misuse of antibiotics hence the emergence of *Salmonella* resistant serovars.

CHAPTER ONE

1.0. INTRODUCTION

Salmonella spp. represents a well- characterized bacterial organism known to cause a number of illnesses and deaths worldwide (Everest *et al*., 2001). Among the many *Salmonella* species, *Salmonella* Typhi has been reported to infect at least 16 million people and about 500,000 die yearly worldwide (Everest *et al*., 2001). In America and in sub-Saharan Africa, non-typhoidal *Salmonella* has emerged as a common cause of invasive disease in both rural and urban areas (Mead *et al*., 1999, Gordon 2008, Oundo *et al.*, 2000, Bachou *et al.*, 2006). In Kenya non-typhoidal *Salmonella* species (NTS) have been reported to cause bacteremia among children and immunocompromised patients (Kariuki *et al*., 2002).

Salmonella species are non-lactose fermenting, non-sporing gram-negative bacteria that belong to the Enterobacteriaceae family. *Salmonella spp*. can be grouped into typhoidal and non-typhoidal based on their clinical importance of the pathogen. Typhoidal *Salmonella* serovar includes *Salmonella enterica* serotype Typhi and *S*. Paratyphi that are host-restricted human pathogens (Uzzau *et al.*, 2000). Non-typhoidal *Salmonella* (NTS) include *Salmonella* Typhimurium, *Salmonella* Heidelberg, *Salmonella* Newport, *Salmonella* Agona, *Salmonella* Enteritidis, *Salmonella* Dublin, *Salmonella* Hadar and (Arora, 2001, Adkins *et al.*, 2006).

The *Salmonella* pathogen inhabits the intestinal tract of the host where it is reported to cause, gastroenteritis, enteric fever and bacteremia (Gray *et al*., 2002) and has also been reported to live asymptomatically in both animals and human hosts (Gray *et al*., 2002). *S.* Typhi and S. Paratyphi cause human enteric fever, whereas gastroenteritis is caused by *S*. Typhimurium, *S*. Enteritidis and *S*. Newport (Gray *et al.,* 2002). However, *S*. Choleraesuis, which has mainly been

reported in pigs, is more destructive in humans than in animals (Slack *et al.*, 1978; Gray *et al*., 2002).

It has been suggested that rapid and robust diagnostic assays are required to avert and curb the spread of *Salmonella spp.* With this in mind various diagnostic tools have been developed for the effective detection of *Salmonella* in a biological matrices (Wattiau *et al.,* 2008). Traditionally, *Salmonella spp.* detection methods are based on culture technique, which involves enrichment in non-selective media, selective enrichment and sample identification on selective media followed by biochemical identification and serological tests (Okamura *et al.,* 2009). Although these procedures are quite sensitive, they are quite labor intensive requiring approximately 5-7 days to validate *Salmonella spp.* presence in the sample (Techathuvanan *et al.,* 2010, Okamura *et al.,* 2009). Immunoassay techniques such as ELISAs have been advanced specifically for *Salmonella* detection (Mansfield *et al.,* 2000): however their low specificity has greatly limited their wide applications (Erickson *et al.,* 2007). Similarly, molecular-based methods such as PCR, real-time PCR, and DNA microarray have also been advanced mainly to satisfy the lack of a rapid diagnostic molecular tool. (Kwang *et al*., 1996, Wattiau *et al.,* 2008). However, most of these techniques have been hampered in their utility, in that they are not able to differentiate between the different *Salmonella* serovars in a sample matrix. This has necessitated the need to develop a more sensitive, specific assay for the diagnosis of *Salmonella species* infections up to serovar level.

High-resolution melting point is a new, rapid molecular diagnostic technique that has been used to detect sequence variation and has thus been applied in species differentiation, genotyping and mutation detection (Lenka *et al*., 2011). In this assay, the region of interest is first amplified and the amplified products then subjected to a steady increase in temperature where the resultant melt

curves are generated by monitoring the fluorescence of a saturating dye. Thus, when combined with real time PCR (rtPCR), HRMA has been reported to be an ideal technique for DNA diagnostics (Wittwer *et al.,* 2003) and has since been applied for rapid diagnosis of *Salmonella* serovars in developed countries (Jeng *et al.,* 2011). In Kenya, the technique has been used in the detection of *Plasmodium* parasites in various biological fluids (Kipanga *et al.,* 2014)

This study was aimed at evaluating the specificity and limit of detection of HRMA as a diagnostic tool for diagnosis of specific circulating *Salmonella* serovars present in biological samples of domestic animals and related animal products in Kenya. This data is important, as HRM will provide an alternative rapid and sensitive method for detection of *Salmonella spp.* in Kenya up to serovar level.

1.1 Problem statement

Salmonella infections in both man and animals have been recognized as a major public health problem worldwide (Akinyemi *et al.,* 2007). More than 90% of non-typhoidal *Salmonella* infections are food borne while the remaining 10% are often due to contact with infected pets (reptiles and birds) or infected persons or contaminated water (Hohmann, 2001). However, the true incidence of *Salmonella* worldwide is difficult to determine (Crump *et al.,* 2004, Arndt *et al.,* 2014) largely due to lack of reliable detection methods, especially in rural outpatient settings.

Several tests including culture and serological methods have been used to detect *Salmonella* infection in both domestic animals and associated products. In Kenya, a serological based-widal agglutination test has been commonly used for the diagnosis of typhoid fever, a disease caused by *S.* Typhi (Kakai *et al.,* 2008). Though it is a simple and rapid test method, for *Salmonella* infection widal test is reported to be associated with low sensitivity (Omuse *et al.,* 2010).

Similarly although, the recently developed molecular based methods such as polymerase chain reaction (PCR) or real time PCR methods are specific, fast and quite sensitive; none of the methods is able to differentiate between the many different types of *Salmonella* serovars. This has necessitated the need to develop a more sensitive and specific molecular tool for rapid detection and identification of *Salmonella* infectious agents. Such an assay would greatly enhance the current diagnostic, treatment, and surveillance methods associated with this global public health problem. This will prevent and control the spread of *Salmonella* pathogens both in domesticated animals that act as a *Salmonella spp*. reservoir as well as in the human population.

1.2. Justification

In sub-Saharan Africa, invasive non-typhoidal *Salmonella* enterica (iNTS) is a main health problem particularly among young children and the immune compromised HIV positives adults. *Salmonella* associated mortality in this population has been estimated to vary from nearly 10–30% (Reddy *et al.,* 2010, Berkley *et al.,* 2005). In Kenya multi-resistant *Salmonella* Typhimurium strains, have been reported as the leading cause of bacteremia among the HIV infected population (Kariuki *et al.,* 1996: Leegaard *et al.,* 1996).

Moreover, a diagnostic gap has been shown to exist in low resource outpatient settings, which has often resulted in varied incidences of invasive typhoidal and non-typhoidal salmonellosis (Crump *et al.,* 2004; Gordon *et al.,* 2008; Sigaúque *et al.,* 2009 Reddy *et al.,* 2010; Buckle *et al.,* 2012; Mogasale *et al.,* 2014, Arndt *et al.,* 2014;). This has often resulted in inaccurate results as a result of either under diagnosis or over diagnosis of enteric fever. Over diagnosis of typhoid fever has often resulted in improper use of antibiotics, which has often resulted in the emergence of resistant bacteria against the conventional antibiotics (Holt *et al.,* 2011, Koirala *et al.,* 2012, Kingsley *et al.,* 2009, Savard *et al.,* 2011). This has threatened to erode the efforts made to

reduce case fatality rates for most bacterial infections. This therefore necessitates the need for a rapid, specific and sensitive *Salmonella* detection method up to serovar level.

Salmonella serotyping being the main method for identifying the different *Salmonella* serovars (Brenner *et al.,* 2000) is time consuming. Therefore a more rapid identification and serotyping tool such as HRMA would prove very useful for in terms of cost and turnaround time especially in an disease outbreak situation and in a clinical setting.

This study was conducted in order to evaluate the specificity and sensitivity of a new HRM technique against the traditional available methods. This assay is a promising molecular tool that is rapid, sensitive and specific for identification of the different *Salmonella* serovars. The validation of this molecular tool is important, as the assay could be adapted in a hospital setting for *Salmonella* detection.

1.3. Objectives

This study was carried out to evaluate a rapid, specific, and sensitive HRM assay technique for the detection of *Salmonella* serovars circulating in domestic animals in Nairobi and its environs.

1.3.1. Specific objectives

1. To characterize and identify circulating *Salmonella* serovars in chicken, eggs, pigs and cattle.
2. To design and evaluate a rapid and sensitive HRM assay as a detection tool for *Salmonella* serovars circulating in chicken, eggs, pigs and cattle.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Morphology and biochemical characteristics of *Salmonella spp.*

Salmonella spp. are non-lactose fermenting, non-spore forming gram negative bacteria within the family of Enterobacteraceae. Members within this family vary in size that range between 0.2 -1.5 x 2-5 µm, and are all actively mobile with the exception of *Salmonella* Pulorum-Galinarium (Cheesebrough *et al.*, 2000, Perilla *et al*., 2003). *Salmonella spp.* are chemoorganotrophic organism as they have the capability to utilize nutrients by both aerobic and anaerobic pathways (Popoff *et al.,* 2005). Hydrogen sulphide (HS) is the major metabolic end product of most of the *Salmonella spp.* with the exception of *S.* Paratyphi A and *S.* Choleraesuis which do not produce hydrogen sulphide (Ziprin *et al.*, 1994).

Salmonella spp. are regarded as non-fastidious organism since they can inhabit and multiply under varying growth conditions outside the living hosts. Majority of the *Salmonella* serotypes thrive at temperature range of between 5°C to 47°C with the peak survival temperature ranging between 35°-37°C. However, a few serovars are known to grow at variable temperatures of as low as 2°C or as high as 54°C (Gray *et al.,* 2002). Some *Salmonella* pathogens are capable of surviving up to seven years under frozen temperature ranges of between -23°C to -18°C (Bell *et al*., 2002). *Salmonella spp.* have been reported to withstand a pH range of 4 to 9 and also exhibit high tolerance to environmental conditions with a salt concentration of up to 20% in the growth medium (Bell *et al*., 2002, Guthrie *et al.,* 1991). This clearly indicates that *Salmonella* can inhabit diverse, in some cases extreme environmental conditions and hence the difficulty experienced in controlling *Salmonella spp.* (Guthrie *et al.,* 1991).

2.2. Classification and nomenclature of *Salmonella spp.*

Salmonella spp. nomenclature is quite elaborate and still changing with time (Breener *et al*., 2000). This has therefore resulted in adoption of several different systems for the classifications of *Salmonella* pathogens. Traditionally, *Salmonella spp.* have been classified on the basis of their place of origin such as *Salmonella* London and *Salmonella* Indiana (Breener *et al.,* 2000). This nomenclatural system has however, been replaced by phage typing, a system based on the susceptibility of *Salmonella* isolates to different selected bacteriophages (Bhunia *et al.,* 2008). Phage typing has generally been used to differentiate isolates of the same serotype especially when the origin and characteristic of an outbreak is to be determined (Hanes *et al.,* 2003).

Epidemiologically, *Salmonella spp.* have also been classified based on host adaptability as either host-restricted or host adapted serotypes (Gray *et al*., 2002). Host-restricted serotypes such as *S.* Typhi have been reported to only infect humans, while host-adapted serotypes such as *S.* Pullorum of the avian species are affiliated with one host species although some have been reported to also cause disease in other hosts (Gray *et al.,* 2002). These also includes serovars such as, *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg, a group that has often been recovered from humans (Gray *et al.,* 2002, Boyen *et al.,* 2008) although they mostly affect their specific host animal.

Salmonella spp. can also be classified on the basis of clinical importance (Arora *et al.,* 2001, Adkins *et al.,* 2006). For example, *S.* Typhi and *S.* Paratyphi cause typhoid salmonellosis while *S.* Typhimurium and recently serotype DT104, *S.* Enteritidis, *S.* Dublin, *S.* Agona, *S.* Newport, *S.* Hadar, and *S.* Heidelberg are etiologic agents of non typhoidal salmonellosis (Arora *et al.,* 2001, Adkins *et al.,* 2006).

Initially *Salmonella spp.* were classified based on their antigenic properties based on the analysis of both the O and H antigens present on the bacterial cell surface (White *et al.,* 1926). This classification was further extended by (Kauffman, *et al.,* 1966) which has given rise to a great number of serovars. *Salmonella* has three major antigenic determinants composed of virulence (Vi) capsular K antigens, flagella H antigens and somatic O antigens. The O antigen is located on the bacterial cell wall and *Salmonella* spp. have been reported to possess 2 or more O antigens on its surface (Poppoff *et al.,* 2005). The H-antigen, which is the flagella antigen, is heat labile and enables the motility of the *Salmonella* bacterium (Slack *et al.,* 1978). On the other hand the capsular Vi antigen enhances the bacterium virulence (Slack *et al.,* 1978). Among all the *Salmonella* sub-species only two, *Salmonella* enteric serovar *S.* Typhi and *S.* Choleraesuis, have the Vi antigen (WHO 2008) and therefore they are considered more virulent. Cross reactivity between the O antigens of *Salmonella* and other genre of *Enterobacteriaceae* has been reported. This has necessitated the adoption of more specific classification of the *Salmonella* serotypes based on the antigenicity of the flagella H antigens (Scherer *et al.,* 2001).

The Center for Disease Control and Prevention (CDC) classification system the genus *Salmonella* contains two species: *S. enterica*, and *S. bongori*. *S. enterica* consists of six subspecies: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae;* IIIa, *S. enterica* subsp. *arizonae;* IIIb, *S. e*nterica subsp. *diarizonae*; IV, *S. enterica* subsp. h*outenae*; and VI, *S. enterica* subsp. *indica* (Table 1). This classification is based on Kauffmann White classification system

Table 2.4: Kauffmann-White classification of *Salmonella species* and their habitats (Brenner *et al.*, 2000).

