Studying *Salmonella* and *E. coli* O157 along beef supply chain in Bishoftu, Ethiopia: Linkage with diarrheal illness in people?

Fanta Desissa Gutema

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Promoters
Prof. Dr. Lieven De Zutter
Department of Veterinary Public Health and Food Safety
Faculty of Veterinary Medicine
Ghent University
Prof. Dr. Sarah Gabriel
Department of Veterinary Public Health and Food Safety
Faculty of Veterinary Medicine
Ghent University
Prof. Dr. Luc Duchateau
Department of Nutrition, Genetics and Ethology
Faculty of Veterinary Medicine
Ghent University
Dr. Reta Duguma Abdi
Department of Veterinary Clinical studies
Faculty of Veterinary Medicine and Agriculture
Addis Ababa University
Department of Biomedical Sciences
College of Veterinary Medicine
Long Island University, New York

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DEDICATION

Desissa Gutema, Sinke Daba, Bachu Daba and Zerihun Bekele

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LIST OF ABBREVATIONS

AIEC	Ad-herent Invasive *E. coli*
aEPEC	Atypical Enteropathogenic *E. coli*
AW	Water activity
BPW	Buffered Peptone Water
CDC	Center of Disease Control of the United States
CFU	Colony-Forming Unit
CHERG	Child Health Epidemiology Reference Group
CI	Confidence Interval
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CSA	Central Statistical agency
DAEC	Diffusely adhering *E.coli*
DALYs	Disability-Adjusted Life-Years
DNA	Deoxyribonucleic Acid
EAEC	Enteroaggregative *E.coli*
ECDC	European Centre for Disease Prevention and Control
ECOFFs	Epidemiological Cut-Off Values
EDHS	Ethiopian Demographic survey
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E.coli*
EPEC	Enteropathogenic *E.coli*
ERIC	Enterobacterial Repetitive Intergenic Consensus
ETEC	Enterotoxigenic *E.coli*
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
FBD	Foodborne Disease
FTDs	Food Transmitted Diseases
HUS	Hemolytic Uremic Syndrome
ID	Infectious dose
ISO	International Organization for Standardization
LEE	Locus of Enterocyte Effacement
MIC	Minimal Inhibitory Concentration
MLST	Multi Locus Sequence Typing
MSRV	Modified Semi-Solid Rappaport-Vassiliadis medium

NTS	Non-typhoidal *Salmonella*
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analysis
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
STEC	Shiga toxin-producing *E. coli*
stx	Shiga toxin
tEPEC	Typical Enteropathogenic *E. coli*
VTEC	Verocytotoxic *E. coli*
WGS	Whole genomic sequence
WHO	World Health Organization
XLD	Xylose lysine deoxycholate agar

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Chapter 1

1. Literature Review

1.1. General Introduction

Foodborne diseases (FBD) are an important public health problem worldwide. For the year 2010, it was estimated that FBD resulted in 600 million cases and 420,000 deaths resulting in nearly 33 million disability adjusted life years globally. The highest mortality burden was in Africa followed by south eastern Asia. Diarrheal diseases are responsible for more than half of the global burden of FBD resulting in 550 million cases and 230,000 deaths every year (WHO, 2015).

Different pathogens such as viruses, bacteria and parasites are associated with FBD in humans (Schmidt, *et. al.*, 2009). Non-typhoidal *Salmonella* and Shiga toxin-producing *E. coli* (STEC) are among the most common bacterial causes of diarrheal illnesses (Walker et al., 2010; WHO, 2015). These pathogens are present in domestic and wild animals and belong to the zoonotic pathogens. Different domesticated animals such as pigs, poultry and to a lesser extent ruminants (cattle, sheep and goats) are reservoirs for *Salmonella*. Ruminants and especially cattle are the main reservoirs for STEC (Gyles, 2007; Ferrari et al., 2019; Hanson et al., 2020; Hoelzer et al., 2011). Primarily, the pathogens propagate among animals and subsequently infect people. Most cases in humans are associated with consumption of raw or undercooked contaminated food products from animal origin such as pork, poultry (including eggs), beef and milk (Bhandare et al., 2007; Cantas and Suer, 2014; EFSA and ECDC, 2021; De Freitas et al., 2010). Outbreak data clearly demonstrated that also contaminated vegetables, fruit and water may cause diseases in humans (CDC,2020). Contact with infected animals (including pet animals) and their fecal materials and contact between humans is also important(Gyles, 2007; Hanson et al., 2020).

Salmonella are among the 31 pathogens displaying the highest capability of triggering intestinal or systemic diseases in humans among diarrheal and/or invasive agents and the third leading cause of death among food-transmitted diseases (FTDs) (WHO, 2015). In 2019, *Salmonella* was one of major causes of bacterial FBD with the vast majority (72.4%) of the salmonellosis foodborne outbreaks caused by *S.* Enteritidis in the European Union (EU) (EFSA and ECDC, 2021) and a major cause of FBD in the United States (USA) (Tauxe et al., 2010).

According to a recent review, *S.* Typhimurium and *S.* Anatum were the most widely distributed and reported serotypes from beef (Ferrari et al., 2019). Beef contaminated with *Salmonella* has been linked with several foodborne outbreaks. For instance, in the USA, among the 1965 *Salmonella* outbreaks where a food vehicle was implicated during the period 1973–2011, 96 were attributed to beef accounting for 3684 illnesses (Laufer et al., 2015).

STEC causes 2,801,000 acute illnesses, 3,890 cases of hemolytic uremic syndrome (HUS), 270 cases of permanent end stage renal diseases (ESRD) and 230 deaths in humans annually across the world (Majowicz et al., 2014). For 2018, there were 8,314 STEC cases, 411 HUS cases and 11 deaths in the EU (EFSA and ECDC, 2019). Different STEC serotypes such as O157, O26, O45, O91, O111, O103, O104, O121, O145 and O146 serogroups are responsible for infections in humans (Gould et al., 2013; Havelaar et al., 2016; Valilis et al., 2018; EFSA BIOHAZ Panel, 2020). However, *E. coli* O157 is the widely known STEC serotype and the most commonly reported cause of human illness (Lim et al., 2010). The incidences of infections caused by strains belonging to non-O157 are also increasing and become of public health concerns (EFSA BIOHAZ Panel, 2020). Cattle are the main reservoir and asymptomatic carriers (Gyles, 2007; Tein and Katz, 2017). Beside beef, which is the common food source, other contaminated food products also attributed to STEC infection globally (Doyle et al., 2013; Lim et al., 2010; Pires et al., 2019).

In Ethiopia, there is no foodborne diseases surveillance program and in consequence, no citable information is available for the incidence of foodborne illnesses. Diarrhea is among the five top leading causes of mortality. In 2015, the death rate in the country due to diarrhea was estimated at 88.6 per 100,000 people in Ethiopia (Misganaw et al., 2017). Different pathogens with predominance of parasites are isolated from diarrheic patients in Ethiopia (Ayenew et al., 2019; Mekonnen et al., 2019). Common foodborne pathogens such as bacteria (e.g. *Salmonella* spp., pathogenic *Escherichia coli* and *Campylobacter* spp.) from animals, foods and humans were reported (Abayneh et al., 2014; Abebe etal., 2020; Eguale et al., 2015; Zelalem et al., 2019;; Tadesse, 2014; Tadesse and Tessema, 2014). In humans other causes of gastro-enteritis such as

viruses (e.g. Norovirus, Sapovirus and Hepatitis A virus) (Sisay et a al., 2016; Belay et al., 2019) and enteric parasites (e.g. *Entamoeba histolytica*, *Hookworm* spp. and *Giardia*) (Tigabu et al., 2019; Ayenew et al., 2019) were reported from diarrheic patients. However, information on the relative importance of etiologic agents responsible for FBD is not available in Ethiopia (Eguale et al., 2015).

Salmonella and STEC have been reported from cattle at slaughterhouses and beef in retail shops in Ethiopia (Abayneh et al., 2014). In Ethiopia, there are over 300 local slaughterhouses with limited capacity and infrastructure supplying meat for local consumption. The basic hygienic standards in the slaughterhouses are generally low. In consequence, the risk of contamination of beef carcasses with pathogens during slaughter is likely (Eshetie et al., 2018). Hygienic practices at slaughterhouses, during distribution and at retail shops are key points in ensuring the quality and safety of meat to safeguard the public health (Lues and Van Tonder, 2007; Rani et al., 2017). The widespread practice of raw or under cooked beef consumption is considered to be a major risk of foodborne infections in Ethiopia (Abayneh et al., 2014; Avery, 2014; Seleshe et al., 2014). However, information regarding the association of cattle and the linkage between the consumption of contaminated beef with *Salmonella* and STEC and diarrheal diseases is lacking. This thesis aimed to gain insights in the potential association between *Salmonella* and *E. coli* O157 in cattle and /or beef and diarrheal diseases in humans in Bishoftu town, Ethiopia. To that end, the occurrence and molecular characteristics of the pathogens were studied. Moreover, the antimicrobial resistance of the pathogens and hygienic practices at slaughterhouses and retail shops were assessed.

1.2. *Salmonella*

1.2.1. Taxonomy and characteristics

The genus *Salmonella* was first discovered by Karl Joseph Eberth and Rudolf Virchow from abdominal lymph nodes and the spleen of typhoid patients in 1879 and confirmed by Robert Koch. It was named *Salmonella* in 1884 when Salmon and Smith isolated the bacillus from hogs succumbed to the disease known as hog cholera (Le Minor, 1991). *Salmonella* are non-spore forming, motile (except *S*. Gallinarum and *S*. Pullorum) due to the presence of peritrichous flagella, facultative anaerobic and Gram-negative rods of 0.7 to 1.5 µm wide and 2.0 to 5.0 µm long belonging to the family *Enterobacteriaceae* (Giannella, 1996). The genus *Salmonella* consists of two species: *Salmonella enterica* and *Salmonella bongori*. Clinically relevant *Salmonella* belongs to *S. enterica* which consists of 6 subspecies: *S. enterica* subspecies *enterica*, *S. enterica* subspecies *salamae*, *S. enterica* subspecies *arizonae*, *S. enterica* subspecies *diarizonae*, *S. enterica* subspecies *houtenae* and *S. enterica* subspecies *indica* (Tindall et al., 2005). According to the Kauffman-White scheme, *Salmonella* are classified into more than 2579 serotypes based on somatic lipopolysaccharide (O), flagellar (H) and capsular (Vi) antigens (Grimont and Weill, 2007).

According to Heyndrickx et al (2005) *Salmonella* serotypes with a name can be reported on different ways: species name followed by subspecies name and serotype name like *S. enterica* subsp. *enterica* serovar Typhimurium or genus name and serotype name, whereby in both cases the serotype name must be written with an initial capitalized letter and not in italic like *S*. Typhimurium. *Salmonella enterica* can be divided into typhoidal *Salmonella* with only humans as host, and non-typhoidal *Salmonella* (NTS) with a broad host range (Tindall et al., 2005). Some *Salmonella* serotypes have different abilities to infect and to cause disease, and are sometimes referred to as host generalists (causing infections in many hosts e.g. *S*. Typhimurium and *S*. Enteritidis, host-adapted (primarily associated with one host e.g. *S*. Dublin in cattle), and host-restricted (associated with one host only e.g. *S*. Typhi in man).

All non-typhoidal *Salmonella enterica*, with the exception of *S.* Pullorum and *S.* Gallinarium, have a zoonotic potential (Sanderson and Nair, 2012). However, only ~50 serotypes are regularly isolated from humans (Harvey et al., 2017). For example, in the USA, the ten serovars most frequently identified in human disease (colored) are listed in Figure 1.1. Those serotypes are frequently present in food products of animal origin.

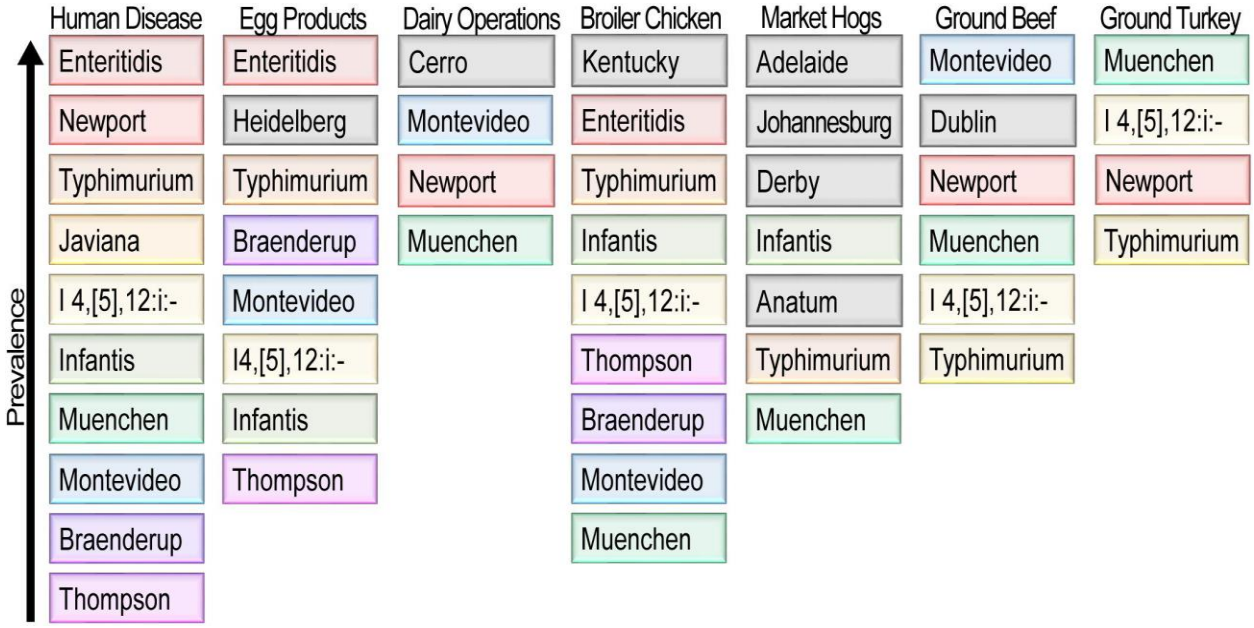

Figure1.1. Ranking of *Salmonella* serovar prevalence from reported human clinical cases of salmonellosis and surveillance of different animal food sources in the USA. (Source: Cheng et al., 2019).

Salmonella grow at a temperature range of 5 to 47°C with an optimal between 35°C and 37°C, pH range of 4 to 9 with the optimum being between 6.5 and 7.5. *Salmonella* require a water activity (A_w) of at least 0.94 for growth, but yet can survive at A_w<0.2 such as in dried foods (Pui et al., 2011; Doyle et al., 2013). The focus of this review and the research work is *S. enterica* subspecies *enterica* and more specifically the non-typhoidal *Salmonella*, causing foodborne infections and will be referred to as *Salmonella* across the document.

1.2.2. *Salmonella* in humans

Salmonella infections in humans are a major public health concern worldwide (Lee et al., 2015; Majowicz et al., 2010). *Salmonella* is an infectious and contagious agent. The risk of illness increases with ingested dose and outbreaks are generally associated with exposure to high infectious doses. Dose-responses for different common *Salmonella* serotypes linked with different food matrix causing infection in humans based on human challenge experiments are not available (Bollaerts et al., 2008). Yet, this information is important to quantify the risk of infection and illness in humans. The available information on dose-response relationship based on data from all forms of outbreaks indicated an ID_{50} of 7 CFU's for infection and an ID_{50} of 36 CFUs for illness (Teunis et al., 2010). All people can get a *Salmonella* infection, but some groups such as adults aged 65 and older, children younger than 5 years and immuno-compromised are more likely to develop disease (CDC, 2020). A susceptible population has a higher probability of illness at low dose levels when the combination of pathogen-food matrix is extremely virulent and at high dose levels when the combination is less virulent (Bollaerts et al., 2008).