Salmonella species and subspecies	No. of serotypes within subspecies	Usual habitat (Animals /Environment)
S. enterica subsp. *enterica* (I)	1,454	Warm-blooded animals
S. enterica subsp. *salamae* (II)	489	Cold-blooded animals.
S. enterica subsp. *arizonae* (IIIa)	94	Cold-blooded animals
S. enterica subsp. *diarizonae* (IIIb)	324	Cold-blooded animals
S. enterica subsp. *houtenae* (IV)	70	Cold-blooded animals
S. enterica subsp. *indica* (VI)	12	Cold-blooded animals
S. bongori (V)	20	Cold-blooded animals.
Total	2,463	

2.3. Occurrence of *Salmonella spp.* worldwide

Animals especially farm animals are the principal reservoir host for *Salmonella spp.* (Winfield *et al.,* 2003). *Salmonella species* live in the intestinal tract of both warm and cold-blooded animals. While some species are ubiquitous, others are specifically adapted to a particular host (Humphrey *et al.,* 2002). Farm animals are frequently asymptomatic carriers following an enteric infection. The animal's shed the bacteria in farm products including milk, eggs, contaminated carcasses and other agricultural products grown on land fertilized with farmyard manure (You *et al.,* 2006).

Chicken is the natural hosts for *S.* Gallinarium and *S.* Pullorum. Both serovars cause systemic infections in chickens that are marked by low morbidity and high mortality (Uzzah *et al.,* 2000). Clinically, while *S.* Gallinarium cause typhoid in adult chicken, *S.* Pullorum affects the very young chickens of between 2−3 weeks old (Shivaprashad, 1997). *S.* Enteritidis is more prevalent in poultry and poultry products as it's associated with salmonellosis in poultry (Betancor *et al.,* 2010).

Chicken egg is an important transmission vehicle for *S.* Enteritidis with most eggs being infected either by horizontal or by vertical transmission (Chen *et al.,* 2005). In horizontal transmission, infection occurs as the bacteria penetrate through eggshells after eggs have been laid while in vertical transmission, they infect the hen's reproductive system and affects the egg contents prior to eggshell formation (Chen *et al.,* 2005). Thus, consumption of contaminated egg products can lead to *Salmonella* infection, especially among the highly susceptible individuals such as the immunocompromised (FDA 2010).

Salmonella Typhimurium is associated with pig salmonellosis (Vieira-Pinto *et al.,* 2006), similarly, host adapted serotype Choleraesuis is also associated with pigs where they generally cause severe systemic disease that result in high mortality rates (Boyen *et al.,* 2008). A previous study in Kenya had estimated the prevalence of *Salmonella spp*. in pigs to be about 13.8% with *S.* SaintPaul being the most frequently isolated serovar followed by *S.* Heidelberg and *S.* Braenderup (Kikuvi *et al.,* 2010).

S. Typhimurium also infects cattle, where it is reported to cause diarrhea, fever, loss of appetite and decreased body weight (Vieira-Pinto *et al.,* 2006). Similarly, *S.* Dublin also infects cattle and is reported to cause intestinal inflammation (Gray *et al.,* 2002). *Salmonella spp.* are host specific serotypes, *S.* Typhi and *S.* Paratyphi only infect humans, *S.* Pullorum infects avian species; *S.* Dublin infects cattle, while *S.* Choleraesuis infects pigs. *S.* Pullorum, *S.* Dublin, and *S.* Choleraesuis rarely infect humans. However, infection of humans by these species is regarded to be extremely dangerous, invasive and accompanied with high rates of mortality (Gray *et al.,* 2002). Despite most of the *Salmonella spp.* being host specific, few of these species including *S.* Enteritidis, *S.* Typhimurium, and *S.* Heidelberg can easily be transmitted from animals and have been shown to be the most common serotypes that infect humans each year (Gray *et al.,* 2002).

2.4. Geographical distribution of *Salmonella spp*.

The occurrence of and prevalence *Salmonella* serovars varies across different regions globally. However, globalization, international travel, human migration, trade involving food, animals and livestock feed has greatly contributed to the spread of new *Salmonella* serovars into most countries (Uyttendaele *et al.,* 1998; Hohmann, 2001). Thus, failure to control *Salmonella* infection in one country presents a potential problem for other countries, particularly because of

the emerging new *Salmonella* strains in the developing countries and in the USA (Farrar, 1985; Miriagou *et al*., 2004). *S*. Enteritidis and *S.* Typhimurium are the two most frequently isolated serovars from human samples (Hendriksen *et al.*, 2011). However, the prevalence of *Salmonella* varies depending on the geographical locality, and time. Some serovars such as *S*. Stanley and *S*. Weltevreden are more prevalent in Southeast Asia (Hendrisken *et al*., 2009a).

Previous studies in various countries (table 2) have revealed *Salmonella* Enteritidis, *S.* Typhimurium, and *S.* Newport as the most common serotype that cause human disease globally (Galanis *et al*., 2006). In Africa, *S.* Enteritidis and *S.* Typhimurium are the most prevalent isolates from humans constituting approximately 25% of the total isolates from humans (Galanis *et al*., 2006). In Kenya, the main *Salmonella* serotypes that have been isolated and characterized from human samples include *S*. Typhimurium 59%, *S*. Enteritidis 28.3% and about 12.7% other serotypes consisting of *S*. Haifa, *S*. Braenderup, *S*. Choleraesuis, *S*. Dublin, *S*. SaintPaul and *S*. Indiana (Kariuki *et al.,* 2006). However, in Kenya the prevalence of *Salmonella* in pigs indicates that *S.* Saintpaul is the most frequently isolated serovar, subsequently followed by both *S*. Heidelberg and *S*. Braenderup (Kikuvi *et al.,* 2010).

Table 5.2: Global rank of countries that ranked top 10 of each of the 20 most common *Salmonella* serotypes among human isolates 2002 (WHO 2002)

Global rank	Serotype	Europe n%	North American%	Oceanic n %	Asia n%	African %	Latin America and Caribbean n %	Total n%
1	Enteritidis	8(100)	2(100)	1(100)	4(80)	4(80)	7(70)	26(84)
2	Typhimurium	8(100)	2(100)	1(100)	5(100)	4(80)	6(60)	26(84)
3	Newport	3(38)	2(100)	0	1(20)	1(20)	1(10)	26(84)
4	Heildelberg	2(25)	2(100)	0	2(40)	0	2(20)	8(26)
5	Infatis	8(100)	2(100)	1(100)	1(20)	1(20)	1(10)	8(26)
6	Hadar	6(75)	2(100)	0	3(60)	3(60)	0	14(45)
7	Virchow	5(63)	0	1(100)	0	2(40)	1(10)	14(45)
8	Javiana	0	1(50)	0	0	0	2(20)	9(29)
9	Saintpaul	3(38)	2(100)	1(100)	1(20)	0	3(30)	3(10)

10	Montevideo	2(25)	2(100)	1(100	2(40)	1(20)	4(40)	10(32)
11	Agona	6(75)	2(100)	0	1(20)	0	3(30)	12(39)
12	Oranienburg	0	2(100)	0	0	0	1(10)	12(39)
13	Thompson	3(38)	2(100)	1(100)	1(20)	0	0	3(10)
14	Typhi	1(13)	1(50)	1(100)	2(40)	4(80)	5(50)	7(23)
16	ParatyphiB d-tartrate	2(25)	2(100)	0	0	0	0	1(3)
17	Braender up	0	0	0	1(20)	1(20)	2(20)	4(13)
18	Blockley	2(25)	0	0	0	0	2(6)	4(26)
19	Anatum	1(13)	0	0	1(20)	0	3(30)	5(16)
20	Weltevreden	0	0	0	2(40)	0	1(10)	3(10)

2.5. Clinical importance of *Salmonella spp.*

In developing countries, non-typhoidal *Salmonella spp*. is contributing to the steadily increasing number of human diseases (Kariuki *et al.*, 1996). For example, in Kenya and Malawi multi-resistant *S.* Typhimurium strains have been reported as the major cause of bacteremia in most of the immune suppressed adults (Kariuki *et al.*, 1996, Leegaard *et al.*, 1996). Nonetheless, the prevalence of *Salmonella* in humans and animals has not been conclusively evaluated in Africa. This has been attributed to the very few number of studies being done in this area, lack of harmonized epidemiological surveillance systems and other diseases being given research priority over *Salmonella* associated diseases. (Ameh *et al.*, 2004).

2.6. Transmission and pathogenesis of *Salmonella spp.*

In order to cause disease, *Salmonella* spp. are generally first ingested and then travels through the digestive system to reach the small intestine where the pathogen generates inflammatory responses in the intestinal cells thus leading to gastroenteritis (Slack *et al.*, 1978). Gastroenteritis, enteric fever, and bacteremia are the three most common clinical manifestations associated with *Salmonella spp*. (Wray *et al.*, 2000). Enteric disease often present itself as bloody or profuse watery diarrhea with pyrexia. Salmonellosis clinical symptoms may also include acute septicemia, abortion, arthritis, necrosis of extremities and respiratory disease (Wray *et al.*, 2000). Upon infection the disease causing pathogen can be isolated from various body fluids such as duodenal aspirates, blood, urine, and stool (Cheesbrough *et al.*, 2000).

2.7. *Salmonella spp.* detection methods in biological samples

Salmonella detection methods can be broadly classified as culture, immunological and nucleic acid based techniques (Carrique-Mas, *et al.*, 2008). All these methods have been associated with various advantages and drawbacks.

2.7.1. Culture methods for *Salmonella* detection

Culture is the most commonly used tool for *Salmonella* identification. Due to their high selectivity and sensitivity these techniques have been regarded as the gold standard for *Salmonella spp.* detection in biological matrices (Alocilja *et al.,* 2003). Culture methods main disadvantage is that they are time consuming, typically requiring between 5–7 days to obtain results, this is due to the fact that identification is based on presence or absence of visible colonies. Culture based techniques involve various procedures including both selective, non-selective enrichment, selective plating, serological and biochemical confirmation (Maciorowski, *et al.,* 2006) to obtain positive results. Culture technique detects live bacterial cells and thus provides an epidemiological advantage over molecular techniques. However, when working on a large sample size this technique is quite labor intensive (Maciorowski, *et al.,* 2006) particularly during an epidemic. The identification of *Salmonella spp.* cells using culture methods from food matrices involves four methodological steps. In the pre-enrichment stage, the sample is grown in buffered peptone water or lactose broth so as to multiply the number of target cells prior to growth on a non-selective broth (Sandel *et al.,* 2003, McKillip *et al.,* 2004). This step is then followed by enrichment step in a selective broth, such as Rasspaport- Vasilliadis (RV) broth, Selenite Cysteine Broth (SC), or tetrationate broth (TT). Finally, the last step involves isolation of the positive isolates on selective Brilliant green agar, Bismuth sulfite

agar, Hektoen agar (HA) or Xylose lysine Deoxycholate (XLD) (Molbak *et al.,* 2006). However, some strains of *Salmonella* have been reported to possess different reactions in response to inhibitory substances, incubation temperatures, selective enrichment broths and media (Cardinale *et al.,* 2005). For example, since some *Salmonella* serotypes such as *S.* Anatum, *S.* Tennessee, *S.* Newington and *S.* Senftenberg are lactose positive bacteria (Bell, *et al.,* 2002), an alternative selective media such as Mannitol Lysine Crystal Violet Brilliant Green or Bismuth Sulphite Agar (Bell, *et al.,* 2002) has been suggested be used together with lactose test to accurately distinguish *Salmonella* from other microorganisms in various food samples.

The common confirmatory test for *Salmonella* includes both biochemical and serotyping tests (Ewing, 1986). Biochemical tests include checking for glucose utilization lysine decarboxylase, urease, indole test, hydrogen sulphide production, and fermentation of dulcitol (Ewing, 1986). Besides, serological confirmatory assays utilize polyvalent antisera for flagella (H) and somatic (O) antigens. In this identification system, isolates of *Salmonella* species are identified based on a typical biochemical reaction that results in the agglutinatination with both the H and O antisera. To identify the *Salmonella* serovar, positive *Salmonella* isolates are further serotyped using specific antisera that recognize 46 O antigens, and 119 H antigens according to Kauffman-White (KW) typing scheme (Shipp *et al.,* 1980). However, serotyping is not routinely done in clinical or food microbiology laboratories but is rather undertaken at reference laboratories (Hyatt *et al.,* 2004).

2.7.2. Immunological based assays

Immunological based techniques available for *Salmonella* species detection are usually centered on the enzyme linked immunosorbent assay (ELISA) however other techniques based on dot blot enzyme assays have also been explored for *Salmonella* (Blias *et al.*, 1998). ELISA assay is based on antigen-antibody reaction with a 'label' often attached to the antibody that allows the reaction to be visualized (Cox, 1988). Depending on the substrates used, ELISA assay can either be colorimetric or fluorogenic (Cox, 1988).

ELISAs are easy to perform, highly specific, sensitive, and scalable technique and have hence been adapted for routine microbial testing by laboratories. However, the performance of ELISA can be affected by factors such as enrichment medium and incubation conditions. Negative results can be obtained within 24 h after an overnight incubation in selective broth, whereas positive results may require further processing to identify the sample based on its serological and biochemical characteristics. However, ELISA has been associated with some limitations, especially in the identification *of Salmonella* in foodstuff has it has low sensitivity (Ricke *et al.*, 1999).

Immunoprecipitation also known as immunochromatography is another antibody assay in a sandwich format (Olsvik *et al.*, 1994) that uses antibodies that are coupled to latex beads for detection of *Salmonella* (Olsvik *et al.*, 1994, Feldsine *et al.*, 1997). Immunoprecipitation assays are advantageous because they are extremely simple, require no purification of the biological sample.

2.7.3. DNA based methods for detection of *Salmonella spp.*

The development of DNA-based methods has necessitated the introduction of novel approaches in the detection of *Salmonella species* from various sample matrices (Witter *et al.,* 2003). All DNA based methods are based on the hybridization of two complementary single stranded DNA molecules. One DNA molecule is usually in the form of a probe; primer, DNA fragment or artificially synthesized oligonucleotide while the other strand corresponds to the target microorganism (Chien *et al*., 1976). This is done in order to obtain a double stranded nucleic acid molecule under defined physical and chemical conditions. Several types of DNA based methods but with varying sensitivities and specificities have been developed for *Salmonella species* detection.