The three most prevalent *Salmonella* serovars associated with foodborne illness were *S.* Enteritidis, *S.* Typhimurium and *S.* Newport in the USA (Luvsansharav et al., 2020) and *S.* Enteritidis, *S.* Typhimurium and monophasic *S.* Typhimurium in the EU (EFSA and ECDC, 2018). Information on the dominant serotypes is not readily available for Africa which could be due to lack of an integrated surveillance and reporting system. According to the meta-analysis study by Tadesse (2014), *S.* Concord (34%), *S.* Typhi (32.5%), *S.* Typhimurium (9.4%) and *S.* Paratyphi (6.1%) are the dominant serotypes reported from humans in Ethiopia.

Salmonella infections have been associated with symptoms ranging from mild self-limiting gastroenteritis characterized by diarrhea, abdominal pain and vomiting in people of all ages to severe invasive diseases with complicated extra-intestinal illness, bacteremia and meningitis in children, elderly and immunocompromized patients (Majowicz et al., 2010). Salmonellosis outcomes differ substantially by serotype, based on virulence factors of the serotypes and the

immune status of the individual. For instance, *Salmonella* serotype Dublin and *Salmonella* serotype Choleraesuis cause severe illness but relatively few people become infected, while other serotypes such as *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport are responsible for a large proportion of the total salmonellosis cases (Jones et al., 2008), but cause less severe illness. *Salmonella* Typhimurium and *S.* Enteritidis together account for approximately 50% of all isolates globally reported from human clinical cases (Freitas et al., 2010).

Approximately 5% of individuals with gastrointestinal illness caused by *Salmonella* develop a bacteremia (Acheson and Hohmann, 2001). Bacteremia due to *Salmonella* also varies with serotypes involved. For instance, *S.* Dublin is more associated with bacteremia than the most common serotypes implicated in foodborne human salmonellosis (Wesley et al., 2018). A systematic study by Stanaway et al (2019) estimated that 535,000 cases of *Salmonella* invasive disease occurred in 2017 with the highest incidence in sub-Saharan Africa (34.5 cases per 100,000 persons/year).

Globally, it was estimated that 93.8 million cases of gastroenteritis occur each year due to *Salmonella* spp. resulting in 155,000 deaths of which 80.3 million cases were foodborne (Majowicz et al., 2010). In 2017, *Salmonella* enterocolitis was estimated to cause 95.1 million cases, 50,771 deaths and 3.10 million disability life adjusted years globally (Stanaway et al., 2019). *Salmonella* is the most common cause of foodborne outbreaks with *S.* Enteritidis causing one out of seven outbreaks in the EU (EFSA and ECDC, 2018) and responsible for approximately 30% in the USA (Dewey-Mattia et al., 2018). In total, 91,662 human salmonellosis cases were reported resulting in notification rate of 19.7 cases per 100,000 populations in 2017 in EU countries (EFSA and ECDC, 2018). According to Center for Disease Control, *Salmonella* cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the USA every year (CDC, 2019a).

Mortality rate of *Salmonella* infection in resource-constrained countries, with frequent invasive infections, is estimated to range from 18 to 24% (Chimalizeni et al., 2010). *Salmonella* was estimated to cause 2,458,000 gastroenteritis cases and 4,100 deaths in Sub-Saharan African

countries and 29,839,000 cases and 49,200 death in South and Southeast Asia per year (Majowicz et al., 2010).

Human salmonellosis is also recognized as an important socioeconomic disease posing considerable economic burden (Ao et al., 2015). According to the basic cost-of-illness model that accounts for the costs of diagnosis, medical care, and treatment as well as losses in productivity due to time away from work and illness-related mortality, a typical case of *Salmonella* is estimated to cost approximately $4,312 implying high economic loss in outbreaks involving large populations in the USA (Scharff, 2012). The overall annual economic burden of human salmonellosis could be as high as €3 billion in the EU (EFSA, 2014) and $3.3 billion in the USA (Hoffmann et al., 2012). Data related to the cost of FBD including salmonellosis are generally not available for developing countries (Shekhar, 2018).

1.2.3. *Salmonella* in cattle

Bovine salmonellosis is an important disease for cattle. In cattle, salmonellosis may manifest clinically as a syndrome of septicemia, acute or chronic enteritis and abortion. Diarrhea with or without fever is the most common clinical sign in adults and pneumonia is a common manifestation of *Salmonella* infection in calves (Holschbach and Peek, 2018; La Ragione et al., 2012). Severity of infection caused by *Salmonella* show variations based on serovars involved and their virulence factors (Cakin et al., 2020). Clinical signs are more frequent among calves than adults (El-Seedy et al., 2016). Calves are frequently colonized by *Salmonella* and are most likely to experience salmonellosis within 2-4 weeks of age (House et al., 2001).

Only a few serovars are of clinical importance. Bovine salmonellosis is caused predominantly by *S.* Typhimurium and *S.* Dublin (Costa et al., 2012). Other serovars are sporadically associated with bovine infections (Mastroeni and Maskell, 2006). *Salmonella* Dublin is often associated with systemic infections which can result in meningoencephalitis, polyarthritis or pneumonia, occasionally in the absence of diarrhea and may result in asymptomatic shedding or in abortion in pregnant cows.

In young calves, *S.* Dublin causes disease that is clinically indistinguishable from *S.* Typhimurium and is characterized primarily by diarrhea (Costa et al., 2012). *S.* Dublin has the ability to establish lifelong infections in cattle, characterized by an asymptomatic carrier status with intermittent periods of bacteremia and shedding especially among dairy cattle (Holschbach and Peek, 2018). Those animals may shed up to 10^6 organisms per gram of feces (Kemal, 2014). Conversely, S. Typhimurium is often associated with acute enteritis and exudative diarrhea, usually affecting calves (Costa et al., 2012). Systemic infections and abortion are not a common clinical manifestation of *S.* Typhimurium (Carrique-Mas et al., 2010).

Salmonellosis also causes a significant economic loss in farm animals because of the cost of clinical disease, which includes cost of death, diagnosis and treatment, missed benefits (e.g. discarded milk or reduced milk yield due to disease) and cost of control and prevention (Nielsen et al., 2013; Pal et al., 2020). A study by Nielsen et al (2013) in Sweden, estimated the gross margin losses caused by *S.* Dublin at 57-315 euros per cow group in the first year after infection, and 9-196 euros per group each of the following ten years based on the hygienic status of the herd.

Most *Salmonella* infected animals show no signs of clinical disease, but may shed bacteria into the environment (Belluco et al., 2015). As a consequence, *Salmonella* is often isolated from healthy cattle (Bosilevac et al., 2009). *Salmonella* reside in the gastrointestinal tract of warm-blooded animals including cattle as transient members of the intestinal microbial population (Callaway et al., 2008; Ferrari et al., 2019). Prevalence of *Salmonella* in beef and dairy cattle has been reported from different countries with variable reports (Hanson et al., 2020).

According to a recent systematic review and meta-analysis, the global pooled prevalence estimate of *Salmonella* in apparently healthy cattle was estimated at 9% ranging from 2% in Europe to 11% in North America (Gutema et al., 2019). According to this study, the most frequently reported *Salmonella* serotypes include *S.* Montevideo, *S.* Typhimurium, *S.* Kentucky, *S.* Meleagridis, *S.* Anatum, *S.* Cerro, *S.* Mbandaka, *S.* Muenster, *S.* Newport, and *S.* Senftenberg in decreasing order of frequency. Another recent systematic and meta-analysis using data from

African countries indicated a higher (15.4%) prevalence estimate of *Salmonella* in cattle feces (Thomas et al., 2020). Cattle may also carry *Salmonella* on their hide. The prevalence on hides ranged from 17.7% at slaughterhouses in England (Reid et al., 2002) and up to 94% in the USA (Brichta-Harhay et al., 2008).

1.2.4. Role of cattle in human salmonellosis

Various domestic and wild animals are reservoirs for *Salmonella*. The most common domesticated animal hosts are chickens, pigs, and cattle (Ferrari et al., 2019; Hanson et al., 2020). Cattle play a significant role in the epidemiology of zoonotic salmonellosis (Ferrari et al., 2019; Hoelzer et al., 2011; Hanson et al., 2020). The presence of *Salmonella* in cattle feces and on hides, contact with infected cattle and cross contamination of carcasses during hide removal and evisceration are the most common sources of *Salmonella* infection in humans (Cummings et al., 2010). *Salmonella* in meat and meat products are the highest risk agent/food pairs in causing outbreaks in humans (EFSA and ECDC, 2018). Beef and beef products are assumed to account for approximately 10% of foodborne *Salmonella* cases (Hanson et al., 2020). For example, the majority of outbreaks due to *Salmonella* are linked mainly to ground beef and continuously result in product recall in the USA (CDC, 2019b).

1.3. *Escherichia coli* O157

1.3.1. Taxonomy and characteristics

E. coli are gram-negative, rod shaped and facultative anaerobic bacteria belonging to the genus *Escherichia* within the family Enterobacteriaceae (Ewing, 1986). *E. coli* belongs to the intestinal microflora of animals and humans. *E. coli* strains can be distinguished by the combination of their O-antigen, H-flagellar antigen, and K-capsular antigen. Serotyping based on O- and H-typing has been considered the standard method (DebRoy et al., 2018; Kaufmann, 1974). At present, 187 O-groups and 53 H-types of *E. coli* species have been identified (DebRoy et al., 2016) and more than 700 serotypes of *E. coli* have been identified (Doyle et al., 2013) based on the Kaufmann classification (Kaufmann, 1974).

Many *E. coli* strains are harmless and even beneficial to the host. However, there are some strains of *E. coli* which are pathogenic and cause diseases in animals and humans. In humans, *E. coli* may cause a range of infections such as urinary tract infections, mastitis, peritonitis, meningitis, pneumonia, sepsis and diarrheal disease (EFSA BIOHAZ Panel, 2020; Nataro and Kaper, 1998). The diarrheagenic *E. coli* are categorized into seven main pathotypes based on the pathogenic mechanisms causing intestinal infections in humans: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), Shiga toxin producing *E. coli* (STEC) and adherent invasive *E. coli* (AIEC) (Croxen et al., 2013). Currently, there is an emergence of cross-pathotypes within *E. coli* strains making distinction between the pathotypes difficult. These are defined as strains harboring pathogenicity genes associated with more than one pathovar, e.g. EAEC strains carrying *stx* genes. Regardless of the original pathotype, once an *E. coli* strain carries the *stx* gene(s) it may be considered as STEC (EFSA BIOHAZ Panel, 2020).

Among the *E. coli* pathotypes, STEC are zoonotic and can be transferred to humans by contaminated food and water (Croxen et al., 2013). STEC infections are characterized by the production of Shiga toxins (Stx), so called because of their similarity with the toxin produced by

Shigella dysenteriae serotype 1. STEC also have been referred to as VTEC, or verocytotoxic *E. coli*, due to the preliminary observation that the toxin(s) kill Vero cells *in vitro* (Mainil and Daube, 2005). STEC strains produce two types of Shiga toxins which are antigenically distinct prototypes: Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). There are variations within each toxin. Shiga toxin 1 consists of 4 subtypes (Stx1a, Stx1c, Stx1d and Stx1e) while stx2 contains 12 subtypes (St*x*2a–Stx2l) (EFSA BIOHAZ Panel, 2020). STEC may also possess other virulence factors like intimin, encoded by *eaeA* gene (Law, 2000).

Intimin and Shiga toxins play an important role in the pathogenesis of STEC infection. The intimin facilitates the intimate attachment of the bacteria to the intestinal epithelium during colonization (Ramachandran et al., 2003). Following the attachment, STEC produce Shiga toxins that enter into the circulatory system. After translocation to the blood circulation, Shiga toxins bind to cells like endothelial cells, possessing globotriaosyl-ceramide (Gb3) in the cell membrane (Lingwood, 1993). Then, the Shiga toxins enter the cells by endocytosis and finally inhibit protein synthesis by cleaving ribosomes resulting in death of the target host cells (Hauser et al., 2020). In general, it is assumed that the presence of *stx*2 and *eae* genes makes STEC more virulent (Lupindu, 2018). Strains encoding subtype *Stx*2a are more likely to cause systemic sequelae (Fitzgerald et al., 2019). Current literature that details the pathogenicity of STEC and the public health risk posed by contamination of food with STEC are available (Castro et al., 2017; EFSA BIOHAZ Panel, 2020).

STEC can grow at optimum conditions of temperature of 37 °C (7 °C to 50 °C), pH of 6-7 (4 to 9) and A_W of 0.995 (0.95 to 0.995). For *E. coli* O157 strains the growth is limited to maximum 44.5°C.The ability of STEC to resist acidic pH (close to 2.5) assists the survival in foods with adverse pH and overcomes the acidity barrier of the stomach allowing the entrance and colonization of the intestinal tract (Castro et al., 2017; Doyle et al., 2013; FSS,2019).

E. coli O157 is the most widely known and well-studied STEC serotype globally (Lim et al., 2010, Scallan et al., 2011). *E. coli* O157 possesses metabolic characteristics distinct from other *E. coli*. Most *E. coli* O157 strains are unable to ferment sorbitol within 24h, cannot hydrolyze 4-

methylumbrelliferyl-D-glucuronide (as lacking β-glucuronidase enzyme) and do not grow well at temperatures > 44.5 °C (Doyle et al., 2013). These characteristics are very important in discriminating *E. coli* O157 from other *E. coli*, including non O157 STEC strains. Currently, the incidence of infections caused by strains belonging to non-O157 such as O26, O45, O91, O111, O103,O104, O121, O145 and O146 serogroups are also increasing (Gould et al., 2013; Havelaar et al., 2016; Valilis et al., 2018; EFSA BIOHAZ Panel, 2020). The focus of this review and the research work is *E. coli* O157.

1.3.2. *E. coli* O157 in humans

Humans get infected with STEC through consumption of contaminated foods and water. Exposure by direct contact with animals or with their feces is also an important route of exposure (Croxen et al., 2013; Heredia and García, 2018). The infectious dose of STEC is considered low. It is estimated that less than 100 cells are required to develop disease (Caprioli et al., 2005). The symptoms usually appear after an incubation period of 3 to 4 days where the patient initially develops diarrhea, accompanied by abdominal pain, which, in most cases, aggravates to bloody diarrhea (Gyles, 2007). In some cases, STEC infections are associated with hemorrhagic colitis, and life-threatening conditions such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Croxen et al., 2013).

A study on the global burden of STEC indicated that STEC causes 2,801,000 acute illnesses with an incidence rate of 43.1 cases per 100,000 person/year, 3,890 cases of HUS and 230 deaths annually of which 10% can be contributed by *E. coli* O157. The incidence rate of STEC infections was estimated to range from 0.6 STEC illnesses per 100,000 person-years in the African sub-regions, to 136 per 100,000 person-years in the Eastern Mediterranean sub-regions (Majowicz et al., 2014).

A recent systematic review of published articles between 1980 and 2015 on reported *E. coli* infections linked with consumption of contaminated meat and meat products, showed a global occurrence of 33 outbreaks with 1966 cases of which 1543 cases were laboratory confirmed, 476

hospitalizations, 233 cases of HUS and 32 deaths (Omer et al., 2018). Most (87.8%) of the outbreaks were caused by serotype O157: H7 and most were reported from the USA and the EU (Omer et al., 2018). In general, infections due to *E. coli* O157 are a major concern worldwide (Lim et al., 2010).

1.3.3. *E. coli* O157 in cattle

STEC live in the gut of different ruminants such as cattle, goats, sheep, deer, and elk. STEC also isolated from other animals, which include wild boars, rabbits, birds, dogs, rodents, and insects (Croxen et al., 2013). Cattle are the primary reservoir for STEC (Gyles, 2007). Colonization by *E. coli* O157 is considered asymptomatic in cattle (except occasional diarrhea in newborn calves) (Pruimboom-Brees et al., 2000; Stein and Katz, 2017; Tourret et al., 2016). This is due to lack of Gb3 and *stx* receptivity in the bovine gastrointestinal tract (Pruimboom-Brees et al., 2000). In cattle, the gastrointestinal colonization of *E. coli* O157 induces an innate immune response and production of specific mucosal antibodies enabling the animals to remain free of infection for most of their lives (Nart et al., 2008).