2.7.3.1. Polymerase Chain Reaction (PCR) for *Salmonell*a detection

PCR is a molecular tool that's based on the amplification of a specific short target sequence of DNA (Mullis *et al.,* 1986). The procedure involves subjecting extracted DNA tohigh temperatures that results dissociation of the DNA into a single stranded DNA template. Denaturation is subsequently followed by annealing of a specific short single stranded DNA fragments (primer) to the sense strand, followed by extension which is aided by a thermo stable DNA polymerase, such as Taq polymerase to form a template strand (Chien *et al*., 1976). The generated DNA template is then targeted for amplification during the next PCR cycle and thus there is an increase in the specific DNA sequence. The amplified product generated is then separated by gel electrophoresis and visualized by staining with fluorescent dyes such as ethidium bromide (Feng *et al.,* 1997, Ferretti *et al.,* 2001).

PCR technique is increasingly being used in laboratory diagnostics to detect various pathogens and particularly its been used in food industry for bacterial identification, characterization, and enumeration thus replacing the known conventional methods (McKillip *et al.,* 2004). The technique generally reduces the time required to detect and identify a pathogen (Cohen *et al.,* 1994; Rahin *et al.,* 1992). The main disadvantage of the technique is the inhibition of the PCR reaction by components of the test sample. For example, in the case of DNA extracted from feces sample matrices of the feces have been shown in some cases to reduce the sensitivity of the test (Jensen *et al.,* 2003). This has often led to the performance of specific and suitable DNA extraction procedures prior to PCR reaction to prevent PCR inhibitions.

Among the PCR techniques, Real Time PCR (qPCR) is another DNA based laboratory technique that simultaneously enables both the detection, and quantification of a specific DNA sequence in a sample in real-time following each amplification cycle. It is a second generation PCR recently developed to offer a reliable estimation of the number of bacteria in different samples (Mackay *et al.,* 2002). The quantification of pathogens in real-time PCR is based on rapid increase in the initial target DNA quantity in association with the number of cycles performed cycles. In real-time PCR, the amount of DNA in a sample is determined using a standard curve derived from a serial dilution of known number of target copies, thereby providing an absolute quantitative data of the target product (Fey *et al.,* 2004).

Real time PCR has proved to be a valuable molecular tool in terms of specificity, sensitivity, rapidity, quantitative measurement, and lower contamination rate compared to the conventional PCR methods (Mackay *et al.,* 2002). Accumulating amplicons in real

time is well monitored by the labeling of the primers or amplicon with fluorogenic molecules and have been reported to offer benefits over radiogenic oligoprobes as they are much easier to dispose, no harmful exposure to radioactive emissions and have an extended shelf (Matthews *et al.*, 1988). In *Salmonella* detection, real-time PCR, technology has been traditionally used due to its rapidity, specificity and sensitivity (Rodriguez-Lazaro *et al.*, 2003, Csordas *et al.*, 2004). *InvA* gene has previous been successfully targeted for the *Salmonella* species detection by real-time PCR (Gallegos-Robles *et al.*, 2009).

Although several methods are available for *Salmonella* detection, none has clearly emerged as the perfect method for *Salmonella* detection because of various constraints. An ideal detection assay should be robust, rapid, sensitive and portable. More importantly should be capable of, differentiating the different *Salmonella spp.* strains from each other. The enforcement of this ideal method should be balanced against the financial constraints continually associated with a technology that's being used routinely in a laboratory. Two innovative nucleic based, LAMP and HRMA assays have been developed for the detection of microorganisms in biological samples. HRMA assay is a new closed tube technique used for detection of DNA sequence variation that's rapid, simple and sensitivity making an attractive tool laboratory diagnosis (Liew *et al*., 2004) and is the basis for this study. LAMP assay on the other hand is also a simple and an inexpensive method that doesn't require the use of a thermocycler (Notomi *et al.*, 2000). It's however limited in that it's not capable of differentiating *Salmonella* upto serovar level unlike HRMA.

2.7.3.2. Loop mediated isothermal amplification test for *Salmonella spp.* detection.

Loop-mediated isothermal amplification (LAMP) is a DNA based amplification method, unlike real time PCR, LAMP does not require expensive thermocyclers since its amplification can be done in a water bath and thus it is a better alternative method for diagnosis in resource limited areas (Notomi *et al.*, 2000). LAMP assay is a technique that amplifies nucleic acids at one constant temperature unlike the conventional PCR. The assay relies on the use of an enzyme *Bst* DNA polymerase, that has an auto-cycling strand displacement DNA synthesis ability (Mori *et al.*, 2001, Notomi *et al.*, 2000). This method is unique in that it occurs at a single amplification temperature of 60 to 65°C, and requires six primers for the amplification of the target gene. LAMP therefore, is considered more rapid, simple and specific to perform than PCR. Unlike the conventional PCR, LAMP method does not require gel electrophoresis for DNA fragment identification as the product can be detected by fluorescence or by turbidity (Notomi *et al.*, 2000). LAMP method can also be used for both DNA and RNA targets, as it is able to detect target RNA by the reverse transcription-LAMP (RT-LAMP) reaction by using the AMV reverse Transcriptase (Fukuta *et al.*, 2003).

LAMP reaction that involves the use of DNA polymerase enzyme is considered to progress through both non-cyclic and cyclic steps with strand displacement activity (Ushikubo, 2004). This assay requires two inner primers forward inner primer, backward inner primer and two outer primers F3 and B3. The inner primers has two unique sequences that match the coding and template strands sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages (Notomi *et al.*,

2000). The forward loop primer (FLP) and backward loop primer (BLP) are additional primers that bind to target regions difficult to access.

LAMP amplification reaction involves three steps: an initial step that involves production of starting material, cycling amplification and elongation, and recycling (Notomi *et al.*, 2000). LAMP method has made is less complex and easier thus it's more applicable for resource-limited laboratories in rural areas of developing countries. Nevertheless, it has also been reported that LAMP exhibits less sensitivity to inhibitory substances present in biological samples than PCR (Kaneko *et al.,* 2007). This robustness of LAMP against inhibitors contributes to saving up time that could be used for sample processing steps. LAMP is also rapid, specific, sensitive and cost effective as the procedure can be carried out using simple equipment such as water bath. Hence with LAMP method, cost-effective genetic experiments have been conducted in a basic laboratory. However, sometimes the difficulty of designing LAMP primers have greatly contributed to the low utility of LAMP as an innovative tool (Mori *et al.,* 2004)

2.7.3.3. Detection of *Salmonella spp*. by LAMP assay

Two detection methods based on LAMP assay have recently been described for *Salmonella spp.* These two assays are based on the detection of either the *invA* gene (Hara-Kudo, *et al.,* 2005) or the *phoP* gene (Li *et al.,* 2009) of the *Salmonella*. In addition other *Salmonella* LAMP tests have also targeted specific serovars or specific O groups (Okamura, *et al.,* 2008, Ohtsuka *et al.,* 2005). However, there is no LAMP assay that has been designed for the detection *Salmonella serovars* is in existence.

2.7.3.4. High Resolution melting point analysis

High-resolution melting point analysis (HRMA) is a powerful and robust DNA based technique that has been recently developed to detect specific variations in an organism's DNA sequence (Wittwer *et al.,* 2003). The techniques works by generating melt curves, based on the fluorescence data collected as the PCR-amplified DNA fragments are heated and disassociate into single strands. The melting curves of the dissociated DNA are then generated and analyzed to ascertain the variation in sequence of the sample DNA (Liew *et al.,* 2004).

HRM is based on the principle of DNA melting, which largely depends on the length and sequence of the target DNA fragment (Lenka *et al.,* 2011). The melting temperature is affected by length due to the hydrogen bonds that hold the two strands together, the more the base pairs the more the energy required to break the bonds. G-C base pairs form 3 hydrogen bonds; the A-T pairs form only two hydrogen bonds between the two bases. This indicates that with the more G-C base- pairs present in a given DNA sample, the more the required energy for melting (Lenka *et al.,* 2011) and hence the specific characteristic melting curve.

The melting curve is generated by plotting the fluorescence data acquired during melting as a function of temperature during heating of the sample. Thus denaturation of a PCR product saturated by a dsDNA dye is observed as a sudden decrease of fluorescence near Tm (Riere *et al.,* 1997). Moreover, prior to this sharp decrease in fluorescence near Tm, a constant slow decrease in fluorescence with temperature increase is observed and usually reflects physical changes in the relative fluorescence intensity caused by increasing temperature. Once a sharp decrease is observed, the dsDNA melting is reflected in the

melt domain of the curve, followed by another constant slow decrease in fluorescence with temperature when dsDNA melting is complete and the dsDNA melting domain flows into post-melt domain (Lenka *et al.,* 2011). HRM analysis is however, affected by the fluorescent dye concentration and the rate of temperature change (Reed *et al.,* 2007). In addition, PCR yield after amplification further influences the melting point analysis. Thus for each HRM assay performed the reaction factors should be optimized and kept constant (Reed *et al.,* 2007).

HRMA's specificity, speed, high sensitivity, flexibility and ease of use makes the method an attractive molecular biology tool with broad applications in both clinical diagnostic and research (Newman *et al.,* 2012, Bougel *et al.,* 2013).

2.7.3.5. HRMA for the detection of microbial infections

High resolution melting analysis has been applied in the diagnosis of a variety of bacterial, viral and parasitic infections (Yang *et al.,* 2009). Assays based on HRMA can be very specific and detect a particular variant within a strain or broad enough to include many species when a specific locus is carefully chosen (Raherison *et al.,* 2009). HRM has been developed in association with detection of *Salmonella enterica* serovars (Slinger *et al.,* 2007). In the Slinger study they used HRMA to detect *gryA* mutations that are associated with quinolone resistance in typhoid and paratyphoid fever. The *gryA* mutations were detected based on the small consistent changes in melting temperature (Slinger *et al.,* 2007). Moreover, HRMA has been used in the USA in the identification of *Salmonella spp.* where *S*. Enteritidis was detected within 6hr of a patient hospitalization (Jeng *et al*., 2013*).* This data reinforces HRMA assay as a rapid molecular tool that is sensitivity sand specific and can thus be applied in laboratory diagnostics. However, there

is no current data that indicates the use of HRMA assay specific to *Salmonella spp.* isolated from domestic animals in Kenya. Hence in this study, HRMA assay specific to *Salmonella* was developed, and its sensitivity and specificity evaluated based on the V1, V3, and V6 hyper variable regions of the 16S rRNA target region.

2.7.3.6. 16S rRNA Hyper-variable region

Bacterial genomes have been shown to contain many genes with specific functions. For example, 16S rRNA in bacteria is known to have nine hyper variable regions V1-V9 that have been used for species identification due to the sequence diversity of these region among different bacterial species (Van de Peer *et al.,* 1996).

Most bacterial species have conserved regions flanking the nine hyper-variable regions and thus the target sequences can thus be amplified using universal primers (Baker *et al.*, 2003; Lu *et al*., 2000; McCabe *et al.,* 1999; Munson *et al.,* 2004). 16S rRNA hyper variable region sequences have been used in several studies to differentiate between different species or identify a single bacterial species (Choi *et al.,* 1996, Kataoka *et al*., 1997, Marchesi *et al.,* 1998, Becker *et al*., 2004; Bertilsson *et al*., 2002, Lu *et al*., 2000, Yang *et al.,* 2002, Rothman *et al.,* 2002, Clarridge, 2004, Maynard *et al*., 20050). V1, V3 and V6 primers previously described in earlier studies are broad based primers that have been used to differentiate the different *Salmonella* species (Masek *et al.,* 2013, Jeng *et al.,* 2011). V1 is about 28 nucleotides long; V3 is 65 nucleotides long while V6 is 58 base pair long (Chakravorty *et al.,* 2007).

In this study the three primers VI, V3, and V6 were used to characterize and subsequently differentiate the different *Salmonella* species found in chicken, pigs, cattle

and egg samples in Kenya against *Salmonella* isolates from KEMRI. The curves generated using HRM were used to identify the circulating *Salmonella serovar* in various regions of Kenya.

CHAPTER THREE

3.0: MATERIALS AND METHODS

3.1 Study design

This was a cross sectional study that was carried out in some selected sites in Nairobi and its environs of Kiambu and Machakos counties. The areas covered in this study included Gikomba, Burma, Kariokor markets and Dagorreti slaughterhouse all located in Nairobi county, Ndumbuini slaughter in Kiambu County and Machakos market in Machakos County.

3.2. Sample collection and processing

Samples collected included eggs and fecal samples from chicken, pigs and cattle. The sample size for each of the domestic animal and eggs sampled was determined using the formula of (Daniel *et al.,* 1999) expressed as:

$$\boldsymbol{n = \frac{Z^2 P(1-P)}{d^2}}$$

Where: n=sample size, z=z statistics for a level of confidence, p =expected prevalence or proportion, d=precision. Thus, on the basis of this formula the numbers of samples collected per each animal species were: Pigs=182 (Kikuvi *et al.,* 2010), chicken=191 (Endris *et al.,* 2013), Cattle=150 (Addis *et al.,* 2011), Eggs=217 (Bayu *et al.,* 2013).

A total of two hundred and seventeen egg samples and one hundred and ninety one chicken clocal swabs were randomly sampled from chicken vendors in Machakos town, Kariakor, Burma and Gikomba markets (Kikuvi *et al.,* 2007).

With the help of the staff at Ndumbuini slaughterhouse, fresh fecal matter was collected using sterile swabs in sterile falcon tubes from the rectum of one hundred and eighty two pigs. Similarly, fresh cattle fecal samples were also collected in sterile falcon tubes from the rectum of one hundred and fifty cattle from Dagoretti slaughterhouse.

Positive control *Salmonella species* isolates including *S.* Typhi, *S.* Typhimurium, *S.* Gallinarium, *S.* Pullorum were provided by the center of microbiology research at KEMRI. All the samples were kept individually in portable frozen boxes and immediately transported to University of Nairobi Chiromo campus laboratory for processing.

3.3. Isolation and identification of *Salmonella spp.*

Samples including chicken clocal swabs, pig and cattle fecal materials were first pre-enriched in nutrient broth, while the egg samples were enriched in peptone broth. Prior to enrichment, each egg sample was first disinfected with 75% alcohol and left to air dry in a sterile chamber for 10 minutes , after which it was cracked with a sterile knife and the contents were thoroughly mixed. One milliliter of each egg mixture was inoculated into nine milliliters of peptone broth (Oxoid, Basingstoke England, UK). For chicken samples, each clocal swab (5grams) previously collected in the field was aseptically inoculated into nine milliliters of nutrient broth (Oxoid, Basingstoke England, UK). Approximately 5 grams swabfull of each of the fresh cattle and pig samples of fecal material, were aseptically inoculated into nine milliliters nutrient broth (Oxoid, Basingstoke England, UK). All the samples were incubated at 37°C for 16-18 hours in a thermo shaker (Gallenkamp, London, England). Similar treatment was done on the pure *Salmonella* positive isolates that were earlier provided by KEMRI.

Following the pre-enrichment treatment, one milliliter of enriched cultures of each of the sample type were transferred to nine milliliters of selenite cystine broth (Oxoid, Basingstoke England, UK) and incubated at 37°C for 18-24h in a thermo shaker (Gallenkamp, London, England). A loopful of the cultured broths were then plated onto XLD agar and incubated at 37°C for 24 h in order to isolate the suspected colonies.

The isolated *Salmonella spp*. were stored as glycerol stocks at -80ºC for future use. The glycerol stocks for each of the isolated strains were prepared by mixing two hundred micro liters of an overnight culture and with 1200µL of 80% sterile glycerol (Sigma, St. Louis, USA)

3.3.1. Morphological characterization of *Salmonella spp.*

Gram stain test was carried out on each of the bacterial isolates. Briefly a bacterial smear from a pure culture of each of the positive controls was prepared and heat fixed on a clean glass slide. The slide was then flooded with crystal violet dye for 1minute and subsequently rinsed with running tap water. The slide was then flooded with Gram's iodine for another 1 minute, and rinsed with running tap water. This was then followed by decolourization with 95% ethanol and then rinsed with tap water for 30 seconds. The processed slide was then counter stained with neutral red dye for 1 minute, rinsed with running tap water and it was left to air dry. The dry slide was then covered with immersion oil and viewed at X100 under a Leica ICC 50 microscope for the presence of gram-negative rods (Leica microsystems, Wetzlar, Germany).

3.3.2. Biochemical characterization of *Salmonella spp.* in samples

Salmonella spp. identified morphologically were biochemically characterized using the Analytical Profile test kit (API 20E bioMe´rieux®, Inc., France). The API 20E test kit consists of 20 micro tubes containing different dehydrated substrates that included glucose, mannose, inositol, sorbitol, rhamnose, sucrose, melibose, amygdalin, arabinose and dehydrated enzymes that include O-nitrophenyl-D- galactopyranoside; arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate, hydrogen sulphide production, urease, tryptophan deaminase, indole, voges proskauer test, gelatinase. First, the strips were prepared by distributing, 5 ml of distilled water into the honeycombed wells of the tray, and then incubated to create a humid atmosphere. A single colony was then isolated from the XLD plate using a sterile swab and emulsified in five milliliters of 0.85% NaCl in order to achieve a homogeneous bacterial suspension. A sterile needle was used to inject the homogenous solution in each of the 20 microtubes of the API 20E kit. Some biochemical reactions were allowed to occur under either anaerobic or aerobic conditions. Arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), Hydrogen Sulphide (H_2S) and Urea (URE) reactions were carried out under anaerobic conditions, by overlaying the wells with a drop of mineral oil. The remaining wells including glucose, mannose, inositol, sorbitol, rhamnose, sucrose, melibose, amygdalin, arabinose and dehydrated enzymes that include O-nitrophenyl-D-galactopyranoside, citrate, urease, tryptophan deaminase, indole, voges proskauer test, gelatinase were allowed to react aerobically thus no mineral oil was added to the wells. The samples were then incubated at 37°C for 18 to 24 h. The results from this experiment were then determined according to API 20E software.

3.4. Extraction of genomic DNA from *Salmonella spp*.

Extraction of *Salmonella* genomic DNA was based on the Qaigen method (QIAprep Spin Miniprep Kit (250) handbook). Briefly, a single bacterial colony of each of the positively identified *Salmonella spp.* samples, including the positive controls was picked from a positive XLD plate and each colony inoculated into a ten milliliters nutrient broth (Oxoid, Basingstoke England, UK). The culture were then grown overnight at 37°C for 12-16 hours in a thermo shaker at 120 rpm (Gallenkamp, London, England). The cultured isolates were then harvested by centrifugation at 13000 rpm (HettichZentrifugen,Tuttlingen, Germany) for 10 minutes according to the manufacturer's protocol (QIAprep Spin Miniprep Kit (250)). The supernatant was then poured off and the harvested cells re-suspended in two hundred and fifty micro liters of P1 buffer (QIAprep Spin Miniprep Kit). Two hundred and fifty micro liters of buffer P2 was then added to the tube and gently inverted 4-6 times to mix and allow lysis of the membrane protein and lipids to take place. Three hundred and fifty micro liters, of N3 buffer was then added and mixed thoroughly by inverting the tube 4-6 times. The supernatant was harvested by centrifugation at 13000 rpm (HettichZentrifugen, Tuttlingen, Germany) for 10 minutes. The clear supernatant obtained after centrifugation was then transferred to QIAprep spin column and subsequently centrifuged at 13000 rpm (HettichZentrifugen, Tuttlingen, Germany) for 2 minutes. The supernatant was discarded, after which, the QIAprep spin column was washed with five hundred microlitres of the PB wash buffer, centrifuged for 2 minutes (HettichZentrifugen, Tuttlingen, Germany) and supernatant discarded. The QIAprep spin column was subsequently washed with seven hundred and fifty micro liters of PE wash buffer, centrifuged for 2 minutes

(HettichZentrifugen, Tuttlingen, Germany) and the supernatant discarded. To elute the DNA, the QIAprep spin column was then transferred to a new 1.5 ml micro centrifuge tube and fifty micro liters of elution buffer added, and allowed to stand for 1minute and then centrifuged for 1 minute. The QIAprep spin column was discarded and the resultant DNA product stored at -20°C prior to analysis.

3.4.1. DNA analysis

The quality of genomic DNA extracted from the *Salmonella* species was analyzed on 1% (w/v) agarose (Sigma, St. Louis, USA) gel in 1X TAE buffer. A 1% TAE /agarose/EtBr gel was prepared by boiling 1.0 g agarose in 100 ml of 1X TAE. The hot agarose solution was then allowed to cool down prior to addition of 5 µL of ethidium bromide. The solution was then poured into the gel casting chamber and a comb placed in position. After polymerization (30 minutes) the gel was transferred into the electrophoresis chamber with the slots facing the cathode and covered with a running buffer (1X TAE buffer). 6X orange DNA loading dye (Fermentas, Pittsburgh, USA) was premixed with the DNA samples in the ratio 1:5 µL of sample (final concentration: 1X) prior to loading of the samples onto the wells in the gel. The sample was then electrophoresed at 5 V/cm (80V) for genomic DNA. The current was supplied by an electrophoresis power supply (Consort EV265, Holliston, USA). The DNA bands were visualized under a UV trans illuminator (Herolab, Wiesloch, Germany).

3.4.2. 16S rRNA PCR coupled HRM

The analysis of 16S rRNA PCR-HRM assay was based on the method of (Yang *et al*., 2009). Positive *Salmonella* specimens, together with the positive controls, were assayed for the presence of hyper variable regions: V1, V3, and V6 using the following primers

V1-F5=-GYGGCGNACGGGTGAGTAA V1-R 5=-TTACCCCACCAACTAGC, V3-F 5=-CCAGACTCCTACGGGAGGCAG V3-R 5=-CGTATTACCGCGGCTGCTG V6-F 5=-TGGAGCATGTGGTTTAATTCGA V6-R 5=-AGCTGACGACANCCATGCA (Inqaba Biotec). Briefly, each HRM analysis was performed in a 10µL total volume consisting of 4 µL of 2.0 Master Mix (Roche Light cycler 480 SYBR Green I Master mix, Basel, Switzerland) and 4 µL of molecular grade water (Roche Technology). A total of 0.5 µL of 1.5 µmol/L forward primer and reverse primer for V1, V3, and V6 regions (Inqaba South Africa) was added to each separate reaction and 1 µL of template DNA. Each PCR analysis contained one primer pair. The PCR was performed at cycling conditions as follows: Denaturation step at 95°C for 30 seconds, followed by 45 cycles repeats at 95°C for 30 seconds, Annealing at 60°C for 60 seconds, and extension temperature at 72°C for 60 seconds and 1 cycle at 95°C for 30 seconds and 28°C for 30 seconds (Hardick e*t al.,* 2012, Won *et al.*, 2010).

Each post qPCR sample amplicon was then subjected to melt curve analysis on Bioneer Exicycler Real time PCR instrument (Daejeon, Korea). The melting temperatures ranged from 60°C to 95°C and data acquisition was done for every 0.1°C increase in temperature (Hardick *et al.,* 2012, Won *et al.*, 2010).

3.4.3. Limit of Detection Test for HRM assay.

HRM assay limit of detection was determined as described previously (Masek *et al.*, 2013). Briefly the DNA concentration of the positively identified *Salmonella* samples, *S.* Enteritidis, *S.* Choleraesuis, *S.* Tyhimurium, *S.* Dublin was determined using a 1000c Nano Drop (Thermo Fisher Scientific Waltham, USA). A tenfold serial dilution was prepared, by adding one microlitre DNA of each sample to nine microliters of elution

buffer. A hundredfold dilution was prepared by adding one microliter of the tenfold dilution in nine microliters of the elution buffer. A thousand-fold dilution was prepared by adding one microliter of the hundred-fold dilution in nine microliters of the elution buffer. The serially diluted samples were each subjected to HRMA assay as previously described.

3.4.4. Specificity tests for HRM assay in detection of *Salmonella spp.*

For specificity testing an artificial sample was prepared to mimic a mixed infection of *Salmonella*. Hence five microliter of each of *S.* Enteritidis, *S.* Gallinarum, *S.* Typhi, and *S.* Braenderup positive controls were mixed in a single tube as described by Masek *et al.*, 2013. This mixed sample was then subjected to HRM assay as earlier described.

3.4.5. Data analysis

The data on the prevalence of *Salmonella spp*. obtained by culture, API 20E strips, was presented as proportions. Results were displayed and described using Microsoft Excel® generated graphs and summary tables. HRM data was analyzed based on the cycle threshold (CT) value (Masek *et al.,* 2013) with the CT value<32 being considered negative as previously described (Masek *et al.,* 2013). The prevalence of *Salmonella* identified using HRM assays were displayed as proportions using Microsoft Excel® generated graphs.

CHAPTER FOUR

4.0. RESULTS

4.1. Isolation of *Salmonella* bacteria species from biological samples

Following culturing process, *Salmonella* like colonies were visually observed on XLD media, as pink colonies with black centers (figure 4.1a). This was evaluated against *E.coli* a negative control culture that had yellow colonies (figure 4.1b). Based on this culture method, eggs had the highest percentage of *Salmonella* infection with a total of 9.2% of the total samples being positively identified as being infected with *Salmonella*. About 8% of the pig's fecal material was infected with *Salmonella* while chicken and cattle sample material each registered a 4% *Salmonella* contamination (figure 4.2).

Figure 4.1: Photographs of bacteria colonies (a) pink colonies with black center of *Salmonella* (b) yellow colonies of *E.coli* (control) following pre-enrichment, and selection on XLD media.

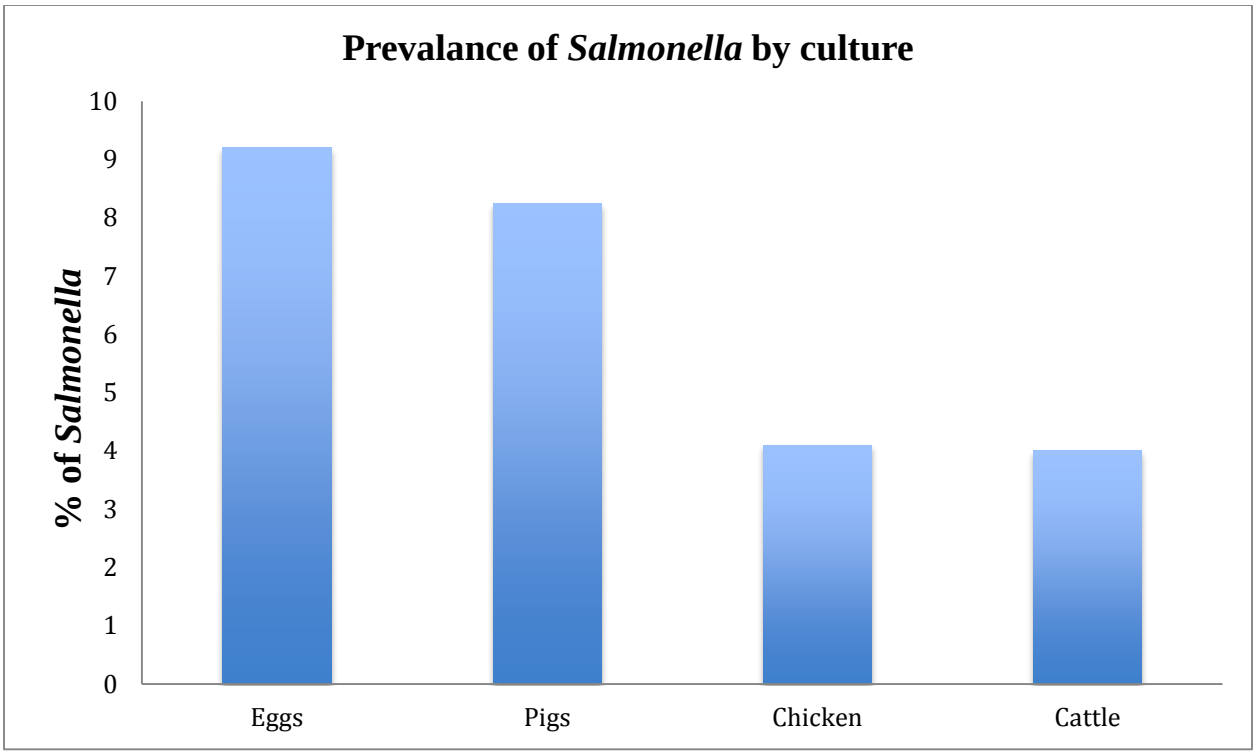


Figure 4.2: Prevalence of *Salmonella* spp. by culture method. The Prevalence of *Salmonella spp*. varied across the different animals sampled with a prevalence of 9.2% in eggs, 8% in pigs and 4% in both chicken and cattle.