The prevalence of *E. coli O157* in cattle is estimated at 5.7% ranging from 0.1% to 61.8% based on studies from different countries (Islam et al., 2014). According to Islam et al (2014), the prevalence of *E. coli* O157 varies substantially, with high prevalence observed in Africa (31.2%), feedlot cattle (19.6%), cattle sampled at slaughterhouses (7.1%) and in samples directly taken from the intestines (12.3%). Previous studies also showed that the *E. coli* O157 occurrence depends on the age of the animal (dominant in young cattle), changes of feed, transportation stress and hot environmental conditions (Dargatz et al., 1997; Meyer-Broseta et al., 2001).

1.3.4. Role of cattle in human *E. coli* O157 infections

Being the major asymptomatic carriers of *E. coli* O157 in the recto-anal junction, cattle play an important role in the epidemiology of diarrheal illness in humans serving as an important source of infection (Cobbold et al., 2007; Griffin and Tauxe, 1991). Cattle shed *E. coli* O157 via feces which can contaminate the hide (Narvaez-Bravo et al., 2013). For example, in a study in USA,

94% of cattle hides were reported contaminated with *E. coli* O157 (Brichta-Harhay et al., 2008). Hides can be an important source of carcass contamination during de-hiding.

Variable occurrences of *E. coli* O157 in beef and beef products have been reported from different countries: 2.2% in Nigeria (Tafida et al., 2014), 0.3% in the EU (EFSA and ECDC, 2013), 6% in Ethiopia (Zelalem et al., 2019), 0.8% in the USA (Hill et al., 2011) and 1.7% in Australia (Kiermeier et al., 2011). Beef contaminated with *E. coli* O157 can potentially lead to diarrheal illness in humans (Heredia and García, 2018), particularly in countries where consumption of raw or undercooked beef is common. Beef and beef products have been associated with several *E. coli* O157 outbreaks (Currie et al., 2019; Furukawa et al., 2018; Pires et al., 2019; Torso et al., 2015). In the EU/EEA, based on outbreaks reported to EFSA from 2012 to 2017, consumption of bovine meat and products thereof was identified as a major source of STEC attributing to 24% of STEC outbreaks (EFSA BIOHAZ Panel, 2020).

1.4. Detection and characterization of *Salmonella* and *E. coli O157*

1.4.1. Detection of *Salmonella*

Since *Salmonella* is generally present in low numbers and accompanied by background flora in samples, detection of the pathogen needs different isolation steps: 1) pre-enrichment: culturing of the sample in a non-selective broth to resuscitate sub-lethally injured *Salmonella* cells, 2) selective enrichment: growth of the target organism and growth suppression of the background flora, 3) selective plating: growth of target organism on selective agar medium in order to obtain pure colonies, and 4) confirmation of suspect colonies.

Over decades a large range of media and different methods were developed for the detection of the pathogen. The ISO 6579:2017 - Horizontal method for detection, enumeration and serotyping of *Salmonella* - Part 1: Detection of *Salmonella*, figures as the golden standard method for the detection of *Salmonella* in the following types of samples: products intended for human consumption and animal feeding stuffs, environmental samples in the area of food production and food handling, samples from the primary production stage such as animal faeces, dust, and

swabs (ISO, 2017). Briefly, it involves the following steps: i) pre-enrichment: adding each test portion to a quantity of the non-selective broth Buffered Peptone Water (BPW) to yield a tenfold dilution and incubation at 37°C ± 1°C for 18h ± 2h; ii) selective enrichment: 1/ for samples from the primary production stage: inoculation of the pre-enriched culture on Modified semi-solid Rappaport-Vassiliadis (MSRV) agar and incubation at 41.5 °C ± 1 °C for 24 h - 48 h ± 3 h and 2/ for all other sample types: inoculation on MSRV or Rappaport-Vassiliadis broth with soya (RVS), the latter incubated at 41.5 °C ± 1 °C for 24 h ± 3 h and on Muller Kauffmann tetrathionate novobiocin (MKTTn) broth and incubated at 37 °C ± 1 °C for 24 h ± 3 h; iii) selective plating: plating out suspect cultures on MSRV agar plates and all other enrichment broths on the selective medium Xylose Lysine Deoxycholate (XLD) agar and incubation of XLD agar plates at 37 °C ± 1 °C for 24 h ± 3 h and a second selective plate of choice; iv) confirmation: at least 1 typical or suspect colony obtained from selective media is picked for biochemical tests.

This microbiological method is generally considered to be the "gold standard method" and serves as the basis for analysis in many food safety and public health laboratories due to the ease of use, reliability of results, high sensitivity and specificity (e.g. MSRV based culture method has 95.5% sensitivity and 96.8% specificity) (Worcman-Barninka et al., 2001), and lower cost compared to some of immunological and molecular-based technologies (Lee et al., 2015). However, these procedures need multiple culturing stages, taking at least 5 days for complete isolation and confirmation.

Several rapid detection methods that include immunological, nucleic acid, miniaturized biochemical and biosensors based assays have been developed. Details of each method are reviewed and described by Lee et al. (2015) and Paniel and Noguer (2019). These methods allow a rapid screening of samples. Yet, positive test results have to be confirmed by traditional culture methods.

1.4.2. Detection of *E. coli* O157

As previously described, typical *E. coli* O157 strains have unique characteristics like the inability to ferment sorbitol and the lack of β-D-glucuronidase activity. These allowed the development of selective media for the detection of the pathogen. For example, by the replacement of the lactose (1%) in MacConkey agar by sorbitol (1%) called Sorbitol-MacConkey agar (SMAC), *E. coli* O157 grow as colorless colonies and can be distinguished from the other *E. coli* strains fermenting sorbitol and forming pink colonies. Several methods for E. coli O157 detection are available. In the EU, the European Food Safety Authority recommends the use of ISO methods for detection of E. coli O157: ISO-16654:2001-microbiology of food and animal feeding stuffs - horizontal method for the detection of E. coli O157 (ISO, 2001) and ISO 13136:2012 - microbiology of food and animal feed – real time polymerase chain reaction– based method for detection and serotyping of O157 (ISO, 2012). The PCR detection method uses real-time PCR as the reference technology for detection of the virulence and serogroup-associated genes of O157. This method is also used for the detection of other STEC strains and the determination of O111, O26, O103 and O145 serogroups. The methods are applicable to detect *E. coli* O157 from 1) products intended for human consumption and the feeding of animals; 2) environmental samples in the area of food production and food handling; 3) environmental samples in the area of primary production. Details on the different detection methods and molecular characterization of E. coli O157 are reviewed by (Castro et al., 2017).

Currently, the standard microbiological method is the ISO horizontal method for the detection of E. coli O157-ISO 16654: 2001 (ISO, 2001). According to this method, the detection of E. coli O157 necessitates four successive steps: i) enrichment: a test portion is enriched in nine times the sample weight of pre-warmed modified tryptone soya broth plus novobiocin at 41.5°C ± 1°C for 6 h and subsequently for a further 12 to 18 h; ii) immunomagnetic separation: *E. coli* O157 are separated and concentrated using immunomagnetic beads coated with antibodies to *E. coli* O157 after 6 h and again, if necessary, after a further 12 to 18 h incubation; iii) selective plating: immunomagnetic particles with adhering bacteria are transferred onto cefixime tellurite sorbitol MacConkey agar (CT-SMAC) and a second selective isolation agar. CT-SMAC is incubated at

37°C for 18 to 24 h and the second agar of choice should be incubated following the manufacturer's recommended procedures and iv) confirmation: sorbitol negative colonies from CT-SMAC and typical *E. coli* O157 colonies on the second isolation agar are streaked onto nutrient agar and incubated at 37°C for 18 to 24 h. Suspect isolates are confirmed by biochemical tests and serological identification is carried out with O157 antiserum.

1.4.3. Characterization of *Salmonella* and *E. coli* O157

Beyond detection of *Salmonella* and *E. coli* O157 strains, there are several methods for the characterization and subtyping of the identified isolates. Litratures on the underlying principles, comparative advantages and disadvantages of each subtyping methods of *Salmonella* (Tang et al., 2019) and *E. coli* O157 (Castro et al., 2017; Fratamico et al., 2016) are available.

Methods used for characterization and subtyping of *Salmonella* isolates include conventional serotyping and molecular subtyping methods. The conventional serotyping method determines *Salmonella* serotypes based on detection of the presence of somatic lipopolysaccharide (O), flagellar (H) and capsular (Vi) antigens. This can be tested by a slide agglutination test with appropriate antisera from pure colonies first for O-antigens and then H-antigen after eliminating auto agglutination. The O-antigens are examined by mixing non-agglutinating pure colony with a drop of anti-O serum first with polyvalent antisera and then with monovalent antiserum to obtain a homogenous and turbid suspension on a glass slide. If agglutination occurs within 1 minute, the reaction is considered as positive for O-antigen. The H-antigens are also determined by using poly- and monovalent anti H-serum with a similar procedure. Test of H antigens involves 2 steps (test for phase 1 and phase 2 antigens). In some cases, capsular antigen is also tested (e.g. *S.* Typhimurium).The serotype is assigned by referring to a reference catalog, such as the Kauffman-White scheme (Grimont and Weill, 2007; ISO, 2017; Tang et al., 2019). Serotyping is widely used for isolate preliminary identification, but it poorly discriminates strains. It also requires the availability of several specific antisera and well-trained personnel to correctly interpret the results (Wattiau et al., 2011). Alternative methods to the classical serotyping method include (i) serotyping based on O (rfb gene cluster) and H (fljB and fliC) antigens loci using

PCR-based methods (Herrera-Leon et al., 2007),(ii) microarray-based methods (McQuiston et al., 2011) (iii) serotyping based on surrogate genomic markers such as virulence genes (Peterson et al., 2010). Various other rapid molecular-based both band and sequence based subtyping methods are available for serotyping of *Salmonella* that include pulsed-field gel electrophoresis (PFGE), repetitive element PCR, multiple locus variable number of tandem repeats analysis (MLVA), multilocus sequence typing (MLST), whole-genome sequencing (WGS) and clustered regularly interspaced short palindromic repeat (CRISPR) as reviewed by Tang et al.,2019. A comparison of ability to provide sensitive subtype discrimination, ability of serovar prediction and turnaround time of these methods is indicated in Table 1.1. WGS has best discrimination among molecular subtyping of *Salmonella*. The serotype prediction based on SeqSero (Zhang et al., 2015) and *Salmonella* in silico Typing Resource (SISTR) (Yoshida et al., 2016) platforms has been reported to be approx. 92 and 95%, respectively suggesting that WGS-based methods may be more reliable than traditional serotyping to assign *Salmonella* isolates to serovars.

Table 1.1. Comparison of *Salmonella* characterization and genotyping methods.

Method	**Ability to identify or predict serovars**	**Ability to provide sensitive subtype discrimination**	**Time to results from single colony**
White–Kauffman serotyping	While *Salmonella* serovars are based on White–Kauffmann serotyping, serotyping does provide frequent misclassification	Very poor subtype discrimination; only valuable as subtyping method for rare and unusual serovars	2–17 days
Pulsed-field gel Electrophoresis (PFGE)	Intermediate ability to predict serovars	Good subtyping discrimination for most serovars. Some PFGE patterns are very common within some serovars (e.g., pattern 4 for *S.* Enteritidis)	4–6 days
Multiple locus variable number of tandem repeats analysis (MLVA)	Intermediate ability topredict serovars	Good subtyping discrimination for most serovars. May perform better than PFGE for some serovars but worse for others	1–2 days
Multilocus sequence typing (MLST)	Intermediate ability to predict serovars	Better than conventional serotyping and riboprinting, worse than PFGE and WG	1–2 days
Whole-genome sequencing (WGS)	Currently available serovar-prediction Software using WGS data work well for less common serovars. May not work for extremely rare serovars	Best discrimination among molecular subtyping approaches	3–17 days

Source: (Tang et al., 2019

The characterization methods for *E. coli* O157 include multiplex PCR to define several virulence and serogroup-associated genes such as *stx*1, *stx*2 and *eae* (ISO, 2012), subtyping of Shiga-toxins using PCR method (Scheutz et al., 2012), MLST, PFGE, WGS, CRISPR (Fratamico et al.,2016).

PFGE is up to now the most commonly used genotyping method for *Salmonella* and *E. coli* O157. A worldwide protocol for PFGE is available making comparisons between laboratories and countries possible. The PFGE approach uses restriction enzymes that recognize specific restriction sites along the genomic DNA and fragment the DNA to sizes normally ranging from 20 to 800 kb. These large fragments are separated in an agarose gel by constantly changing the direction of the electric current (pulsed field), which allows to separate the DNA fragments by size, generating a specific "fingerprint pattern" for a given isolate. The restriction enzymes XbaI, NotI, SpeI, and SfiI have been typically used for Gram-negative bacteria including *Salmonella* and *E. coli* O157. The primary restriction enzyme used for *Salmonella* and *E. coli* O157 PFGE is XbaI (CDC, 2017).

Currently, WGS and CRISPR are recognized as the best subtyping techniques for *Salmonella* and *E. coli* O157. Whole genome sequencing generates a unique "fingerprint" of the bacterium by capturing the DNA sequence across the entire genome of single microbial isolates and is used in outbreak investigations to determine the source of the pathogen. The WGS method has the potential to become the new "gold-standard" for pathogen subtyping (Banerji et al., 2020; Tang et al., 2019). WGS supports epidemiological investigations with a high-level of precision. However, it is not effective for food safety management if used only in a single sector. The application of this technology in national food safety management needs multidisciplinary collaborative works among all pertinent sectors such as on standardizing the guidelines and data sharing system to be effectively used by all developed and developing countries within the framework of One Health approach. In countries with limited capacity and resources, a feasibility assessment conducted jointly with potential national partners in the regulatory framework will be a vital starting point for competent food safety authorities (FAO, 2016). CRISPR are short, highly conserved DNA repeats separated by unique sequences of similar length, and they have been used for subtyping, identification, and detection of bacteria including

Salmonella and *E. coli* O157 (Shariat and Dudley, 2014). Based on spacer content or sequencing of CRISPR loci, CRISPR-based typing analyses can be used to differentiate strains for epidemiological investigations or for pathogen detection (Fratamico et al., 2016; Shariat and Dudley, 2014; Tang et al., 2019).

1.5. Antimicrobial resistance

Antimicrobial resistance is a serious public health problem ultimately resulting in therapeutic failure which in turn can lead to death. According to the CDC report (2019a), more than 2.8 million antibiotic-resistant infections occur and more than 35,000 people die each year in the USA. Antimicrobial resistance is due to mutations following the selective pressure of bacterial populations exposed to antimicrobials and by the introduction of antibiotic resistance genes such as plasmids (Vidovic and Vidovic, 2020). The use of antibiotics in animal production is incriminated as a major risk factor for antimicrobial resistance leading to transmission of antimicrobial resistant bacterial strains from livestock to humans (Hammerum and Heuer, 2009).

Gastroenteritis caused by *Salmonella* usually resolves without treatment. However, it can be systemic in severe cases and require antimicrobial treatment (Antonelli et al., 2019). Antibiotics such as ciprofloxacin, azithromycin, and ceftriaxone are sometimes needed to treat patients with severe *Salmonella* infections (CDC, 2019a). Several factors have been implicated in the acquisition and dissemination of resistant *Salmonella* strains and often have been associated with farm environment, food industry, household settings, vectors and reservoirs such as flies and migratory birds, clinical settings and consumers (Figure 1.2). The prevalence of single and multi-drug resistant *Salmonella* isolates to clinically important antimicrobial agents have been reported (Gebreyes et al., 2004; Hur et al., 2012; Nair et al., 2018). Moreover, there is an enormous challenge with using antibiotics as *Salmonella* is one of the ‘superbugs’ which are resistant to several classes of antibiotics (Ashbolt et al., 2013). According to the EFSA/ECDC report, in 2017–2018, *Salmonella* isolates from humans, animals and food in EU countries showed high levels of resistance to commonly used antimicrobials such as ampicillin, sulfonamides,

tetracycline, and (fluoro)quinolones. Carbapenemase-producing *Salmonella* also reported. Resistance to colistin is generally low (EFSA and ECDC, 2020).