4.2. Morphological characterization of *Salmonella spp.*

Gram staining was subsequently used to morphologically characterize the samples that were positively identified by culture method. Consistent with the literature the *Salmonella* isolates were characterized as small gram-negative rods that appeared as singly rod or paired form on the microscope slide.

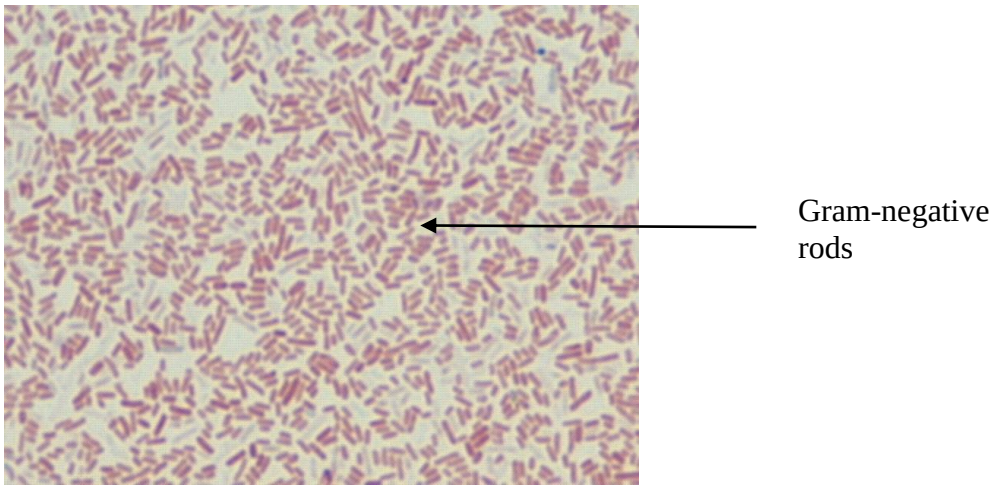


Figure 4.3: A photograph of gram stain of rod shaped *Salmonella* spp. found in the positively identified culture samples.

4.3. Biochemical characterization of *Salmonella spp.*

Salmonella spp. were identified biochemically based on the ability of the pathogen to act on the end product produced during the reaction using the following enzymes arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase. The pathogen was also characterized based on its capability to utilize citrate, hydrogen sulphide production, glucose, mannose, sorbitol, rhamnose, melibose, and arabinose as the substrates. This was confirmed with the colour change of the reaction in the API 20E strips as shown in figure 4.4. Based on the API 20E method of characterization the percentage of *Salmonella spp.* contamination of the samples varied among the different animal species waste material sampled in the study. About 5.99% of the total eggs sampled were infected with *Salmonella* while, 3.85% of the total pigs fecal materials were contaminated with the pathogen. Whereas 3.66% of all the chicken swabs were infected with *Salmonella,* only 2.67 % of the cattle fecal material was contaminated with the *Salmonella* as shown in (figure 4.5).

Figure 4.4: An API 20 E strip kit system for *Salmonella* identification in chicken swabs, eggs and the fecal materials of pigs and cattle. *Salmonella* is identified based on the following; ONPG: O-nitrophenyl-D- galactopyranoside –VE, ADH: arginine dihydrolase, +VE, LDC: lysine decarboxylase +VE, ODC: ornithine decarboxylase +VE, CIT: citrate +VE, H_2S: hydrogen sulphide production +VE, URE: urease -VE, TDA: tryptophan deaminase -VE, IND: indole -VE, VP: Voges Proskauer test -VE, GEL: gelatinase -VE, GLU: glucose +VE, Man: mannose +VE, INO: inositol +VE, SOR: sorbitol +VE, RHA: rhamnose +VE, SAC: sucrose- VE, MEL: melibose +VE, AMY: amygdalin-VE, ARA: arabinose +VE.

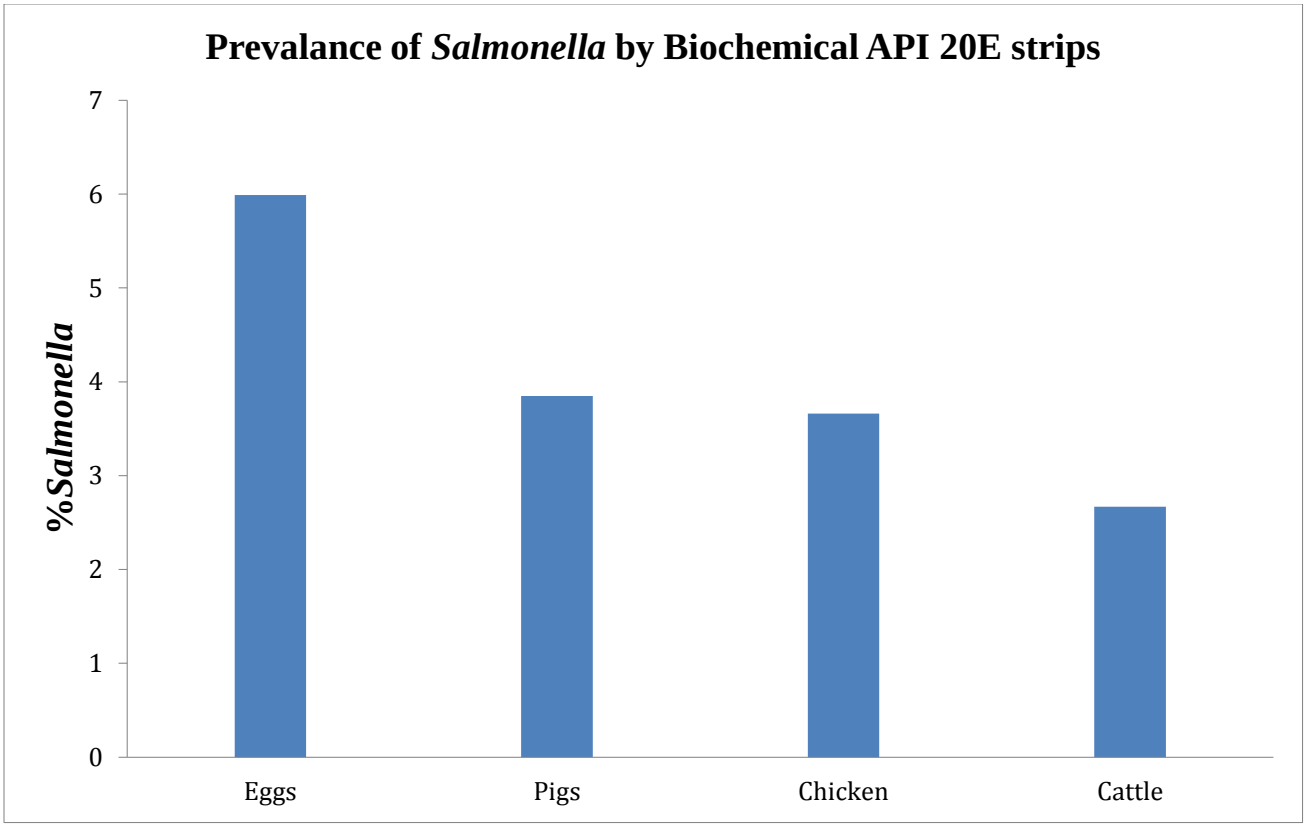


Figure 4.5: Prevalence of *Salmonella spp.* using API 20E strips. The prevalence varied across the different samples with eggs recording a 5.99%, Pigs 3.88%, chicken recorded 3.66% and cattle 2.7%.

4.4. Molecular characterization of *Salmonella spp.* using 16S rRNA real time PCR.

The DNA extracted fro *Salmonella spp*. positive samples were subjected to real time PCR using V1, V3 and V6 primers. A sample characterized to be positive was defined as having a cycle threshold (CT) value of <32 (Masek *et al*., 2013). A CT value of <32 was observed in all the samples using the V1 primers, 96% of the samples amplified with V3 primers and 93% of the samples amplified using V6 primers (Figure 4.6).

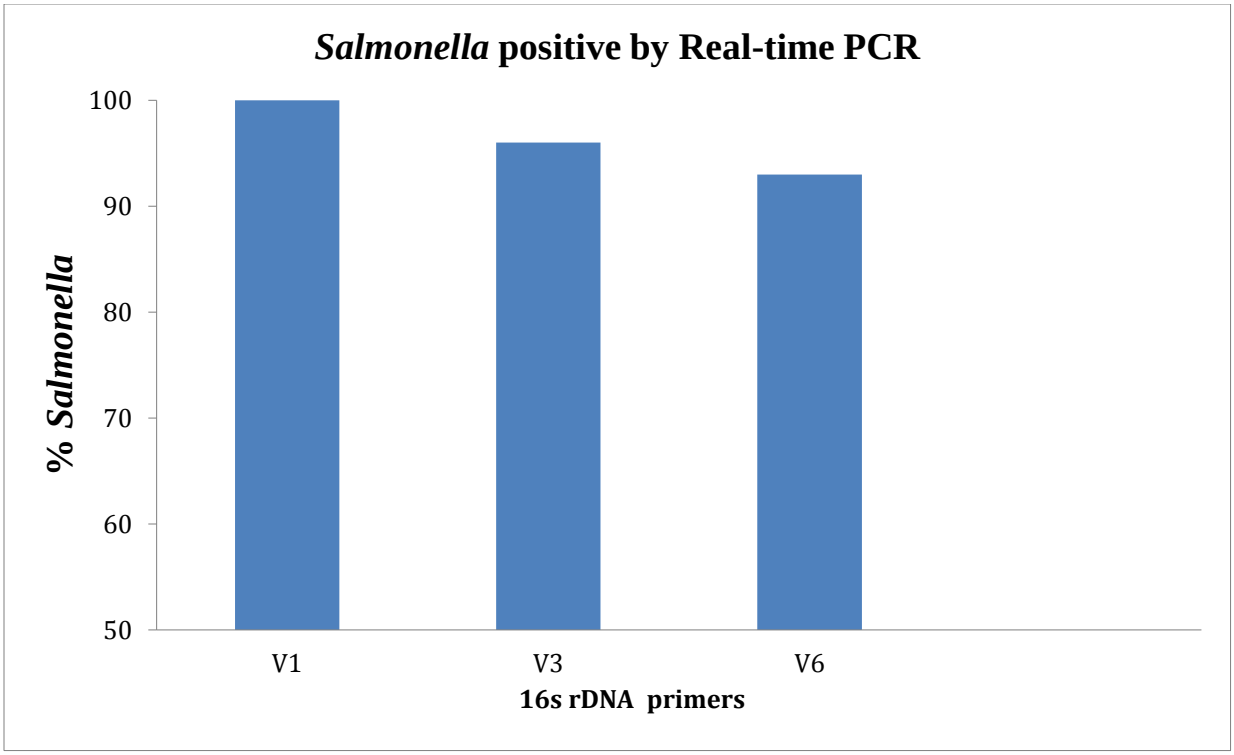


Figure 4.6: Proportion of samples that were positive by PCR using the three primers. All samples amplified successfully with V1 primers, 96% of the samples were positive for V3 and 93% of the samples were positive for V6 primers.

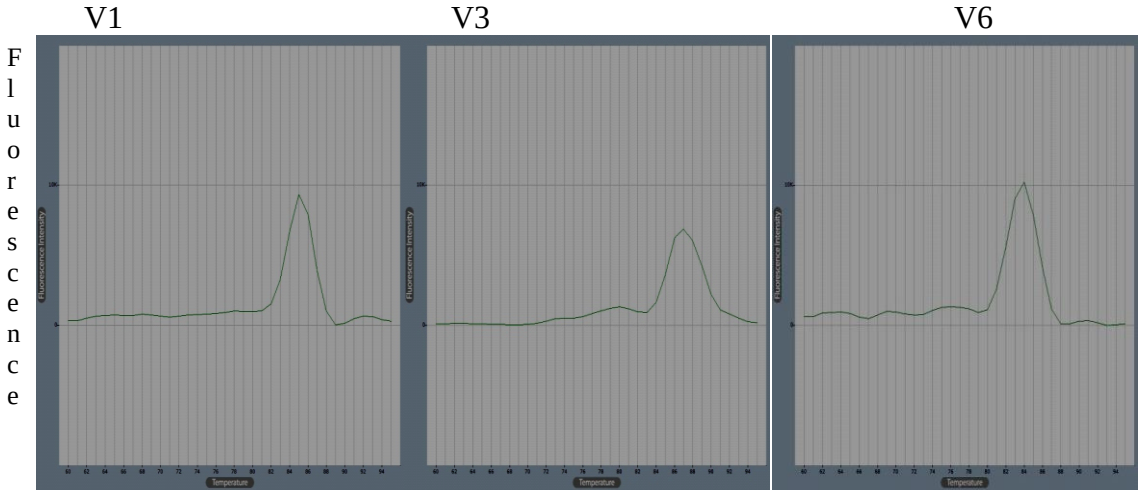
4.5. Identification of *Salmonella spp*. based on HRM curve similarity

Identification of the *Salmonella spp.* was based on curve shape similarity between the control sample and the isolates from the sampled animals. The curve generated is based on the GC content, the length, the sequence and the heterozygosity of the DNA sample thus different *Salmonella spp*. will have different curves.

In this study curve matching of the unknown samples was done against control. Figure 4.7a shows melt profile of *S*. Enteritidis a control from KEMRI, using this criteria four chicken sample and five egg samples were identified as *S*. Enteritidis as shown in (figure 4.7b) and (figure 4.8).