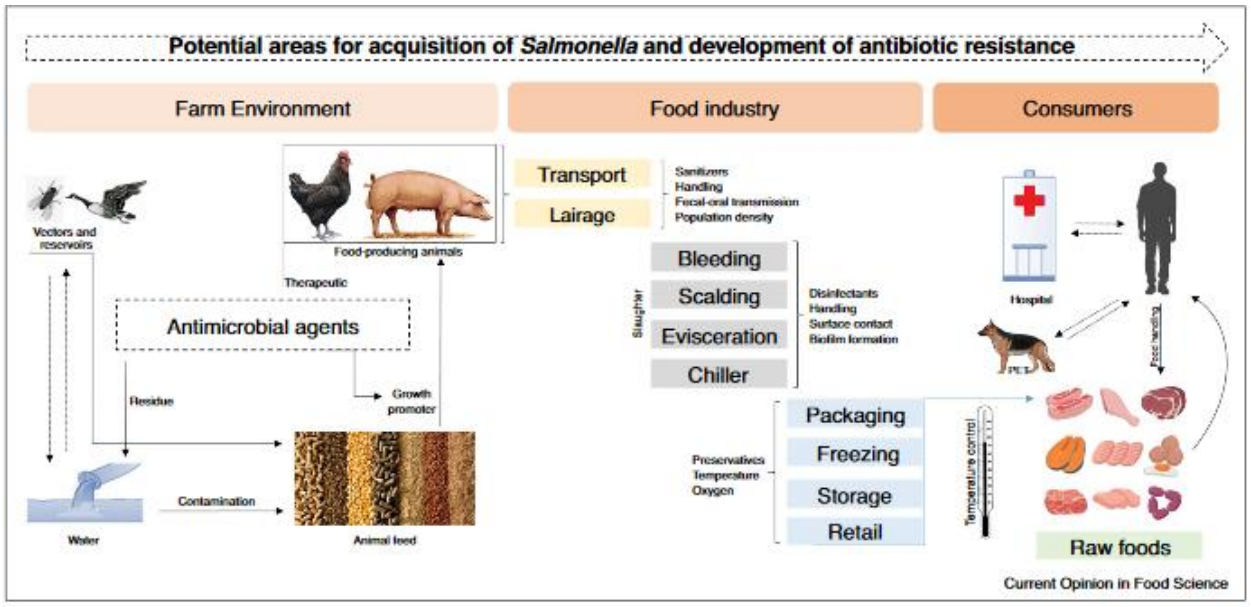

Figure 1.2. Potential areas for acquisition of Salmonella and development of resistance to antimicrobials (Source: Monte et al., 2019).

The use of antibiotics for treatment of STEC infection has long been controversial due to reports indicating that antibiotics may increase the production of Shiga toxin. The recommended therapy is mainly supportive such as rehydration therapy and dialysis in some cases (Mühlen and Dersch, 2020; Wong et al., 2000). Being one of the organisms in the gastrointestinal tract of food animals, STEC may be subject to antibiotic resistance selection and can readily acquire antimicrobial resistant genes (Losada et al., 2016).

Despite the less frequent use of antimicrobials for treatment of diarrheal illness in humans, there has been an increasing report on the development of antimicrobial resistance to *E. coli* O 157 (Mir and Kudva, 2019). Occurrence of resistant *E. coli* O157 strains isolated from animals, foods, and humans has been reported (Abdissa et al., 2017; Mir and Kudva, 2019; Mukherjee et

al., 2017). A recent review by Mir and Kudva (2019) indicated a variable resistance of *E. coli* O157 against antimicrobials such as sulfonamides, streptomycin, tetracycline and ampicillin.

Modeling based on available data on antimicrobial resistance levels developed by *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus*, predicted that by 2050 an estimated 10 million deaths per year will be attributable to antimicrobial resistance with a cumulative economic cost of US$ 100 trillion globally (O'Neill, 2014; O'Neill, 2016). The model assumed that if resistance is left unchecked or no interventions are put in place, the impact will get bigger through time. The model underestimates the real impact of antimicrobial resistance as it based on only on subset of drug-resistant bacteria and public health issues (HIV, tuberculosis and malaria) for which resistance is a concern. Moreover, the prediction is no longer a reality given the current ongoing efforts toward controlling antimicrobial resistance. Antimicrobial resistance has been listed on the priority agenda of several countries and international organizations such as WHO, FAO, OIE and World Bank in order to combat the continuously emerging and further spreading of antimicrobial resistance (Founou et al., 2020).

1.6. Status of *Salmonella* and *E. coli* O157 in Ethiopia

Within Ethiopia, information on the relative importance of major etiologic agents responsible for diarrhea is not readily available (Eguale et al., 2015). *Salmonella* is among the pathogenic bacteria isolated from diarrheal patients, mainly from children less than 5 years of age and also from adults. A systematic review and meta-analysis estimated the pooled prevalence estimates of *Salmonella* in stool samples of diarrheic children, diarrheic adults and carriers to be 8.72%, 5.68%, and 1.08%, respectively, with invasive infections of 5.71% in children and 0.76% in adults. According to this study, *S*. Concord (34%) and *S*. Typhimurium (9%) accounted for 57.9% of the total *Salmonella* isolated (n=329) from human patients (Tadesse, 2014).

Similarly, the pooled prevalence of *Salmonella* is estimated at 7.1% in cattle (Tadesse and Tessema, 2014) and 10% in beef (Zelalem et al., 2019). Some available studies reporting the occurrence/prevalence of *Salmonella* in diarrheic patients, beef and cattle in Ethiopia are summarized in Table 1.2, 1.3 and 1.4, respectively.

Table 1.2. Prevalence and serotype distributions of *Salmonella* in diarrheic patients in Ethiopia.

Study site	Year	Age group	N	P (%)	Serotype (n)	Authors
Ambo	2014	Children	239	1.3	Chicago, Caracas, Saintpaul	(Tosisa et al., 2020)
Robe and Goba	2016	Children	422	6.9	NA	(Assefa and Girma, 2019)
Arba Minch	2017	Children	167	12.6	NA	(Ameya et al., 2018)
Hossana	2017	Children	204	0.9	NA	(Abebe et al., 2018)
Nekemte	2015-2016	Children and adult	422	7.1	NA	(Terfassa and Jida, 2018)
Addis Ababa	2013-2014	Children and adult	957	6.2	Typhimurium (22), Virchow (20), Kottbus (6), Miami (2), Kentucky (2), Newport (2), Enteritidis (1), Braenderup (1), Saintpaul (1), Concord (1), S.V: ROUGH-O;−:- (n=1)	(Eguale et al., 2015)
Jimma	2012	Children	206	6.2	NA	(Beyene and Tasew, 2014)
Hawasa	2011	Children	158	2.5	NA	(Mulatu et al., 2014)

NA: not available; N: number examined; P: prevalence; n: number of serotypes

Table 1.3. Prevalence and serotype distributions of *Salmonella* beef contamination in Ethiopia.

Study site	Year	N	P (%)	Serotype (n)	Authors
Bishoftu	1999-2000	646	2.9	Mishmarhaemek (10), Typhimurium (2), Enteritidis (2), Guildford (3), Dublin (2)	(Alemayehu et al., 2003)
Addis Ababa	2002-2003	160	14.4	Infantis (10), Dublin (1), Anatum (3), Saintpaul (1), Vejle (1), *Salmonella* I:8,20:-(2), Bovismorbificans (2), Braenderup (3),	(Ejeta et al., 2004)
Addis Ababa	2003-2004	142	8.5	Newport (3), Dublin (2), Anatum (2), Typhimurium (1), Infantis (1), Kentucky (1), Saintpaul (1), *Salmonella* 1:9,12:- (1)	(Zewdu and Cornelius, 2009)
Bishoftu	2006-2007	100	2	Eastbourne, Urbana	(Sibhat et al., 2011)
Jimma	2009	120	0.83	NA	(Tassew et al., 2010)
Hawassa	2015-2016	100	4	Muenchen (4)	(Kore et al., 2017)
Wolita sodo	2015-2016	448	12.5	NA	(Wabeto et al., 2017)
Haramya and Dire dawa	2014-2015	290	2.75	NA	(Mengistu et al., 2017)
Bahirdar	2012-2013	300	7.6	NA	(Muluneh and Kibret, 2015)
Gondar	2013	90	35.6	NA	(Garedew et al., 2015)
Bahir Dar	2015	30	21	NA	(Azage and Kibret, 2017)

NA: not available; N: number examined; n: number of serotypes; P: prevalence

Table 1.4. Prevalence and diversity of *Salmonella* serotypes in cattle reported in Ethiopia.

Study sites	Year	N	P (%)	Serotype (n)	Author(s)
Bishoftu	1999/2000	323	0.62	Mishmarhaemek (2)	(Alemayehu et al., 2003)
Bishoftu	2005/6	100	6	Anatum (2), Newport (2), Typhimurium (1), II 40:b:- (1)	(Sibhat et al., 2011)
Addis Ababa	2010	195	7.7	NA	(Addis et al., 2011)
Bahirdar	2006/7	186	5.9	Newport (3), Infantis (2), Typhimurium (1), Haifa (1), Heidelberg (1), Mishmarhaeme (1) Untypable (2)	(Alemu and Zewde, 2012)
Gondar	2013	152	14	Bredeney (11), Uganda (3)	(Hailu et al., 2015)
Central Ethiopia	2013	1203	2.5	Typhimurium var Copenhagen (7), Saintpaul (6), Kentucky (5), Virchow (5), Dublin (3), Livingstone var.14+ (1), I: 6, 7, 14:-: I, w (1), Mikawasima (1) and Aberdeen (1)	(Eguale et al., 2016)
Addis Ababa	2011/12	34	23.5	Saintpaul (2), Larochelle (2), Dublin (1), Kastrup (1), unidentified (2)	(Hiko et al., 2016)
Hawasa	2015/16	150	2.7	1:4,5,12:i:(1), Korovi (3)	(Kore et al., 2017)
Modjo	2016	91	7.7	NA	(Abunna et al., 2017)
Addis Ababa	2014/15	567	4.1	Dublin(10), Virchow (5), Braendrerup (2), Saintpaul (2), Haifa (2), Kottbus (1), Kentucky (1), Mikawasima (1), Typhimurium phage type 3 (1), Typhimurium phage type 193 (1), Typhimurium phage type 4 (1), I:ROUGH-O:g,p:- (1)	(Ketema et al., 2018)*
Jimma	2016	195	5.6	NA	(Takele et al., 2018)

* Serotypes were identified from fecal and carcass swab samples with no clear distinction in the type of serotypes between the two samples*;* NA: not available; N: number of cattle examined; n: number of serotypes; P: prevalence.

The status of STEC is not well studied in Ethiopia. A prevalence of *E. coli* O157 in cattle was reported at 1.9% in Addis Ababa and Debre Berhan (Abdissa et al., 2017), 4.7% in Hawassa (Atnafie et al., 2017) and 7.3% in Jimma (Haile et al., 2017). The pooled prevalence of *E. coli* O157 in beef was estimated at 6% (Zelalem et al., 2019). In humans, only few studies reported a prevalence of 4.5% in Addis Ababa (Ayenew et al., 2019) and and 0% in all age groups of diarrheic patients in Gondar (Huruy et al., 2011). However, these studies did not use latex agglutination test for confirmation.

1.7. Beef production and beef consumption practices in Ethiopia

1.7.1. Cattle and beef production

Ethiopia is largely a rural country with an agrarian economy. Livestock are of economic and social importance both at the household and national level (Shapiro et al., 2017). The contribution of the livestock sector to the Ethiopian economy accounted for 45% of the agricultural gross domestic product, 18.7 % of the national gross domestic product and 16-19 % to the total foreign exchange earnings of the country (Eshetie et al., 2018). The livestock production systems in Ethiopia are categorized as pastoral, agro-pastoral, mixed crop-livestock farming, urban and peri-urban farming and specialized intensive farming systems (Halala, 2015).

Among livestock, cattle are a very common asset in Ethiopian households and 70% of the Ethiopian total population depends on cattle for their livelihoods (FAO, 2018). According to the Central Statistics Agency, the total cattle population of Ethiopia is about 60.39 million; smallholders account for 98% of cattle production and supply in Ethiopia (CSA, 2018).

The Ethiopian cattle populations comprise four groups: humpless shorthorn and longhorn (Bos taurus), humped Zebu (*Bos indicus*), Sanga (interbreed of Zebu and humpless cattle) and the Zenga (interbreed of Sanga and Zebu type) (Mekonnen et al., 2020). There are 28 genetically diverse cattle breeds of which 98.2% are indigenous (Zebu) cattle. These cattle breeds are found

across the country, in the rift valley highlands as well as below sea level in the Afar depression (Assefa and Hailu, 2018; Hagos, 2017).

Cattle are kept for various socioeconomic reasons such as draught power (male cattle), source of milk and meat production, source of income, asset saving etc. (Assefa and Hailu, 2018). The emphasis on the use of cattle varies with the type of production system. For instance, in both crop–livestock and agro-pastoral systems, animal traction ranked first, followed by milk and meat production. In contrast, in pastoralist systems, reproduction/breeding requirements received higher ranks and for female animals breeding outranked the importance of milk production (Ayalew and Rowlands, 2004).

Fattening or conditioning of animals for slaughter usually takes place at well-organized commercial feedlots or simply in the backyard of smallholder farmers. Fattening of cattle concentrates mainly on male animals and a few females which are either infertile or have finished their reproductive cycle (Halala, 2015). Young or old oxen are fattened depending on the supply source. For instance, farmers close to pastoral areas tend to purchase younger stock for feeding but in the heartland of the highlands older oxen are fattened at the end of their productive life. Feedlot operators, on the other hand, generally fatten young and intact males (FAO, 2018).

The estimated average live and carcass weight of cattle is 250 kg and 110 kg with 44 % dressing percentage, respectively (AGPLMD, 2013). Ethiopia produces about 1 million tons of beef valued at $ 5.1 billion per year (FAO, 2018). Based on the intensiveness of the production system, number of holdings of cattle, geographical location and the availability of feed resources, there are four types of beef cattle production system in Ethiopia: the commercial feedlot system, peri-urban small-scale fattening, backyard fattening in the mixed crop-livestock system, and the pastoral/agro-pastoral livestock production system (FAO, 2018) (Table 1.5).

Table 1.5. Beef cattle production systems in Ethiopia.

Production system	Description
Mixed crop-livestock	Subsistence oriented farming concentrated in the mid-and high-altitude agro-ecological zones where cereals and cash crops are the dominant farm activities. Cattle are primarily kept to supply draft power. Old oxen that retire from ploughing are commonly sold or conditioned and finished.
Pastoral/Agro-pastoral	Rangeland based beef cattle production system aimed at exploitation of the natural or semi natural vegetation. The main function of livestock is subsistence. Excess young males are sold to highlanders, where they are used as draught oxen, or to feedlot operators.
Urban/Peri-urban	Smallholder farmers and landless households around urban areas fatten a few animals at a time. Fattening is mostly done after the oxen in the mixed crop livestock system have retired from farm work/ploughing in order to replace them with younger animals.
Commercial feedlots	There are more than 300 feedlots operating in Ethiopia, predominantly in East Shoa (Oromia). Animals are entirely confined in a yard with watering and feeding facilities for a finishing duration of 3-6 months.

Adapted from FAO, 2018.

The supply and marketing of livestock involves several actors. The marketing of live animals in Ethiopia is largely a personalized business with irregular buyers and sellers and with several brokers (Brasesco et al., 2019). Within Ethiopia, more or less actors in the livestock production such as cattle marketing, include input of suppliers, producers, collectors, processors, traders and consumers (Figure 1.3). Butchers buy fattened cattle usually from open/secondary markets and bring them to slaughterhouse for slaughter. The slaughterhouse offers the service of examining the health of the animals, slaughtering and distribution of carcass and offals to the butcheries (AACCSA, 2015; Brasesco et al., 2019).