Similarly *S.* Braenderup was identified in cattle samples by curve matching of the control *S.* Braenderup melt curve melt profile (figure 4.9a) to the cattle samples (figure 4.9b). Pig samples on the other hand harbored *S.* Choleraesuis (figure 4.10b) and *S.* Typhimurium (figure 4.11b) this was based on melt curves generated that were similar in height and shape with curve generated for the respective controls (figure 4.10a) (figure 4.11a). The similarity between the field sample and *Salmonella* isolates control from KEMRI was indicative of sequence similarity in terms of GC content and length. Based on the melt profiles generated for each *Salmonella* serovar by HRM *Salmonella* was identified in all the species; (figure 4.12) with *S.* Enteritidis being the most prevalent in chicken and eggs at 47.3%, followed by *S.* Choleraesuis and *S.* Typhimurium in pigs at 31.5% and 5% respectively, and *S.* Braenderup in cattle. However, samples that lacked the gene of interest generated a flat curve, (figure 4.13).

A. *S.* Enteritidis control



B. Chicken sample

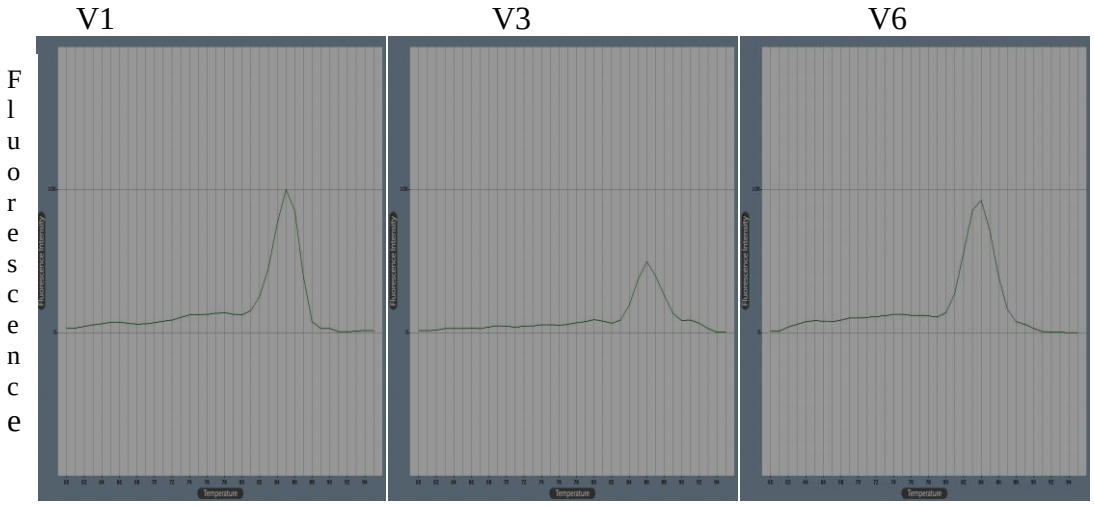


Figure 4.7a: Unique melting profiles illustrated by change in fluorescence units with increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for a control *S.* Enteritidis.

Figure 4.7b: Unique melting profiles, illustrated by change in fluorescence units with increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for chicken sample, which is similar to *S.* Enteritidis. The melt curve of the sample (figure 4.7b) is

similar in shape and height to the melt curve of the control (figure4.7a), which is indicative of sequence similarity between the chicken sample and *S.* Enteritidis.

B. Egg -sample

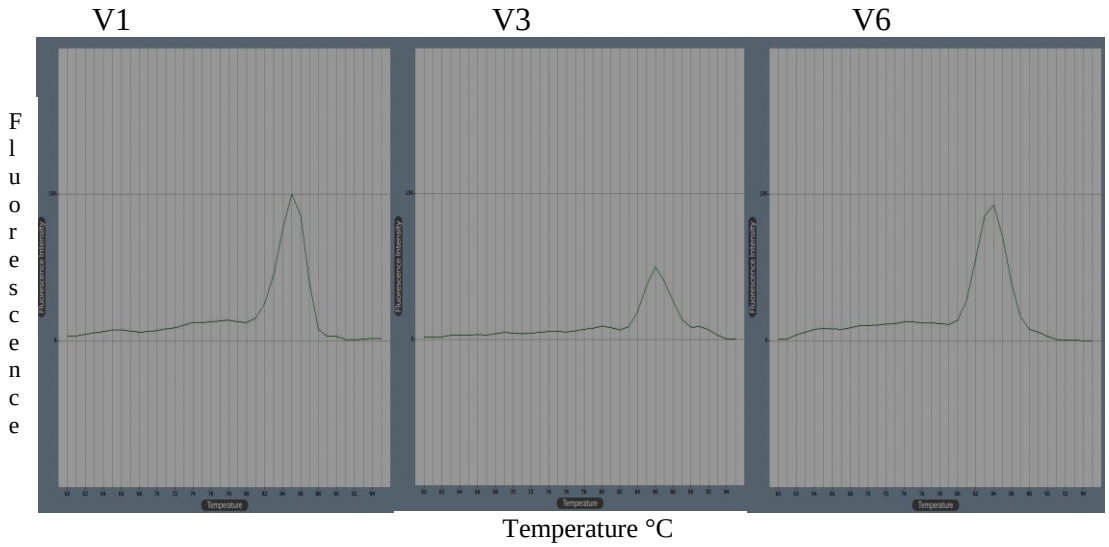
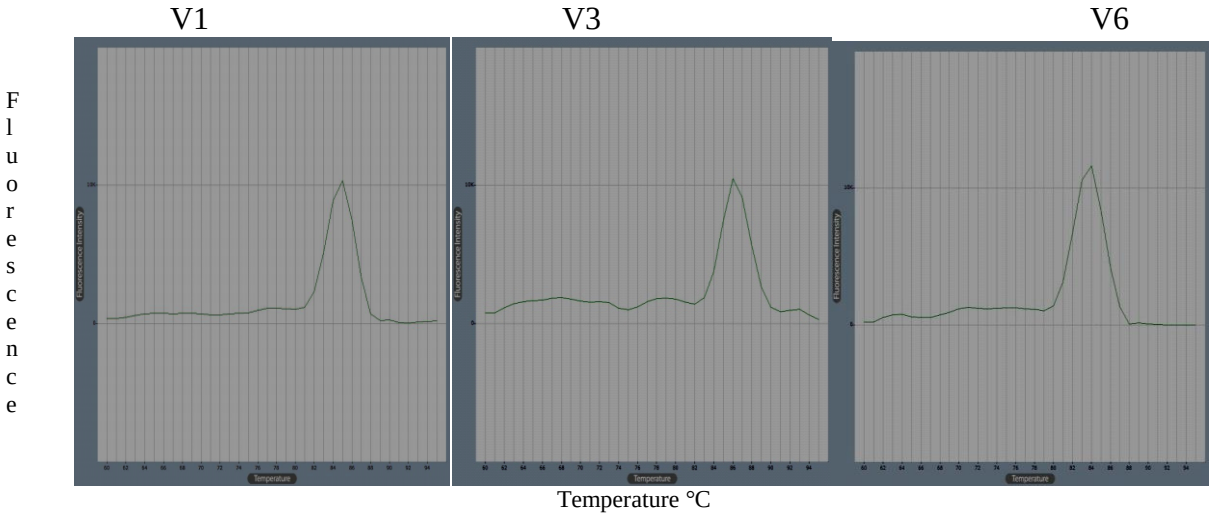


Figure 4.8: Unique melting profiles, illustrated by change in fluorescence units with increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for egg sample, which is similar to *S.* Enteritidis. The melt curve of the sample (figure 4.8) is similar in shape and height to the melt curve of the control (figure 4.7a), which is indicative of sequence similarity between the chicken sample and *S.* Enteritidis.

A. *S.* Braenderup Control



B. Cattle sample

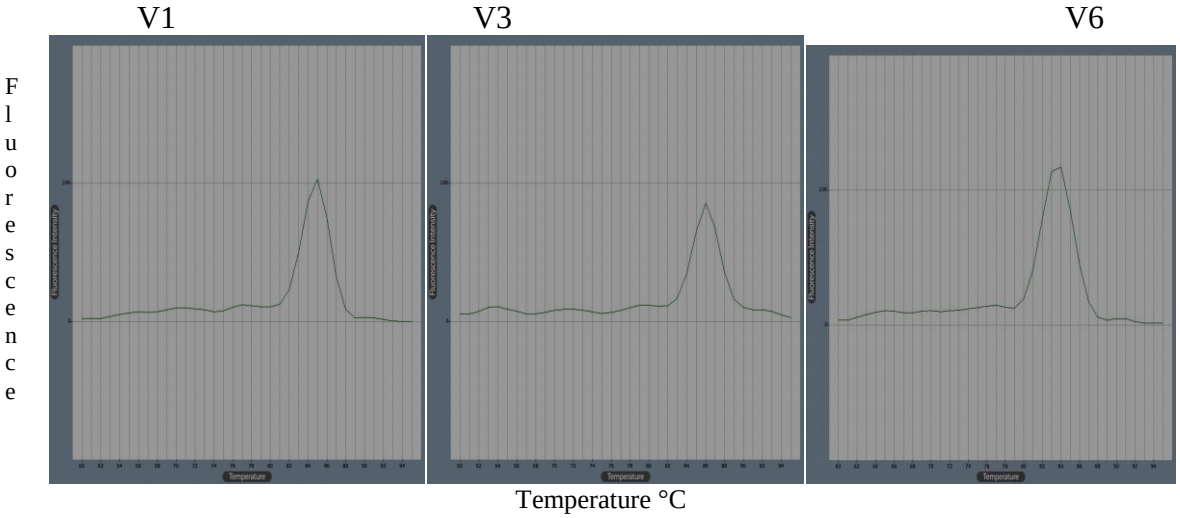
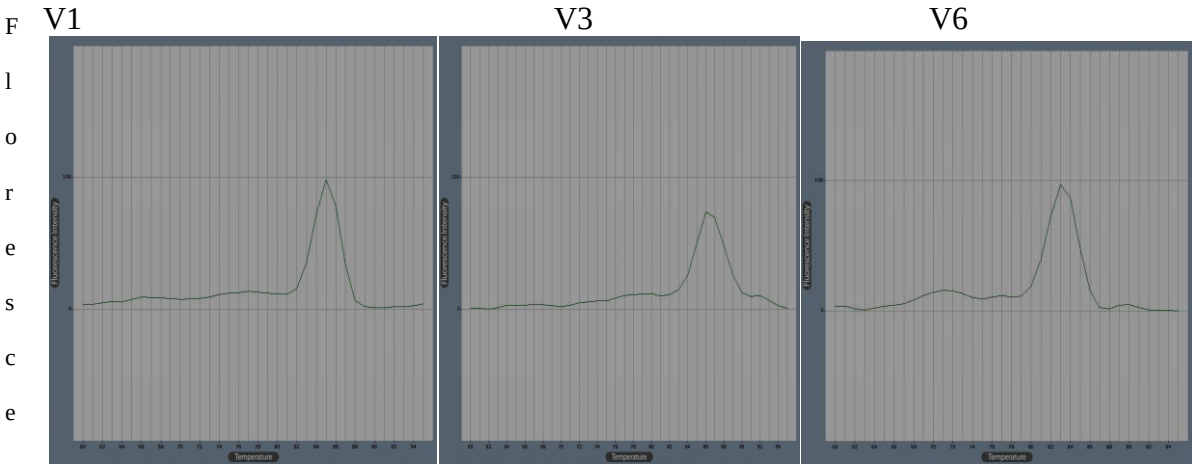


Figure 4.9a: Unique melting profiles, illustrated by change in fluorescence units with increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for a control *S.* Braenderup.

Figure 4.9b: Unique melting profiles, illustrated by change in fluorescence units with increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for cattle sample, which is similar to *S.* Braenderup. The melt curve of the sample (figure 4.9b) is

similar in shape and height to the melt curve of the control (figure 4.9a), which is indicative of sequence similarity between the cattle sample and *S*. Braenderup.

A. *S*. Choleraesuis -control



B. Pig sample

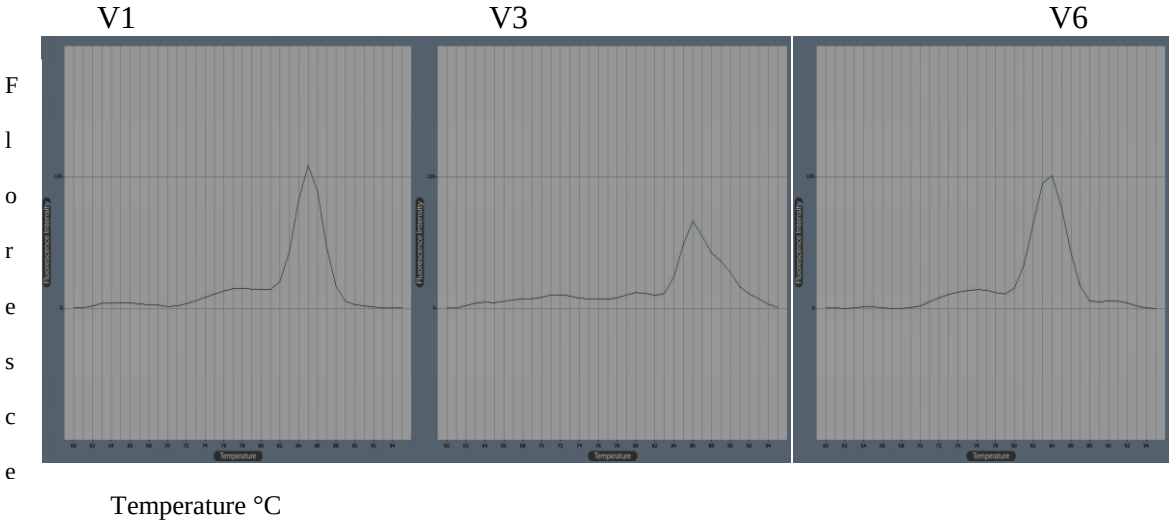
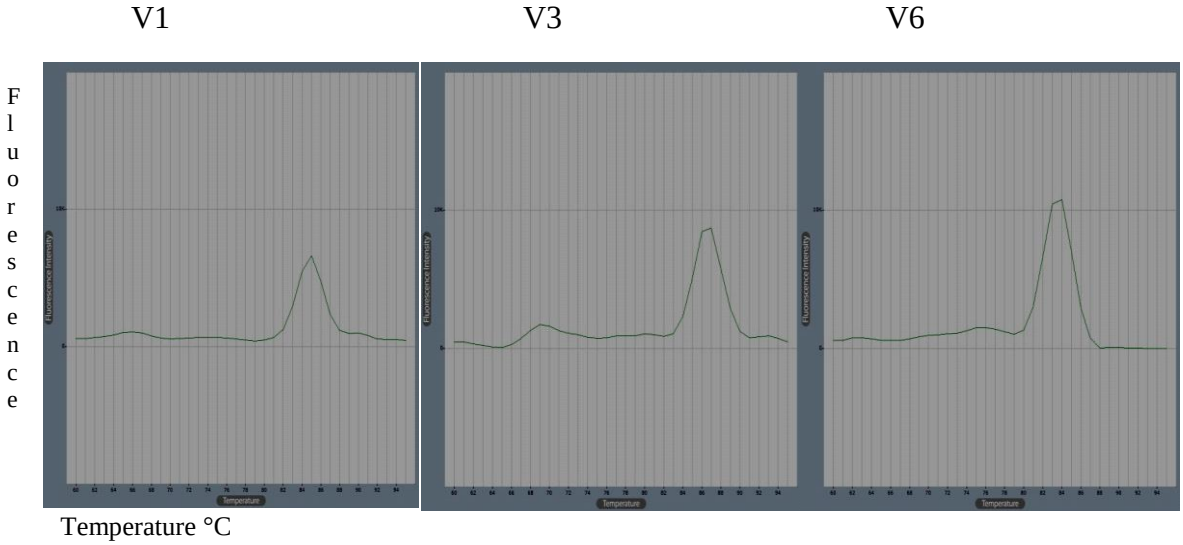


Figure 4.10a: Unique melting profiles, illustrated by change in fluorescence units with increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for a control *S*. Choleraesuis

Figure 4.10b: Unique melting profiles, illustrated by change in fluorescence units with increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for pig sample, which is similar to *S.* Choleraesuis. The melt curve of the sample (figure 4.10b) is similar in shape and height to the melt curve of the control (figure 4.10a), which is indicative of sequence similarity between the cattle sample and *S.* Choleraesuis.