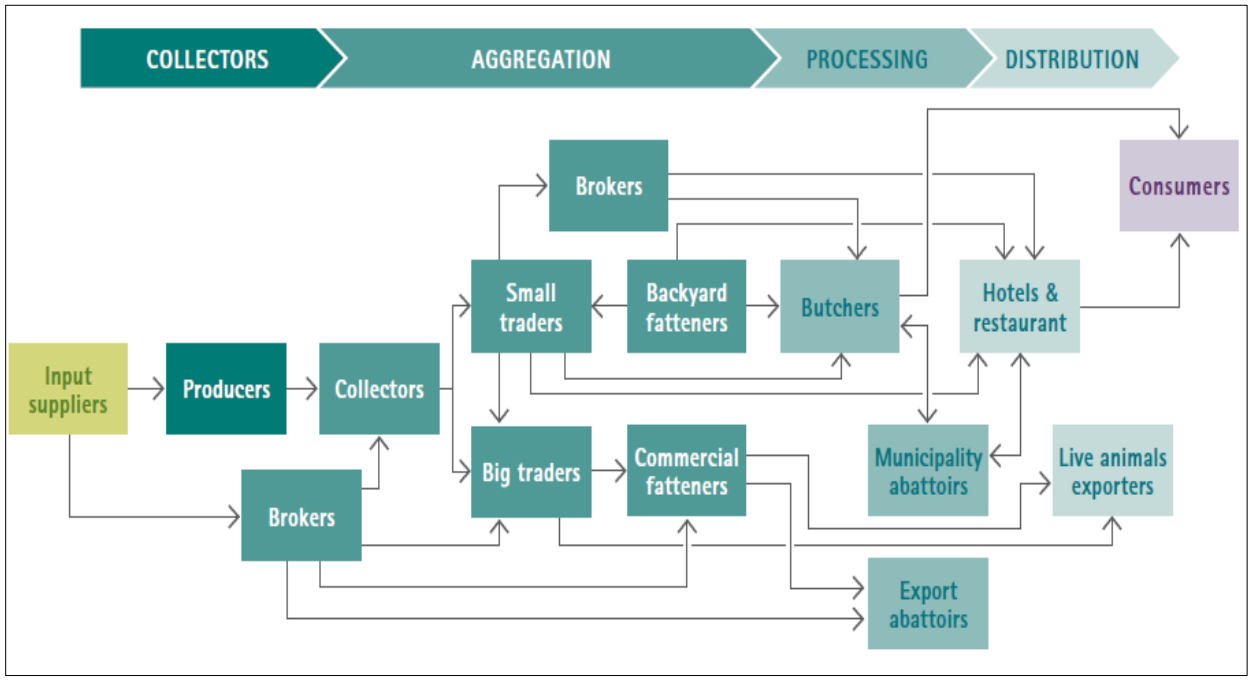

Figure 1.3. Livestock market flow in Ethiopia. Source: (Brasesco et al., 2019).

In Ethiopia, there are over 300 local slaughterhouses with limtted capacities and facilities supplying meat for local consumption (Eshetie et al., 2018). According to the existing proclamations of the country, production and marketing of sound, wholesome and quality meat and meat products for consumer's protection are required. Regulating food hygiene and safety is a shared responsibility of Ministry of Health, Ministry of Agriculture and Quality and the standard Authority of Ethiopia (FAO/WHO, 2005).

The Ministry of Agriculture is empowered by Proclamation No.274 of 1970 to carry out meat inspection in export and local abattoirs. This proclamation also gives power to the ministry to issue regulations and establish criteria useful to determine livestock products as fit for human consumption (EFNG, 1970). Public health proclamation (No. 200/2000)(EFNG, 2000) and Food, Medicine and Health Care Administration and Control Proclamation No. 661/2009 (EFNG, 2010) of the Ministry of Health enable controlling of the safety and quality of food. The Codex standards such as the general principles of food hygiene and code of hygienic practice for meat are the basic reference materials for standard settings and serve as enforcing tools for food safety

where there are no developed Ethiopian standards (FAO/WHO, 2005). However, the basic hygienic standards of most of the slaughterhouses are generally low (Eshetie et al., 2018).

The bulk of meat for domestic consumption comes from backyard slaughtering of animals and some from municipal slaughterhouses that supply to the consumers through butcheries and hotels/restaurants (Brasesco et al., 2019). Despite the immense availability of livestock, sometimes meat is imported to meet the quality requirements of consumers like in the capital city, Addis Ababa. For instance, in 2011, the value of imports for all types of meat was estimated at USD 201, 000 (Brasesco et al., 2019).

1.7.2. Beef consumption

Meat consumption is often an indicator of the economic status of an individual. Ethiopians with higher economic status will demand a greater amount of meat products. In Ethiopia, beef is the most widely consumed meat type, followed by mutton, goats, camel and poultry meat (Halala, 2015). According to OECD (2021), the estimated per capita consumption for the year 2020 was 2.5 kg beef, 0.5 kg mutton and 0.1 kg poultry. The consumption of beef is very low compared to other developing and developed countries (Birhanu, 2019). For instance, the 2.5 kg/capita beef and veal consumption is very low as compared to the consumption of 26.2 kg/capita in the USA in 2020 (OECD, 2021). For the year 2020, the total beef and veal consumption was estimated at 400.4 tons (OECD, 2021). Long fasting periods of the Ethiopian Orthodox Church (over 200 days per year) assumed to contribute to the low consumption of meat. In addition, Ethiopia's domestic red meat consumption is reported to be low due to high prices, which are unaffordable for low-income households (approximately 30 percent of the population still lives below USD 2 per day). Relatively, the consumption of red meat is higher in Addis Ababa than in the rest of the country (Brasesco et al., 2019). Consumption of meat in the form of raw or undercooked is a very common traditional practice in Ethiopia (Avery, 2014; Seleshe et al., 2014) which can expose consumers to foodborne pathogens that in turn likely leads to foodborne illness (Heredia and García, 2018).

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Chapter 2

2. Aims

FBD are an important public health concern globally. A number of zoonotic pathogens such as *Salmonella* and *E. coli* O157 are among the most common bacterial causes of FBD. Consumption of meat and meat products is a major source of FBD and meat and meat products are implicated in several foodborne outbreaks. Cattle are reservoirs of *Salmonella* and *E. coli* O157 and play an important role in the epidemiology of human infection. People acquire foodborne infections through consumption of raw or undercooked contaminated beef products and by contact with infected animals and their fecal materials. In Ethiopia, beef is the common meat type consumed and eaten frequently raw or under-cooked in the form of steak ("diimina") or beef tartare ("kitfoo") made from raw minced beef. In consequence, consumption of raw beef can be source for *Salmonella* and *E. coli* O157 infections in the country. However, data on the potential association of cattle with the occurrence of human diarrheal illness due to *Salmonella* and *E. coli* O157 through consumption of beef is lacking in Ethiopia.

Information on occurrence of theses pathogens and sources for beef contamination along the beef supply chain and their potential linkage with diarrheal illness in humans is necessary to develop efficient preventive measures to ensure beef safety.

Therefore, the following specific objectives were formulated:

- To estimate the prevalence of *Salmonella* in healthy cattle based on the available literature in the period 2000-2017 (Chapter 3).
- To investigate the prevalence and genetic relatedness of *Salmonella* in cattle at slaughterhouses, in beef at retail shops and in diarrheic patients (Chapter 4).
- To investigate the occurrence and genetic relatedness of *E. coli* O157 in cattle at slaughterhouses, in beef at retail shops and in diarrheic patients (Chapter 5).
- To assess the hygienic handling practices in slaughterhouses and retail shops (Chapter 6).
- To assess sources of *Salmonella* and *E. coli* O157 carcass contamination during slaughter process (Chapter 7).

Chapter 3

3. Prevalence and Serotype Diversity of Salmonella in Apparently Healthy Cattle: Systematic Review and Meta-analysis of Published Studies, 2000-2017

Based on: Gutema, F.D., Agga, G.E., Abdi, R.D., De Zutter, L., Duchateau, L. and Gabriël, S. (2019). Prevalence and Serotype Diversity of *Salmonella* in Apparently Healthy Cattle: Systematic Review and Meta-Analysis of Published Studies, 2000–2017. Frontiers in Veterinary Scince, 2019, 6:102. https://doi.org/10.3389/fvets.2019.00102.

3.1. Abstract

Salmonellosis is a leading cause of foodborne illnesses in humans with cattle being one of the reservoirs for *Salmonella*. We estimated a pooled prevalence of *Salmonella* in apparently healthy cattle and examined serotype diversity through systematic review and meta-analysis of studies published between 2000 and 2017. Peer reviewed publications reporting the prevalence of *Salmonella* in cattle were searched through five electronic databases (PubMed, Google scholar, Agricola, Scopus, CAB direct) and through manual search. We obtained 71 publications with 75 datasets consisting of a total of 52,766 animals examined and 5,010 *Salmonella* positive cattle from 29 countries in six continents (except from Antarctica). Pooled prevalence of *Salmonella* in cattle was 9% (95% confidence interval: 7-11%). Significantly high heterogeneity (I^2=98.7%, $P < 0.01$) was observed among all studies as well as within continents. Prevalence varied from 2% (Europe) to 16% (North America). Overall, 143 different serotypes were reported with the most diverse serotypes being reported from Africa (76 different serotypes) followed by North America (49 serotypes). The ten most frequently reported serotypes (Montevideo, Typhimurium, Kentucky, Meleagridis, Anatum, Cerro, Mbandaka, Muenster, Newport and Senftenberg) accounted for 65% of the isolates for which specific serotype information was reported. *Salmonella* Montevideo and *S*. Dublin are the most frequently reported serotypes in North America and Europe, respectively, while *S*. Typhimurium was the most frequent in Africa, Asia and Australasia. Our results indicate variability both in the prevalence and serotype diversity of *Salmonella* in cattle across continents. Although all *Salmonella* serotypes are potentially pathogenic to humans, five (Montevideo, Typhimurium, Anatum, Mbandaka, and Newport) of the top 10 serotypes identified in this study are among the serotypes most commonly associated with clinical illnesses in humans.

3.2. Introduction

Foodborne illnesses pose public health and economic burdens both in developed and developing countries (Glavin, 2003; Kirk et al., 2015). Annually, foodborne illnesses are responsible for an estimated 600 million cases, 420,000 deaths, and 33 million disability adjusted life years lost worldwide (WHO, 2015). *Salmonella* is a major cause of foodborne illnesses in humans (D'Aoust, 1999; Rosel and Delia, 2014; Schlundt et al., 2004). *Salmonella* are Gram-negative, non-spore forming, mostly motile, facultative anaerobic bacilli within the family *Enterobacteriaceae*. The species *S. enterica* consists of six subspecies and more than 2,579 serovars (Tindall et al., 2005; Andino and Hanning, 2015). Based on the clinical profiles of infections caused in humans *Salmonella enterica* can be divided into typhoidal - which are human specific -and non-typhoidal *Salmonella* (NTS) - having a broad host range (Tindall et al., 2005). The NTS serotypes are leading causes of bacterial diarrhea and invasive bacterial infections in young children, the elderly and the immune-compromised individuals throughout the world. *Salmonella* Typhimurium and *S.* Enteritidis together account for approximately 50% of all isolates globally reported from human clinical cases (Feasey et al., 2012; Freitas Neto et al., 2010; Tennant et al., 2016). The global incidence of diarrheal disease due to the NTS accounts for about 94 million enteric infections each year, of which 80.3 million cases are considered foodborne and resulting in 155,000 human deaths annually (Majowicz et al., 2010). Human salmonellosis is also recognized as an important socioeconomic disease posing considerable economic burden in the world (Tauxe et al., 2010; Ao et al., 2015).

Salmonella colonizes mainly the intestinal tracts of humans and animals including cattle. Foods of animal origin are important sources of *Salmonella* infections in humans (Buncic and Sofos, 2012; EFSA, 2009; Ejeta et al., 2004; Pires et al., 2009; Scallan et al., 2011; Tauxe et al., 2010). Humans acquire the infection mainly through consumption of contaminated food products including beef and beef products, by direct contact with infected animals or their environment, and by direct human-to-human transmission (Hoelzer et al., 2011; Laufer et al., 2015; Pui et al., 2011).

The transfer of NTS to food processing plants and equipment used for food preparation also plays an important role ultimately leading to the risk of salmonellosis after the consumption of contaminated foods (Pui et al., 2011). Carcass contamination with *Salmonella* during slaughter particularly under unsatisfactory hygienic operations poses a significant public health risk (Abdunaser et al., 2009; Agga et al., 2016; Chaney et al., 2017; Mannion et al., 2012). Knowledge about the overall occurrence of *Salmonella* and the diversity of serotypes in cattle provides important information for decision making and to promote reliable efforts towards prevention and control of foodborne salmonellosis associated with cattle. Therefore, the objectives of this study were to estimate the prevalence of *Salmonella* in apparently healthy cattle, and to assess the diversity of *Salmonella* serotypes associated with cattle production systems through a systematic review and meta-analysis of peer-reviewed publications between 2000 and 2017.

3.3. Methods

3.3.1. Systematic review of the literature

Preferred reporting items for systematic reviews and meta-analysis protocols (PRISMA-P) 2015 checklist was followed for the systematic review and meta-analysis of studies reporting *Salmonella* serotypes and prevalence in cattle (Moher et al., 2016). Five electronic databases were searched: PubMed (http://www.ncbi.nlm.nih.gov/pubmed), Google scholars (https://scholar.google.com/), Agricola (http://agricola.nal.usda.gov/), Scopus (http://www.scopus.com/) and CAB direct (http://www.cabdirect.org/). Additional publications were obtained by manual search from the retrieved publications. *Salmonella*, cattle and prevalence were the main key words used for the search. The search was conducted with alternative terms for each key term using the general protocol ((*Salmonella* AND (cattle OR bovine OR heifer OR bull OR bullock OR ruminant OR steer OR cow OR cull OR calf OR calves OR yearling OR beef OR dairy OR feedlot) AND (prevalence OR isolation OR identification OR “antimicrobial resistance” OR “antimicrobial susceptibility”)) that was modified and tailored to search strategies of each database when needed.

3.3.2. Relevance screening

The retrieved articles were imported to Refworks to manage and exclude duplicated studies (ProQuest, 2016). The duplicated records were excluded manually after making the bibliography list and prior to the eligibility assessment. The eligibility criteria were: (i) articles published in English between January 1, 2000 (since full articles could not be available online, publications prior to 2000 were not considered) and January 4, 2017 (the last date of literature search); (ii) reported on apparently healthy cattle (i.e. articles not mentioning the disease status of cattle) from different production categories (dairy, beef, mixed) and sample sources (slaughter plant/abattoir/slaughter house, dairy farm, beef farm, ranch, feedlot, grazing point, market place, mixed cattle farm); (iii) samples collected from the intestinal content (feces from the rectum and other intestinal contents); (iv) prevalence report from any part of the world and; v) cross sectional study in which animal level prevalence was reported or could be calculated from the information provided in the publication during data extraction. The exclusion criteria were: i) irrelevant records to the objective of the review;(ii) articles on sick or diseased cattle; (iii) non-cross-sectional study design (iv) report on inappropriate samples such as ground and pen fecal samples or pooled fecal samples from which animal level prevalence was unknown, lymph nodes, rumen contents or other body parts of cattle; (v) when only citations or abstracts were available.

3.3.3. Data extraction

A peer-reviewed publication that describes prevalence of *Salmonella* in cattle was considered as a study unit. Cattle were considered positive for *Salmonella* when samples from the intestinal contents were tested and confirmed positive. When different prevalence reports in the content from various sites of intestinal tract were observed in a single study, we considered this one with the highest proportion for better precision to minimize under estimation. From each eligible publication we extracted the following information: author, year of publication, year of study, study location (country and continent), detection method, production type (beef, dairy and mixed), sampling location (abattoir, farm, market, ranch, grazing points, feedlot), age (calves and

adults), amount of tested samples, sample size, number of *Salmonella* positive samples and serotypes identified and number within each serotype. The extracted information was entered to a Microsoft excel spread sheet for quality assessment and data preparation for analysis.