A. *S.* Typhimurium-control



B. Pig sample

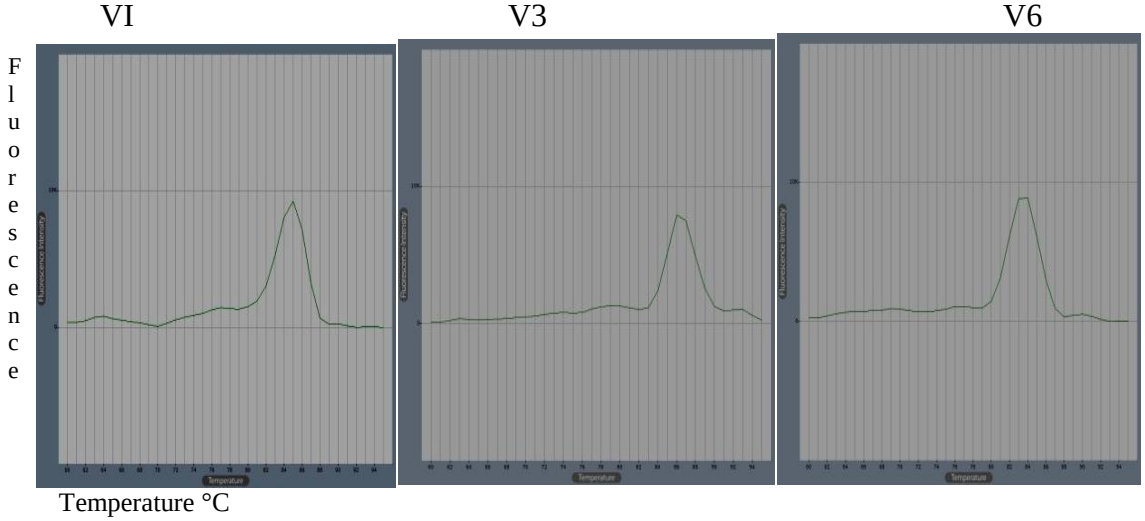


Figure 4.11a: Unique melting profiles, illustrated by change in fluorescence units with

increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for a control *S.*Typhimurium.

Figure 4.11b: Unique melting profiles, illustrated by change in fluorescence units with increasing temperatures for V1, V3 and V6 primers of 16S rRNA region for pig sample, which is similar to *S.* Typhimurium. The melt curve of the sample (figure 4.11b) is similar in shape and height to the melt curve of the control (figure 4.11a), which is indicative of sequence similarity between the pig sample and *S.* Typhimurium.

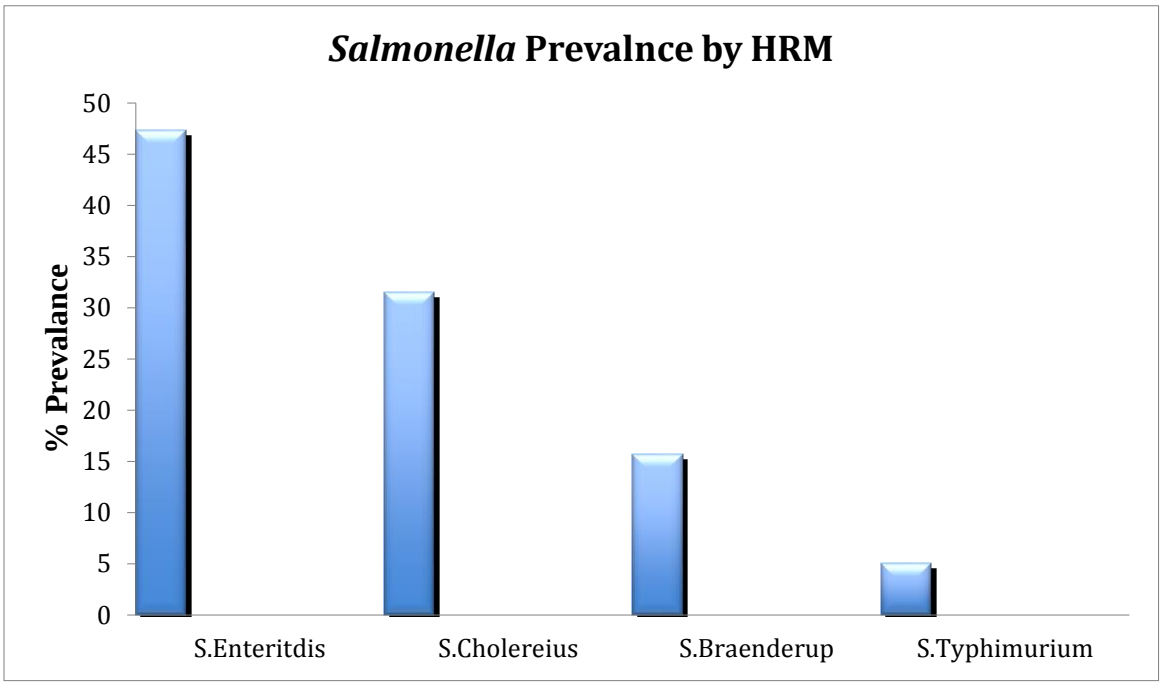
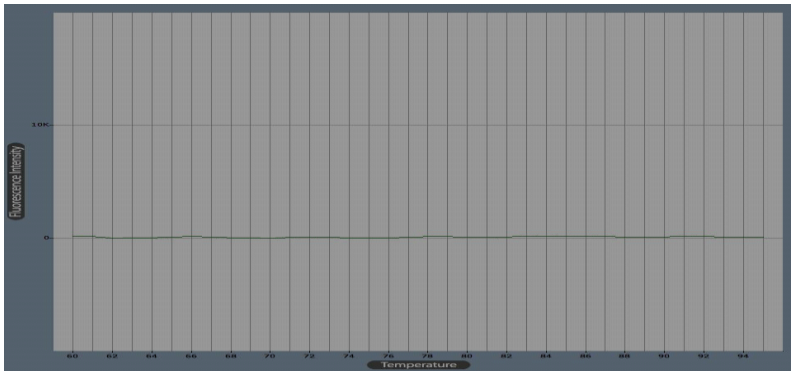


Figure 4.12: Prevalence of *Salmonella* by high-resolution melting point assay. *S.* Enteritidis was most prevalent at 47.3%, *S.* Choleraesuis at 31.5%, *S.* Braenderup at 15.7 and *S.* Typhimurium at 5%.



Temperature °C

Figure 4.13: Melt curve of a non-template. The melt profile is flat, as it does not have the gene of interest that should have been amplified.

4.6. Limit of Detection of HRM assay.

The limit of detection of the HRM assay was 0.031ng/µl of DNA of the *Salmonella* sample. However, the assay could not detect samples that had a lower concentration of DNA below 0.03ng/ul.

Table 4.1: DNA concentration of the diluted samples.

Sample	DNA Concentration ng/µl		
	10^{-1}	10^{-2}	10^{-3}
Egg mixture	3.05	0.305	**0.031**
Chicken swab	2.89	0.289	0.029
Pig fecal matter	3.47	0.35	0.035

4.7. Specificity Test for HRM assay

HRMA exemplified ability to differentiate the different *Salmonella* serovars, that is *S.* Typhi, *S.* Braenderup, *S.* Dublin, *S.* Typhimurium and *S.* Choleraesuis based on curve shape and height (figure 4.14) and (figure 4.15). Using the V1 primers *S.* Braenderup had the highest peak followed by *S.* Choleraesuis, *S.* Dublin, *S.* Typhimurium and finally *S.* Typhi, which had the lowest peak. Similar results were observed for V6 primers with *S.* Braenderup having the highest peak followed by, *S.* Typhimurium, *S.* Choleraesuis, *S.* Typhi and finally, *S.* Dublin. The difference in height and shape is attributed to sequence variability between the different *Salmonella species.* Although the HRM assay was capable of differentiating the different *Salmonella* upto the serovar level it however, failed to differentiate a mixture of different *Salmonella* serovars in case of a poly microbial infection. Only one single curve was observed for each primer instead of three different curves for each of the three *Salmonella spp*. serovars used in the study (figure 4.16).

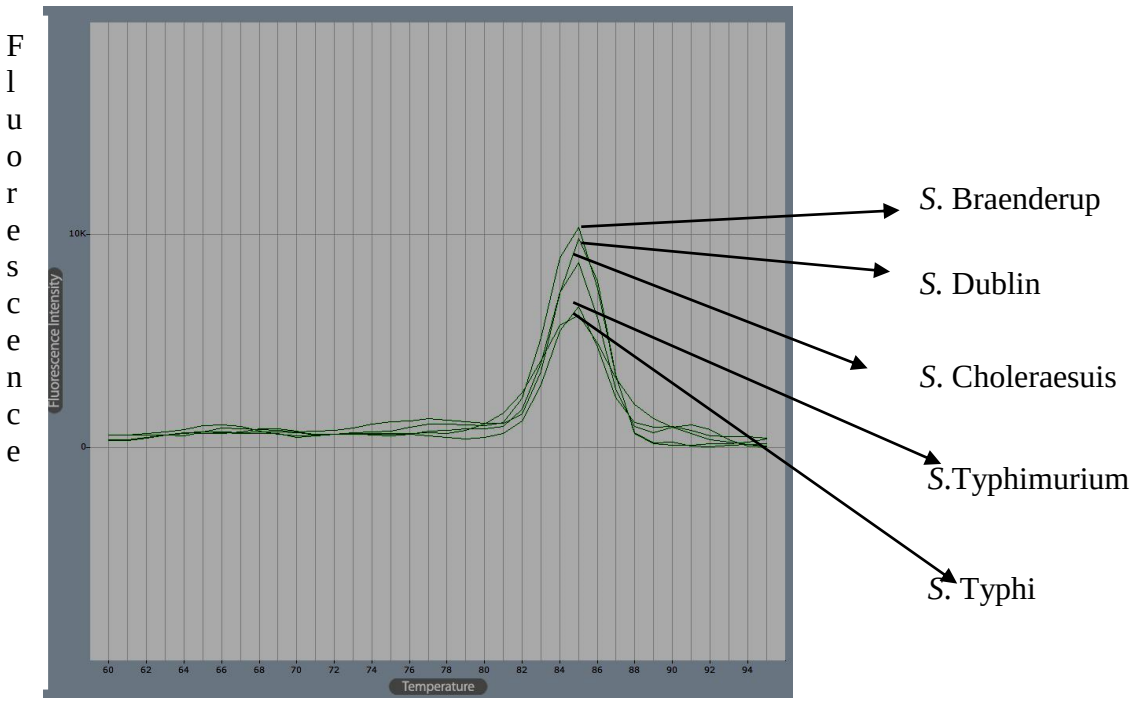


Figure 4.14: Unique melting profiles based on height, illustrated by change in fluorescence units with increasing temperatures for *S.* Braenderup *S.* Choleraesuis, *S.* Dublin, *S.* Typhimurium and *S.* Typhi using V1 primers.