3.3.4. Data analysis

Frequency distributions were used to describe the characteristics of the eligible publications and the diversity and proportion of *Salmonella* serotypes. Meta-analysis was conducted using the meta prop-one package (Nyaga et al., 2014), a Stata based program specifically designed for binominal data, that allows the computation of studies with 0% or 100% prevalence. Analysis was done in STATA version 14 (STATACorp, 2015). The prevalence of *Salmonella* in cattle was defined as the proportion of *Salmonella* positives based on the intestinal content samples. The pooled prevalence of *Salmonella* was computed by meta-analysis from the prevalence values of the individual publications by accounting for potential heterogeneity between studies and weighted on sample size (Borenstein et al., 2009). A logistic-normal random-effects model was used to model the within-study variability. The 95% confidence intervals (CIs) for the proportion of cattle *Salmonella* positive for the separate publications and their pooled prevalence was computed with the exact binomial method with the Freeman-Tukey double arcsine transformation which gives the CIs within admissible values. Further analysis of sub-groups of the overall estimate was performed according to age, production type, detection method and continent categories. Heterogeneity of the effect sizes among the publications was assessed by Cochrane Q test and inverse variance index (I^2) test and quantified as recommended by Higgins and Thompson (Higgins, 2002). A *P* value of < 0.01 was set as an indication of a statistically significant heterogeneity. The basic results from the meta-analysis were visually presented using forest plots. Frequency distributions were used to describe the characteristics of the eligible studies and the diversity and proportion of *Salmonella* serotypes.

3.4. Results

3.4.1. Systematic review of the literature

A flow chart showing the systematic literature search procedure is shown in Figure 1. A total of 2,655 records were retrieved from the five search engines (PubMed, Agricola, CAB direct, Google scholar) and by manual search. After de-duplicating the references, 1,753 publications were retained for further screening. After relevance screening of the titles and abstracts, 1,625 articles were excluded resulting in 128 potentially eligible full articles. Further in-depth eligibility assessment of the full articles resulted in 71 eligible publications for data extraction and analysis.

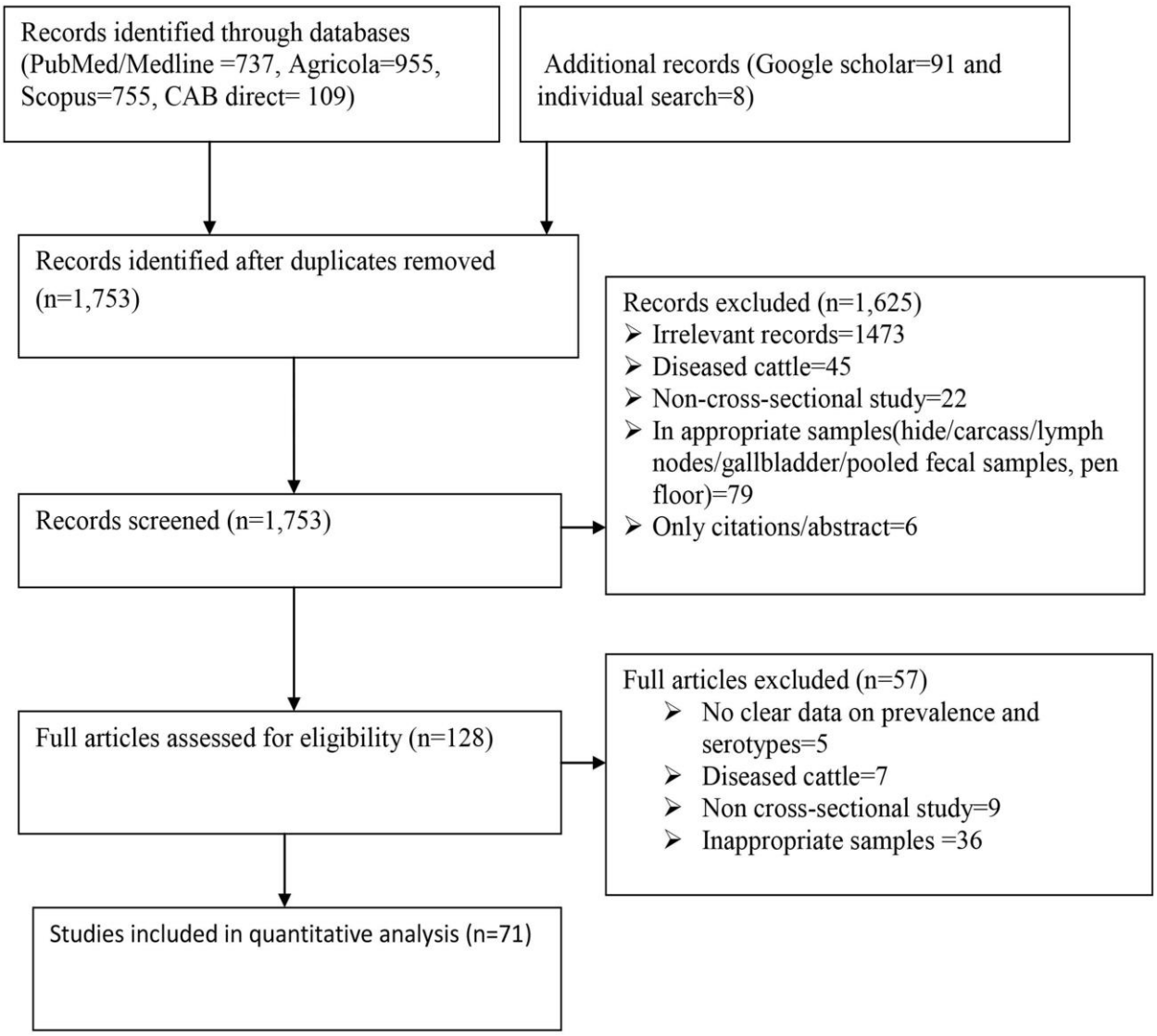

Figure 3.1. Flow diagram for the selection of studies included in the meta-analysis for the prevalence of *Salmonella* in apparently healthy cattle.

3.4.2. Data extraction and meta-analysis

Data were extracted from the 71 peer-reviewed publications comprising 75 data sets. Two separate datasets were extracted from three publications (Al-Saigh et al., 2004; Hanson et al., 2016; Tarazi and Abo-Shehada, 2015) based on age and from one study (Barham et al., 2002) based on sampling points. Therefore, 75 data sets (hereafter referred to as studies) comprising fecal samples or swabs from 52,766 animals were included in the meta-analysis. *Salmonella* was detected in 5,010 of the animals. Over two-thirds (68%) of the studies used ≤ 10 g of feces, and 91% of the studies used traditional culture methods for the detection of *Salmonella*. The publications represented 29 countries across six continents except Antarctica. While 80% of the countries were represented by one or two publications, the United States was the most represented with 25 publications. Forty percent of the studies were conducted on samples collected at processing plants. Detailed characteristics of the publications are shown in Table 3.1.

Table 3.1. Description of the eligible publications included in the systematic review and meta-analysis of *Salmonella* in apparently healthy cattle.

Characteristics	Number of datasets (n=75)	Percentage
Fecal amount (g or ml)		
≤ 10	51	68.0
> 10	7	9.3
Swabs/ loopful	12	16.0
Not specified	5	6.7
Sampling point		
Dairy farm	25	33.3
Abattoir	30	40.0
Feedlot	7	9.3
Grazing point	2	2.7
Mixed farm	8	10.7
Not specified	2	2.7
Market	1	1.3
Detection methods		
Traditional culturing	68	90.7
IMS	6	8.0
PCR	1	1.3
Age		
Adult	63	84.0
Calves	12	16.0
Production type		
Beef	18	24.0
Dairy	28	37.3
Mixed	14	18.7
Not specified	15	20.0
Continent		
Africa	16	21.3
Asia	15	20.0
Australasia	6	8.0
Europe	9	12.0
North America	28	37.3
South America	1	1.3

IMS: Immunomagnetic separation; PCR: Polymerase chain reaction

Overall pooled prevalence of *Salmonella* in cattle was 9% (95% Confidence interval: 7-11%). Results of individual studies along with the effect of sizes are shown in Figure 3.2. Study prevalence values ranged from 0% to 95%. Test of heterogeneity demonstrated the presence of a high degree of heterogeneity (I^2=98.7%, P<0.01) among the studies. To account for some of the variability separate stratified meta-analyses were performed by age, production type, detection method and continent (Table 3. 2). The pooled prevalence of *Salmonella* is higher in the adult cattle (9%) than in the calves (6%), in beef cattle (14%) than other production types, and in North America (16%) than other continents. Studies within each category of the strata defined by detection method and continent, showed significantly high degrees of heterogeneities (P < 0.01). However, no significant heterogeneity was observed between the age groups, among production types and when comparing only between IMS and non-IMS detection methods (P > 0.01).

Table 3.2. Pooled prevalence of *Salmonella* in apparently healthy cattle determined by meta-analysis of 75 datasets studies by age, production type, detection method and continent.

Subgroups	No. of publications	No. of datasets	No. of animals tested	No. of animals positive	Pooled prevalence (95% confidence interval)	Heterogeneity test	
						I^{2*} (%)	*p-value*
Age							
Adult	62	63	45289	4624	9 (7-12)	98.7	<0.01
Calves	12	12	7477	386	6 (2-11)	97.4	<0.01
Production type							
Beef	17	18	5085	366	14 (7-23)	98.3	<0.01
Dairy	26	28	30970	3746	10 (7-13)	98.7	<0.01
Mixed	13	14	10154	588	5 (2-9)	98.0	<0.01
Not specified	15	15	6557	310	5 (2-11)	97.9	<0.01
Detection method							
Non-IMS	64	68	50311	4696	8 (6-11)	98.7	<0.01
PCR	1	1	50	25	50 (37-63)	-	-
IMS	6	6	2405	289	10 (5-16)	92.1	<0.01
Continent							
Africa	16	16	3153	314	9 (3-16)	98.2	<0.01
Asia	14	15	3116	202	4 (1-8)	94.9	<0.01
Australasia	6	6	6370	287	4 (1-11)	98.8	<0.01
Europe	8	9	6470	88	2 (0-3)	92.0	<0.01
North America	26	28	33577	4108	16 (12-20)	99.0	<0.01
South America	1	1	80	11	14 (8-23)	-	-
Total	71	75	52766	5010	9 (7-11)	98.7	<0.01

*Inverse variance index that describes the percentage of variation across studies attributed to heterogeneity rather than chance.

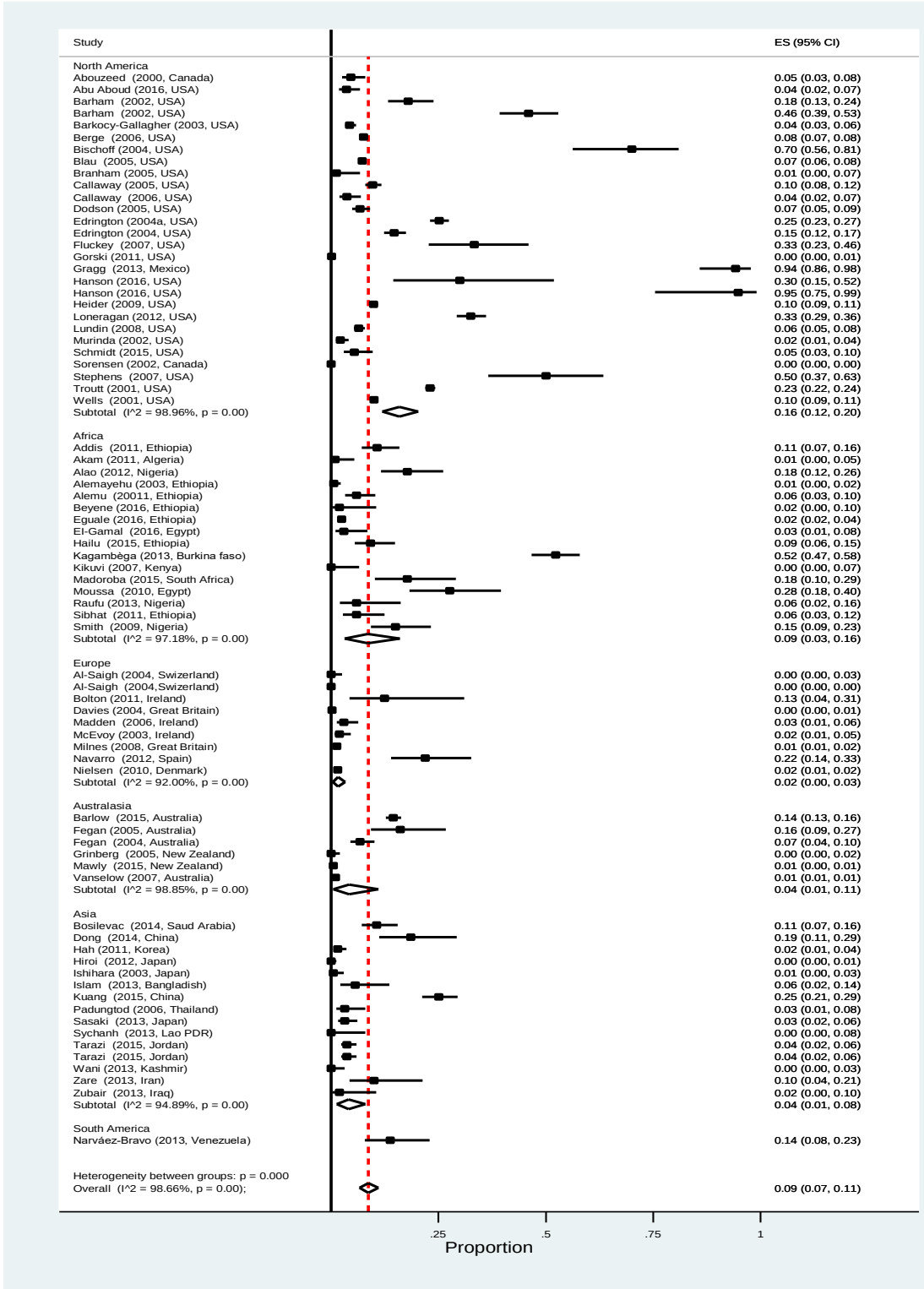

Figure 3.2. Forest plot showing estimated individual and overall *Salmonella* prevalence in apparently healthy cattle (ES: effect size; *CI: confidence interval; I^2:* Inverse variance index).

3.4.3. Diversity of serotypes

Serotype information was not reported for 1,926 *Salmonella* positive cattle from a total of 16,175 cattle examined in 27 publications representing 29 data sets. In the remaining 44 publications representing 46 datasets for which serotype information was available, 3,191 *Salmonella* isolates were reported from 3,084 *Salmonella* positive cattle from a total of 36,591 cattle examined. Among the 3,191 isolates with serotyping information, specific serotypes were reported in 91.6% (2,923/3,191) of the isolates while 2.8% of the isolates were untypable, and the remaining 5.6% were reported as "other serotypes" where the list of which was not stated in the publication.

Overall, 143 different serotypes were reported among the 2,923 *Salmonella* isolates listed in the data sets included in the meta-analysis. The most frequently (with ≥ 1%) reported serotypes are shown in Table 3.3. The 10 most frequently reported cattle associated serotypes across all studies were *S.* Montevideo, *S.*Typhimurium, *S.* Kentucky, *S.* Meleagridis, *S.* Anatum, *S.* Cerro, *S.* Mbandaka, *S.* Muenster, *S.* Newport and *S.* Senftenberg. These 10 most frequently isolated serotypes comprised 69.5% (2,032/2,923) of total isolates for which specific serotypes were reported. There were variations in the frequency and diversity of *Salmonella* serotypes in the six continents for which publications were retrieved (Table 3.4). *S.* Montevideo was the most frequent reported serotype from North America while this serotype did not belong to the five most frequently reported serotypes in most other continents. *S.* Typhimurium was the most frequently reported serotype in Africa, Asia and Australasia, while *S.* Dublin was the most frequently reported serotype in Europe. The most diverse serotypes were reported from Africa (76 different serotypes) followed by North America (49 different serotypes), Australasia (39 serotypes), Asia (23 serotypes), Europe (12 serotypes) and South America (2 serotypes).

Table 3.3. *Salmonella* isolates by serotype in descending order of frequency across studies reporting specific serotypes.