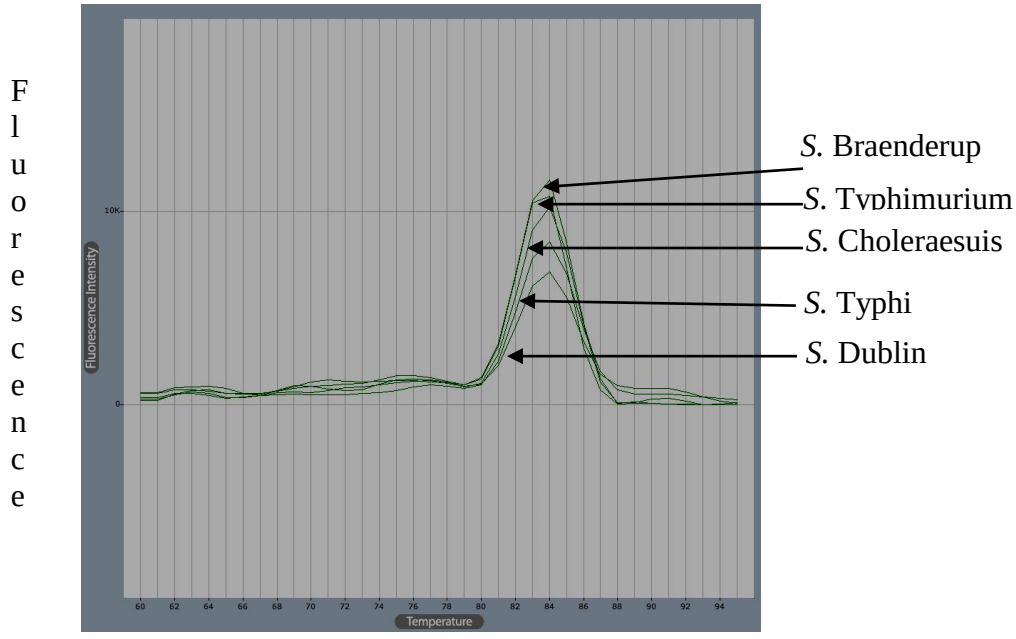
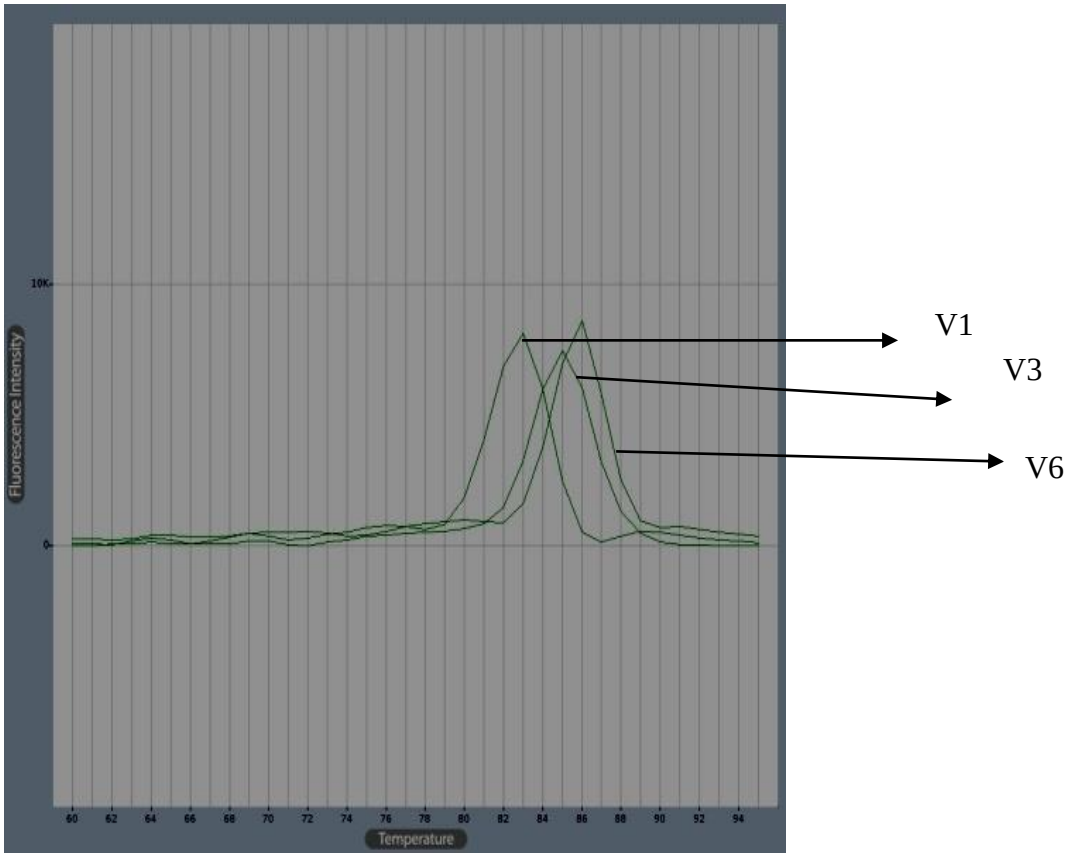


Figure 4.15: Unique melting profiles based on height, illustrated by change in fluorescence units with increasing temperatures for *S.* Braenderup *S.* Choleraesuis, *S.* Dublin, *S.* Typhimurium and *S.* Typhi using V1 primers.



Temperature °C

Figure 4.16: No distinct melting profiles for the sample that had a mixture of different *Salmonella* serovars, only a single peak was observed for each of three primers used.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSSION AND RECOMMENDATIONS.

5.1. DISCUSSION

Invasive *Salmonella* associated diseases are known causes of morbidity and high mortality rates, in developing countries especially in the sub-Saharan Africa (SSA) as well as some parts of the Indian and Asian sub-continents (Kariuki *et al.*, 2010). In the advent of multi-drug resistant *S.* Typhi is one of the key problems currently facing the effective treatment of *Salmonella* associated diseases especially in resource-limited settings (Kariuki *et al.*, 2010). It is reported that the emergence of multidrug resistant *Salmonella* has been partially caused by under diagnosis, inaccurate or over diagnosis of *Salmonella* infection leading to antibiotic drug abuse (Savard *et al.,* 2011).

In this study a new nucleic acid based-HRMA assay was developed for the diagnosis of *Salmonella* in domestic animals feces in order to determine the sensitivity and limit of detection of the technique for *Salmonella serovar* identification. *Salmonella spp.* were first isolated from the fecal materials of cattle, pigs and chicken as well as in the egg content. Using the culture method, about 9.21% of all the eggs sampled were positive for *Salmonella,* 8.24 % of the pig fecal materials were positive while chicken swabs had 4.1% while only 4% of the sampled fecal material of cattle was contaminated with *Salmonella*. This was visually identified based on the pink with black centers colored *Salmonella* colonies as previously reported by Molbak *et al.,* 2006. The color change observed has been related to the ability of *Salmonella spp.* to degrade xylose and utilize lysine in the growth media. During lysine utilization, the decarboxylation of the amino

acid results in the generation of an alkaline metabolite, cadaverine, and a reversion of the medium color back to red (Molbak *et al.,* 2006). Although culture method has been regarded as the gold standard in the identification of *Salmonella* in most biological samples, the specificity of *Salmonella* detection was enhanced by biochemical characterization using the API 20E strips. Using these strips only 5.99% of the egg samples were contaminated with *Salmonella*. On the other hand only 3.85% were positive in the pigs fecal material, 3.66% in chicken swabs while, 2.67% of cows fecal materials were contaminated with *Salmonella.* This clearly indicated that API 20E technique should be used hand in hand with culture method. This is in accordance with WHO standards. This clearly indicates that characterization of *Salmonella spp*. by culture only is likely to result in in false positives. This can clearly explain the increased incidences of *Salmonella* that has been recently reported when the widal test has been used in Nairobi and Embu (Kariuki *et al*., 2004). Culture being the gold standard for identification of *Salmonella spp.* in hospitals it would be best if biochemical identification would be included as part of routine diagnosis of *Salmonella spp.*

The prevalence of *Salmonella* in pig's feces was 3.8% using the biochemical screening method. This is in contrast to earlier studies that have reported higher values like 13.8% (Kikuvi *et al.,* 2007). The lower percentage reported in this study compared to 13.8% found previously could be attributed to the stringent methodology used in this study. Samples used in this assay were first subjected to culture method, biochemical and thus any false negative that would have occurred due to the use of one methodology was eliminated. The prevalence of *Salmonella* in chicken was 3.66% this agrees with a study done in Nigeria where a prevalence of 3.2% (Onunkwo *et al*., 1978) and disagrees with

an Ethiopian study where a prevalence of 9.3% (Endris *et al*., 2013) was reported. These significant differences in these two studies could be attributable to the differences in environ-mental contamination, management systems used, breed and parent stock difference of chickens used in the present and those done by other researcher (Botteldoorn *et al.,* 2003).

In this study eggs had a prevalence of 5.99% perfectly in agreement with an Ethiopian study, which indicated a 4.69% *Salmonella* contaminations (Bayu *et al.,* 2013). Prevalence of *Salmonella spp*. in cattle was 2.67%, which was the lowest across the different animal species. This prevalence disagrees with a study in Ethiopia, which had a prevalence of 10.96% (Addis *et al.,* 2011). This difference could also be attributed to the stringent method used in our study to identify *Salmonella spp.* The lower prevalence of *Salmonella* in cows is clinically important as it indicates lower incidences of Salmonellosis not only in cattle but also in human population.

Upon identification of *Salmonella* in the different sample types used in this study it was important to further identify the specific serotype. This was done using HRM, which is rapid unlike the gold standard serotyping, (Brenner *et al.,* 2000) which is time consuming. HRM assay has been used in many studies for molecular characterization of many bacterial infections (Reed *et al.,* 2007) as well as in the differentiation of *Salmonella* serovars (Jeng *et al.,* 2011, Masek *et al.,* 2013). Thus, in this study HRM assay was developed and evaluated against circulating *Salmonella* serovars among some domestic animals in Kenya. The samples were first subjected to real time PCR; positive samples were defined as having a cycle threshold value of <32 as previously (Masek *et al.,* 2013). In the current study 100% of the samples were successfully amplified using

the V1 primers, 93% of the samples amplified using V3 while 86% of the samples amplified using V6 primers. This means that V1 primers are more specific for the *Salmonella* genes as compared to V3 and V6 primers. Each of the 44 samples evaluated in this study generated a characteristic melt curve for each of the three primers set V1, V3 and V6. Each melt curve generated by the three primers revealed a single dominant peak, indicating presence of the gene of interest, which was absent in the non-template control that appeared flat as shown in (figure 4.13).

Different *Salmonella* species were observed to have similar curve characteristics when using a single primer set. However, each species was uniquely identified based on curve shape and height when using the three primer sets, (figure 4.7 4.8, 4.9, 4.10, and 4.11) this agrees with a previous study (Yang *et al.,* 2009; Masek *et al.,* 2013). Specifically these same three primers V1, V3, V9 were used for S*almonell*a identification of nine *Salmonella* serovars (Masek *et al*., 2013). Notably the V1 region of *S.* Typhi, *S.* Typhimurium, and *S.* Gallinarum generated similar melt curves, which were in agreement to the findings in a similar study (Masek *et al.*, 2013) where VI region of *S.* Typhi, *S.* Typhimurium and *S.* Newport had similar curves. The similarity in curve shape and height denoted similar gene sequence, GC content and length (Reed *et al.,* 2007).

S. Typhi, *S.* Typhimurium, *S.* Enteritidis, *S.* Braenderup, *S.* Gallinarum, *S.* Dublin all previously confirmed by serotyping at KEMRI were used as positive controls. The main circulating serovars identified in this study were *S.* Enteritidis, *S.* Choleraesuis, *S.* Braenderup and *S.* Typhimurium. These controls were matched against thirty-one field samples for curve similarity. Six of the pig's samples were identified as *S.*Choleraesuis, while one of the pig samples was identified as *S.* Typhimurium which agrees with a study

that also isolated *S.* Choleraesuis from Kenyan pigs (Onyango *et al.,* 2014). Three samples from cattle were identified as *S.* Braenderup, these finding are in agreement with a study that isolated *S.* Braenderup cows (Kariuki *et al.,* 2002). *S.* Enteritidis was also isolated in chicken samples this finding agrees with a previous study in Kenya that identified *S.* Enteritidis in chicken at 52.2% (Kariuki *et al.,* 2002).

HRM assay was able to detect the different *Salmonella* serovars found in domestic animals however, culture and API 20 E strips could only identify the organism as *Salmonella,* without identifying the serovar. Conventional *Salmonella* serotyping based on the White-Kauffmann-Le Minor scheme could have been used to identify the serovars, however the method is time consuming and laborious for a large sample set (Breneer *et al.,* 2000). Thus, HRM is a better diagnostic assay for rapid detection of *Salmonella*.

HRM assay limit of detection was determined across the different *Salmonella* harbored in the different animal species. All the serially diluted samples had a CT value of between 12.66 and 31.63 with an exception of a sample diluted a thousand fold (10^3) *S.* Gallinarum from a chicken sample that had a CT value of 32.90 and thus was defined as negative. Thus the limit of detection of this assay was determined 0.031ng/µl (table 4.1).

The HRM assay was also specific in the identification of different *Salmonella* serovars, as exhibited by the unique melt profiles generated for each different *Salmonella* serovar while using the three primers, however use of a single primer would not differentiate the different *Salmonella spp*. (Yang *et al.,* 2009). A potential limitation of the HRMA assay is in it's inability to differentiate closely related organisms that have identical sequences within the amplified region. Therefore analysis of the melt curves generated using this

method was based on three instead of one of the 16S, hyper-variable regions (Yang *et al.*, 2009).

The HRMA assay failed to generate a unique profile for an artificial sample that had a mixture of the different *Salmonella* serovars. This was also observed in other studies, where the HRM assay failed to resolve specimens with poly microbial infections (Masek *et al*., 2013). This limitation of the assay's inability to detect a mixed infection could be attributed to the primers, thus better biomarkers could be developed. A potential drawback with targeting 16S rRNA gene in the detection of closely related species such as *Salmonella* with similar sequences within the amplified DNA target region is that they may not be easily differentiated.

5.2. CONCLUSION

The presence of circulating *Salmonella spp.* in animals and egg poses a major public health concerns and calls for measures to control the spread of *Salmonella* in animals. In this study culture, API 20E strips and HRM assays were used for *Salmonella* detection. Culture, the gold standard detection method used routinely in hospitals for *Salmonella* detection has to be used hand in hand with API 20E strips which increases it's specificity. Though culture and API 20E strips were sensitive, HRM emerged as a rapid, specific and sensitive for the detection of *Salmonella spp.* serovars detection. Based on the HRMA *S.* Enteritidis, *S.* Braenderup, *S.* Typhimurium and *S.* Choleraesuis were identified as the main *Salmonella* serovars circulating in domestic animal and eggs in Kenya.

5.3. RECOMMENDATIONS

HRM is therefore a more rapid, simple, closed tube assay that can be applied in clinical settings to provide physicians with early detection of pathogens causing salmonellosis. A major disadvantage of HRMA is that closely related species with identical sequences within the 16s rRNA hyper-variable region V1, V3 and V6 may not be readily differentiated. In addition the assay failed to differentiate the different *Salmonella* serovars in a poly-microbial infection. Thus further research should be done in developing primers capable of differentiating the different *Salmonella* serovars.

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