Serotypes	No. of isolates	Percentage (n=2,923)	No. of datasets (%)	Continent (number of isolates representing each serotype)
Montevideo	524	17.9	14 (30.4)	Africa (1), Asia (6), Australasia (2), North America (515)
Typhimurium	294	10.1	28 (60.9)	Africa (45), Asia (49), Australasia (96), Europe (12), North America (91)
Kentucky	214	7.3	11 (23.9)	Africa (5), Asia (1), North America (208)
Meleagridis	186	6.4	11 (23.9)	Asia (5), Australasia (2), Europe (4), North America (175)
Anatum	179	6.1	17 (36.9)	Africa (2), Asia (7), Australasia (24), Europe (10), North America (136)
Cerro	176	6.0	7 (15.2)	Australasia (3), North America (173)
Mbandaka	169	5.8	12 (26.1)	Australasia (6), Europe (10), North America (153)
Muenster	113	3.9	6 (13)	Africa (17), North America (96)
Newport	92	3.1	10 (21.7)	Africa (3), Australasia (1), North America (86)
Senftenberg	85	2.9	9 (19.6)	Asia (4), Australasia (9), North America (72)
Dublin	64	2.2	10 (21.7)	Africa (6), Australasia (9), Europe (38), North America (11)
Agona	62	2.1	13 (28.3)	Asia (21), Australasia (3), North America (38)
Menhaden	59	2.0	1 (2.2)	North America (59)
Muenchen	53	1.8	5 (10.9)	North America (47), Australasia (6)
Infantis	51	1.7	1 (2.2)	North America (51)
Give	47	1.6	1 (2.2)	Australasia (47)
Others #	555	18. 9		

Table 3.4. *Salmonella* isolates by serotype within six continents in descending order of frequency in studies reporting specific serotypes

Rank	Serotypes (% of isolates) *					
	North America	**Africa**	**Asia**	**Australasia**	**Europe**	**South America**
1	Montevideo (24.0)	Typhimurium (15.8)	Typhimurium (40.0)	Typhimurium (34.4)	Dublin (44.7)	Javiana (50.0)
2	Kentucky (9.7)	Drac (26, 9.1)	Agona (17)	Anatum (8.6)	Typhimurium (14.1)	Weltevreden (50.0)
3	Meleagridis (8.2)	Enteritidis (8)	Derby (6.5)	Orion (6.8)	Anatum (11.8)	
4	Cerro (8.1)	Muenster (5.9)	Anatum (5.6)	Bovismorbificans (6.1)	Mbandaka (11.8)	
5	Mbandaka (7.1)	Bredeney (5.6)	Montevideo (4.8)	Saintpaul (5.4)	Derby (4.7)	
6	Anatum (6.3)	Urbana (4.5)	Meleagridis (4.0)	Dublin (3.2)	Meleagridis (4.7)	
7	Muenster (4.5)	Ruiru (2.8)	Enteritidis (3.2)	Zanzibar (3.2)	London [10+]	
8	Typhimurium (4.2)	Dublin (2.1)	Kunduchi (3.2)	Infantis (2.9)	6,7: D: - (1.2)	
9	Newport (4.0)	Saintpaul (2.1)	Senftenberg (3.2)	Thompson (2.5)	Agama (1.2)	
10	Senftenberg (3.4)	Virchow (2.1)	Fyris (1.6)	Havana (2.5)	Kedougou (1.2)	
11	Menhaden (2.8)	Hato (1.8)	Kingston (1.6)	Senftenberg (2.5)	Kiel (1.2)	
12	Muenchen (2.2)	Kentucky (1.8)	Rissen (1.6)	Mbandaka (2.2)	Othmarschen (1.2)	
13	Give (2.1)	Newport (1.8)		Muenchen (2.2)		
14	Infantis (1.9)	Tennessee (1.8)		Bredeney (1.8)		
15	Agona (1.8)	Chomedey (1.4)		Adelaide (1.4)		
16	Minnesota (1.4)	Lagos (1.4)		Chester (1.4)		
17	Kinshasa (1.0)	Soumbedioune (1.4)		Agona (1.1)		

18		Eko (1.1)		Cerro (1.1)		
19		Farakan (1.1)		Charity (1.1)		
20		Mishmarhaemek (1.1)		Ruiru (1.1)		
21		Nima (1.1)				
22		Uganda (1.1)				
Other	32serotypes (7.5)	55serotypes (19.3)	9 serotypes (7.3)	19 serotypes (8.6)	-	-
Total	2148	285	124	279	85	2

*only serotypes with ≥ 1% frequency are reported

3.5. Discussion

To the best of our knowledge, this is the first estimate of the overall *Salmonella* prevalence and the diversity of serotypes in apparently healthy cattle. We used a systematic method to identify articles reporting the prevalence of *Salmonella* and the serotypes in such cattle, followed by a quantitative meta-analysis to estimate the overall prevalence of *Salmonella* at the global level.

Salmonella colonizes the gastrointestinal tract of food animals (Andino and Hanning, 2015) and is shed via feces (Abouzeed et al., 2000; Callaway et al., 2005; Loneragan et al., 2012; Narváez-Bravo et al., 2013). Cattle are reservoirs for *Salmonella* and may function as a source of foodborne infection (Feasey et al., 2012; Agga et al., 2016 ; Chaney et al., 2017). A number of serotypes frequently isolated from humans have been isolated from sick or apparently healthy cattle and some human cases have also been linked to direct exposure to cattle (Hoelzer et al., 2011). Knowing the prevalence and diversity of *Salmonella* serotypes in cattle can provide important information necessary to develop preventive measures and strategies at different stages of food chain such as application of hazard analysis and critical control point (HACCP) programs in beef and milk production industries to ensure food safety (Tietjen and Fung, 1995).

There was high heterogeneity in the estimated *Salmonella* prevalence among the studies included in the analysis. The *Salmonella* prevalence can vary depending on the detection method used, the amount of sample processed, production type, number of farms and geographical variation in the distribution of the *Salmonella* (Al-Saigh et al., 2004; Ishihara et al., 2009). The overall pooled prevalence of 9% is higher compared to other reported national level prevalence values ranging from 0.2 % to 7.1% (Dargatz et al., 2000; Davies et al., 2004; Fegan et al., 2004; Mawly et al., 2015; Tadesse and Tessema, 2014). This is not surprising since our meta-analysis provides a precise estimate (with narrow confidence interval) as it includes a higher amount of samples and total number of positive cattle for *Salmonella* by pooling 75 datasets from 71 publications.

The prevalence was higher in the adult cattle (9%) than in the young age group (6%). Although the effect of age needs further investigation, this variation can presumably be in part due to variation in the number of studies included in the meta-analysis in each age group. In the young age group there were 12 publications representing only 14.2% (n=7477) of total cattle examined compared to 63 publications in the adult cattle with 86% of the total cattle examined. Over 70% of the publications were conducted at processing plants and in culled dairy cows destined for slaughter perhaps because of the higher public health significance at the final stage of production chain that is close to consumers (Sofos and Geornaras, 2010). Even though *Salmonella* colonizes the intestinal tracts of cattle, there is no difference in the colonization and shedding of *Salmonella* between healthy calves and adult cattle (Andino and Hanning, 2015). However, a higher prevalence of *Salmonella* shedding animals occurs when asymptomatic chronically infected carrier cattle are present on the farm and stay on the farm for long periods (Narelle Fegan et al., 2004) which may contribute to transmission and persistence of *Salmonella* on the farm.

Although not statistically significant, the prevalence was higher in beef cattle compared to dairy cattle. This apparent difference can be attributed to how the animals were sampled. In most of the studies culled dairy cows were sampled at farms before shipment as opposed to beef cattle which were commonly sampled at the processing plants. Temporary restriction or complete feed withdrawal and transport stress can result in increased fecal shedding of *Salmonella* in feedlot cattle prior to slaughter (Tadesse and Tessema, 2014; Beach et al., 2002; Corrier et al., 1990 ; Millemann et al., 2000).

Variations in serotypes and prevalence estimates that ranged from 2% (Europe) to 16% (North America) in various continents of the world could partly be attributed to the differences in the number of publications, the number of cattle samples and number positive for *Salmonella* included in the analysis. For North America, 26 publications (28 data sets) were retrieved consisting of 33,577 cattle sample, being the majority of the articles. In contrast, the very low prevalence estimate observed in Europe, was estimated only from 8 publications (9 data sets) in which 6,470 cattle sampled and 88 were positive. The diverse serotypes in Africa could be due to

extensive and/or pastoral farming practices and free mobility of animals in the environment which likely expose animals to diverse wildlife, ecologies and interfaces harboring diverse serotypes. The differences might also be associated with the differences in the monitoring and surveillance mechanisms among the continents (Hendriksen et al., 2011). Arguably, in most developing countries where there is no national monitoring and surveillance scheme, the prevalence estimate, based on few number of studies either under estimate or overestimate the true prevalence. Moreover, the difference in the estimate could be due to difference in the livestock farming systems and geographical variation in the distribution of *Salmonella* (Al-Saigh et al., 2004; Ishihara et al., 2009).

Difference in the prevalence was also observed among categories of detection methods. In the majority (91%) of the studies, *Salmonella* was detected using traditional culturing methods which are in general considered less sensitive methods. Limited number of studies used immunomagnetic separation beads or PCR. Variation in the sensitivity of culture detection methods can influence the prevalence estimate and consequently the observed heterogeneity (Eriksson and Aspan, 2007). However, in this study a comparable prevalence was observed based on detection with IMS (10%) and without IMS (8%) which could be due to small number (n=6) of studies that used IMS.

In this systematic review, *S.* Montevideo and *S.* Typhimurium were the two most frequent and dominant serotypes reported where *S.* Montevideo was majorly reported from North America. *S.* Typhimurium is one of the major serotypes that accounted for human clinical cases globally (Tennant et al., 2016). Human infections and outbreaks due to *S.* Montevideo is also increasing around the globe (Lalsiamthara and Lee, 2017) and reported in the USA, Europe, Australia, and Asia (CDC, 2010; Harada et al., 2011; Kim et al., 2004). There were differences in the most commonly reported serotypes and their proportions among different continents. *S.* Typhimurium which is historically associated with cattle ranked number one in Africa, Asia and Australasia. In North America and Europe, however, Montevideo and Dublin ranked number one, respectively. The implication of the shift in serotype with respect to public health requires further study. Interestingly, among the top 10 *Salmonella* serotypes identified in this study, *S.* Montevideo, *S.*

Typhimurium, *S.* Anatum, *S.* Mbandaka and *S.* Newport are among the World Health Organization's top 20 serotypes associated with human salmonellosis across the world (Eriksson and Aspan, 2007).

Spatial and temporal effects on the distribution and diversity of *Salmonella* have been reported (Besser et al., 2000 ; Galland et al., 2001) which may explain the observed differences in the serotype diversity among the studies reporting *Salmonella*. Some of the serotypes reported in the present review were identified as the dominant serotypes elsewhere in cattle at varying proportions. For instance, in the USA, *S.* Newport (48.7%) and *S.* Typhimurium (7.1%) (Jackson et al., 2007); in Ethiopia, *S.* Typhimurium (17.4%), *S.* Newport (13%) and *S.* Anatum (5.8%) (Tadesse and Tessema, 2014) and in Europe, *S.* Typhimurium (38.6%) were reported to be the most frequent and dominant serotypes (EFSA and ECDC, 2015). On the contrary, none of these serotypes were reported from the national survey of *Salmonella* serotypes in cattle carried out in Japan (Ishihara et al., 2009).

All non-typhoidal *Salmonella* serotypes except a few serotypes which are host-specific, can potentially cause disease in humans and reside in one or more animal species (Uzzau et al., 2000). Different domestic animals such as poultry, pigs, poultry and ruminants (cattle, sheep and goats), are reservoirs of *Salmonella* playing an important role in the epidemiology of human infection (Hanson et al., 2020). Pork, poultry, eggs, beef and milk are commonly implicated foods. Ingestion of contaminated water, fruit and vegetables are also other possible sources of infection. Eggs and poultry are the most common sources of *Salmonella* infection (CDC, 2020; Ferrari et al., 2019; Schneider et al., 2011). *S.* Enteritidis and *S.* Typhimurium are the two most important serotypes transmitted from animals to humans in most parts of the world (EFSA and ECDC, 2015; Eng et al., 2015; Greig and Ravel, 2009; Hendriksen et al., 2011). In the USA, 29 cases of diarrheal illness caused by *S.* Typhimurium were associated with the consumption of raw milk or raw-milk products from dairy cattle (Yousef and Carlstrom, 2003). During the period 1973–2011, of the 1,965 *Salmonella* outbreaks where a food vehicle was implicated, 96 were attributed to beef, accounting for 3,684 illnesses in USA. *S.* Newport and *S.* Typhimurium accounted for 18% and 17% of illnesses, and 29% and 18% of hospitalizations, respectively

(Laufer et al., 2015). The multidrug-resistant *S.* Typhimurium DT104 has also been associated with outbreaks related to beef contamination and resulted in hospitalization rates twice as that of other foodborne salmonellosis cases (Yousef and Carlstrom, 2003). From a total of 1,168 foodborne outbreaks of human salmonellosis in 2013 reported by the European member states, 1.6% of the cases were attributed to beef and beef products (EFSA and ECDC, 2015). This evidence supports the importance of cattle and cattle associated serotypes for human salmonellosis, but the role of other animals as a source of FBP should not be overlooked.

Besides the datasets from the publications included in this review and meta-analysis, other relevant information was available in new articles that were published in the years between 2017- 2018 while the manuscript was under preparation by the authors. During this period, 6 full articles and three published abstracts representing 11 datasets were retrieved using the search engines. The majority of these studies were reported from Africa (Ball et al., 2018; Cetin et al., 2018 ; Ketema et al., 2018; Nouichi et al., 2018; Takele et al., 2018; Fuenmayor et al., 2019; Kore et al., 2017) except for two studies from Europe (Cetin et al., 2018) and South America (Fuenmayor et al., 2019). Among the total of 5868 cattle examined, 9.2% (554 /6018), which is nearly equal to the pooled prevalence estimate, were reported to be positive for *Salmonella* species with different serotypes. The global level pooled prevalence of *Salmonella* in cattle was higher (9%) as compared to the pooled prevalence estimates of *E. coli* O157 (5.68%), which is also excreted by cattle showing the relative public health importance of *Salmonella* (Islam et al., 2014).

3.6. Conclusions

This study based on systematic reviews and meta-analysis provides an overall prevalence of *Salmonella* and serotype diversity in apparently healthy cattle at a global level. The results indicated variations in the level of *Salmonella* carriage in cattle across the world, and the presence of a diverse number of *Salmonella* serotypes. The estimated *Salmonella* prevalence was higher in North America. The predominant detection method is traditional culturing. Because of the possibility of *Salmonella* contamination of carcasses during slaughter and milk during

milking, cattle can be a potential source of *Salmonella* and can lead to public health risk and economic loss if the necessary hygienic measures are not properly followed.

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Chapter 4

4. Prevalence, Molecular Characteristics and Antimicrobial Resistance of *Salmonella* in Cattle, Beef and Humans in Bishoftu, Ethiopia

Based on: Gutema, F.D., Rasschaert,Geertrui., Agga, G.E., Olana, M., Addisu, B.D., Abdi, R.D., Mattheus,W., Duchateau, L., Gabriël, S., De Zutter, L. Prevalence, Molecular Characteristics and Antimicrobial Resistance of *Salmonella* in Cattle, Beef and Humans in Bishoftu town, Ethiopia.Foodborne Pathogens and Diseases, 2021,18:283–289**.** https://doi.org/10.1089/fpd.2020.2869.

4.1. Abstract

Within Ethiopia, there is a lack of information on the genetic relatedness of *Salmonella* from cattle, beef and diarrheic patients and its potential transmission from cattle to humans via consumption of contaminated beef. The objective of this study was to assess the prevalence and determine the serotypes, genetic relatedness and antimicrobial resistance of *Salmonella* in cattle (n=240) in two local slaughterhouses, in beef at retail shops (n=127) and diarrheic patients (n=216) in the only hospital in Bishoftu, Ethiopia. *Salmonella* was detected in 2.5% of cattle samples, in 8.7% of beef samples, and in 2.3% of the diarrheic patients. Four *Salmonella* serotypes: *S.* Typhimurium, *S.* Eastbourne, *S.* Saintpaul and *S.* Cotham were identified. *S.* Typhimurium and *S.* Eastbourne were isolated from cattle and beef while *S.* Saintpaul and *S.* Cotham were isolated only from diarrheic patients. Except for serotype *S.* Saintpaul, all isolates were grouped into five pulsotypes of which two pulsotypes contained isolates from cattle and beef. Isolates from humans represented unique pulsotypes. Among the 22 *Salmonella* isolates tested, 95.5 % were resistant to at least one of the 14 antimicrobials tested. Three *Salmonella* isolates originating from cattle were multidrug resistant. One human isolate was susceptible to all antimicrobials tested. More specifically, resistance to ampicillin, sulfamethoxazole, tetracycline, tigecycline and trimethoprim were observed. The most frequently observed resistance was to sulfamethoxazole (90.9%) followed by trimethoprim (22.7%). The study revealed considerable *Salmonella* contamination of beef at retail shops, antimicrobial resistance to commonly used antimicrobials, and shared genetically similar *Salmonella* serotypes between cattle and beef, the link with humans could not be established. Still, the findings of *Salmonella* in cattle and beef, the propensity of transfer of *Salmonella* from cattle to beef coupled with the common consumption of raw/undercooked beef is likely to pose public health risk in Ethiopia.

4.1. Introduction

Human *Salmonella* infection is a major public health concern worldwide (Majowicz et al., 2010). It is mainly manifested by gastroenteritis characterized by diarrhea (Lee et al., 2015). *Salmonella* was estimated to cause 95 million cases, 50,771 deaths and 3 million disability adjusted life years globally in 2017 (Stanaway et al., 2019). *Salmonella* is responsible for 30% of foodborne outbreaks in the USA (Dewey-Mattia et al., 2018). For 2018, 94,203 confirmed salmonellosis cases were reported with a notification rate of 20.1 cases per 100,000 population within Europe (EFSA and ECDC, 2019a). Mortality rate from *Salmonella* infection in developing countries is estimated to be 24% higher than in developed countries (Chimalizeni et al., 2010). The majority of the infections are often associated with ingestion of contaminated foods (Hur et al., 2012).

Cattle are among the reservoirs of animals for *Salmonella* with a global estimated prevalence of 9% ranging from 2% in Europe to 16% in North America, while for Africa the estimated prevalence was 9% (Gutema et al., 2019). Cattle play a significant role in the epidemiology of zoonotic salmonellosis (Hoelzer et al., 2011). The presence of *Salmonella* in cattle, contact with infected cattle and cross contamination of carcasses during hide removal and evisceration are common sources of *Salmonella* infection to humans (Cummings et al., 2010). *Salmonella* in meat and meat products such as pork, poultry and beef are the highest risk agent/food pairs causing foodborne outbreaks in humans (EFSA and ECDC, 2018). Beef contaminated with *Salmonella* has been indicated as the source of infection in several outbreaks. For example, in the USA, among the 1965 outbreaks of *Salmonella* where a food vehicle was implicated during 1973–2011, 96 were attributed to beef accounting for 3,684 illnesses (Laufer et al., 2015).

In Ethiopia, the *Salmonella* prevalence was estimated to be 8.7% in children and 5.7% in adults with diarrhea (Tadesse, 2014), 7.1% in cattle (Tadesse and Tessema, 2014)and 10% in beef (Zelalem et al., 2019) based on meta-analysis. A high level of multidrug resistance was also reported in *Salmonella* isolated from slaughtered cattle (Eguale et al., 2017; Ketema et al., 2018) raising serious concerns of increased transmission risk of resistant strains in the beef supply chain.

A recent study conducted in Ethiopia indicated genetic relatedness among *Salmonella* isolates from humans and animals including cattle that were collected from Addis Ababa and surrounding districts (Eguale et al., 2018). However, this study did not compare the genetic relatedness of *Salmonella* isolates from beef with that of cattle and diarrheic patients to investigate the potential transmission of *Salmonella* from cattle to humans through beef consumption.

Establishing any possible transmission and epidemiological association between the occurrence of *Salmonella* in cattle, beef and diarrhea in humans is essential to devise control options. Therefore, the objective of this study was to compare the occurrence, serotype distribution, antimicrobial resistance profile and genetic relatedness of *Salmonella* in cattle, beef and diarrheic patients in Bishoftu, Ethiopia.

4.2. Materials and methods

4.2.1. Study site

The study was conducted from June 2017 to May 2018 in Bishoftu town, East Shewa Zone, Oromia, Ethiopia (Figure 4.1). Mixed crop livestock production is the predominant agricultural system in the zone where cattle are primarily kept for draft power. Bishoftu is located at 45 kilometers southeast of Addis Ababa at 9^0N latitude and 40^0E longitude with an altitude of 1850 meters above sea level. The area receives an average annual rainfall of 866 mm, 84% of which happens during the long rainy season (June to September). The dry season extends from October to February. The mean annual minimum and maximum temperatures were 14 °C and 26 °C, respectively with a mean relative humidity of 61.3%. According to the 2007 Ethiopian census report, the total human population of Bishoftu town was estimated at 100,114 (CSA, 2007). In the town, there are two small cattle slaughterhouses (one municipal and one privately-owned) and 127 retail shops serving the local community.

There is one public hospital, Bishoftu hospital, with a catchment population of approximately 1.2 million people.

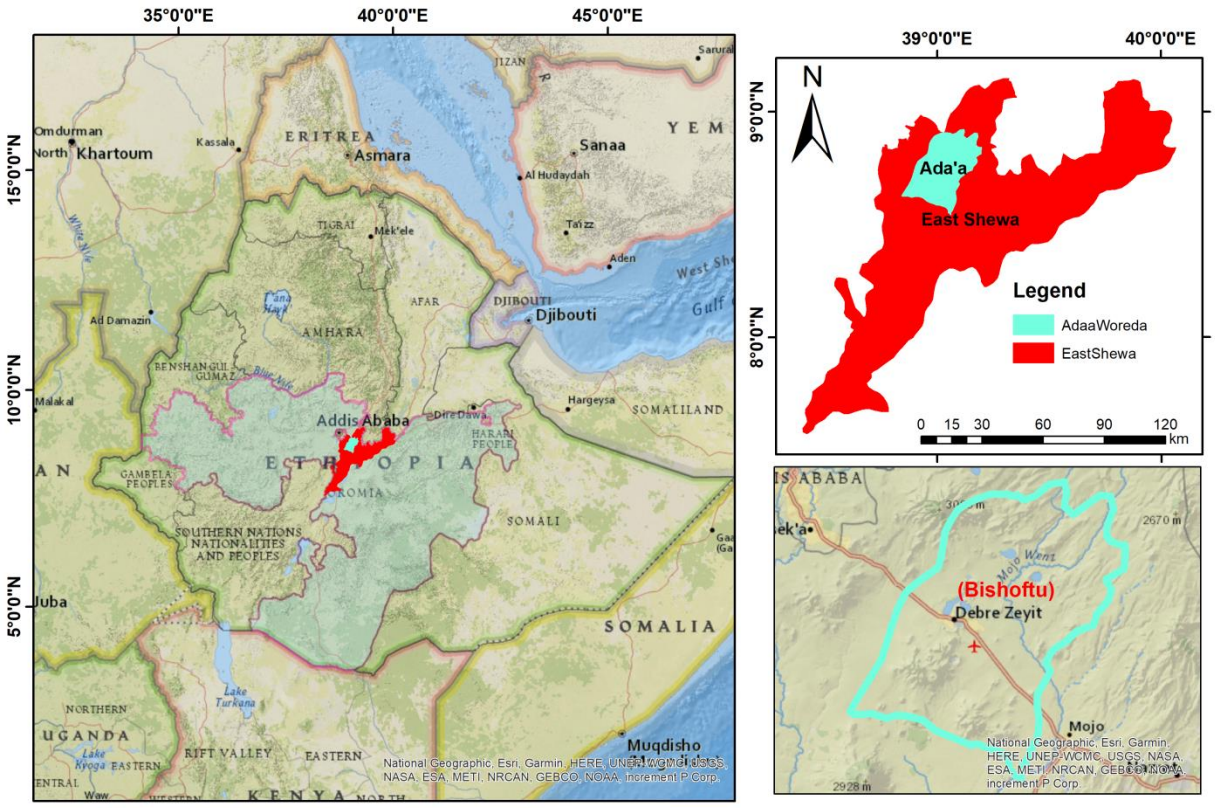


Figure 4.1. Map of the study area.

4.2.2. Study design and sample collection

In order to estimate the prevalence in the three types of samples (rectal content from cattle at slaughterhouses, beef at the retail shops and stool samples from diarrheic patients), the number of samples to be collected was calculated using the formula of Thrusfield (2005) to estimate prevalence in large populations. Based on Ethiopian data for the types of samples (7.1% in cattle (Tadesse and Tessema, 2014) and 9% in diarrheic patients (Tadesse, 2014)), a maximum expected prevalence of 9% with a confidence interval of 95% and an accepted error of 4% was used for the calculation. This resulted in the collection of at least 197 units/sample type. We

included 240 rectal content and 216 stool samples. For beef samples, it was decided to visit all retail shops selling beef in the town ones and to collect one sample from the available beef. Rectal content samples (at least 25g) from 240 cattle, were collected from the two slaughterhouses (the municipal and the private one, 120 cattle each) in the town. The cattle were brought to slaughterhouses directly from open market by beef retail shop owners with no information on the origin of the cattle. Both slaughterhouses were small in capacity where the municipal slaughterhouse and the private slaughterhouse usually slaughtered 5-15 and 15-30 cattle per day, respectively. Sampling was performed from available number of cattle at the slaughterhouses using systematic random sampling technique at private slaughterhouse and due to small number (usually less than 10) consecutively at municipal slaughterhouse. Rectal contents were collected on 14 occasions at the municipal slaughterhouse (June to September, 2017) and on nine occasions at the private slaughterhouse (October to December 2017). The samples were collected from the rectum using rectal gloves after restraining available animals in a crush located in the lairage.

Meat samples were collected from all of the 127 retail shops selling beef in the town (January to April 2018). On average, 10 beef samples were collected once per week. From each retail shop, one pooled sample of beef cuts (at least 25 g, representing about 200 cm^2) from the surface of the exterior of the carcass (fat tissue) and the surface of lean meat available at the time of the visit was collected and placed into a sterile polyethylene bag.

Diarrheic patients of one year or older with a history of passing at least three loose or liquid stools per day visiting Bishoftu hospital were included in the study. Samples were collected from consecutive diarrheic patients identified during each visit at the outpatient wards of the hospital (January to May 2018). One gram of stool sample was transferred into 9 ml buffered peptone water (BPW) from the stool samples submitted to the clinical laboratory of the hospital for routine testing from eligible and consenting patients who agreed to donate a stool sample. Data on age, sex, type of diarrhea and beef consumption history were recorded during sample collection. All collected samples were transported in an icebox to the laboratory and stored at 4^oC until processed within 24h.

Ethical clearance was obtained from Addis Ababa University, College of Veterinary Medicine and Agriculture VM/ERC/06/05/09/2017), Ministry of Science and Technology of Ethiopia (3/10/006/2018) and University Hospital Gent, Belgium (2017/0612). During sample collection, all diarrheic patients were informed about the purpose of the study. Samples were collected after obtaining consent; for minors, assent was requested from the children and written consent from their parents/guardians.

4.2.3. Detection and molecular characterization of *Salmonella*

For the detection of *Salmonella* the ISO method 6579-1: 2017 (ISO, 2017) was applied with a minor modifications. Briefly, 25 g of beef cuts or 25 g of rectal content samples were transferred into a sterile stomacher bag and mixed with 225 ml of buffered peptone water (BPW; Difco, BD, Sparks, MD, USA). The mixture was homogenized using a stomacher blender for 1 minute at 200 rpm and incubated at 37 °C for 18 h. The 1 g stool samples which were collected and transported in 10 ml BPW were directly incubated at 37 °C for 18 h. After the incubation of the pre-enrichments, 0.1 ml of each culture medium was spotted in 3 drops onto modified semi solid Rappaport-Vassiliadis medium (MSRV; Oxoid, Basingstoke, UK) and incubated at 41.5 °C for 24-48 h. After incubation, each plate was examined for the presence of migration zones. A loopful from the edge of a migration zone was streaked onto xylose lysine deoxycholate (XLD, Difco) agar plate and incubated at 37 ºC for 24 h. Then, XLD agar plates were examined for the presence of suspect *Salmonella* colonies (colonies with black center and a lightly transparent zone of reddish color or dome shaped colonies with a black center surrounded by a small red zone). Suspected colonies (1 to 2 colonies) were transferred onto tryptone soya agar slant (Oxoid). After incubation at 37 °C for 24 h, the cultures were subjected to biochemical tests using triple sugar iron agar slants (Difco, BD), lysine decarboxylase test (BBL, BD), and indole test (BBL, BD) for confirmation. Isolates were stored on tryptone soya agar slants (Oxoid, Basingstoke, UK) at -21 °C for further characterization.

For serotyping, one *Salmonella* isolate from each positive sample was first clustered using enterobacterial repetitive intergenic consensus (ERIC) PCR as described by Rasschaert et al

(2005). Based on the obtained ERIC profiles at least one isolate/profile was selected for serotyping according to the Kauffmann-White scheme (Grimont and Weill, 2007) at the Belgian National Reference Laboratory for *Salmonella*. Furthermore all isolates were subjected to pulse field gel electrophoresis (PFGE) after digestion with *Xba*I enzyme (CDC, 2017).

Salmonella Braenderup H9812 was used as reference strain. The fingerprints were grouped according to their similarity with Bionumerics 7.6 software (Applied Maths, Biomérieux, Sint-Martens-Latem, Belgium) using the band-based dice coefficient with a 2% position tolerance and unweighted-pair group method using arithmetic averages (UPGMA). Pulsotypes were assigned on the basis of the difference in the presence of at least one band in the XbaI fingerprint and identified by capital letter.

4.2.4. Antimicrobial susceptibility test

All serotyped *Salmonella* isolates were tested for their antimicrobial susceptibility to 14 antimicrobial drugs (with tested concentration range (µg/ml) in brackets): ampicillin (1-64), azithromycin (2-64), cefotaxime (0.25-4), ceftazidime (0.5-8), chloramphenicol (8-128), ciprofloxacin (0.015-8), colistin (1-16), gentamicin (0.5-32), meropenem (0.03-16), nalidixic acid (4-128), sulfamethoxazole (8-1024), tetracycline (2-64), tigecycline (0.25-8) and trimethoprim (0.25-32). The resistance profiling was evaluated based on the minimum inhibitory concentration (MIC) using Sensititre EU surveillance *Salmonella*/*E. coli* (EUVSEC) plates (Thermo Fisher Scientific, Merelbeke, Belgium). The standard reference strain *E. coli* ATCC 25922 was used as quality control. The tests were performed according to the manufacturer's instructions. European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological breakpoint values were used to categorize the isolates as resistant or susceptible (EUCAST, 2019). For sulfamethoxazole, tigecycline and colistin, the MIC values of *E. coli* were used. Isolates exhibiting resistance to more than two different antimicrobial classes were recorded as multidrug resistant (Magiorakos et al., 2012).

4.2.5. Data Analysis

Data were recorded in Microsoft Excel spread sheet (Microsoft Corp., Redmond, Washington, USA) and imported to STATA version 15.1 (STATA corp., College Station, TX, USA) for statistical analysis. Apparent prevalence of *Salmonella* was calculated as the percentage of positive samples from the total number of samples tested and presented with 95% confidence interval (95% CI). The difference in the prevalence of *Salmonella* among the three sample types and between the slaughterhouses was analyzed using the Pearson's chi-squared test. A $p < 0.05$ was considered significant. Antimicrobial resistance and molecular profiles of *Salmonella* isolates were expressed descriptively using frequency distributions and percentages.

4.3. Results

In total, 22 *Salmonella* positive samples were detected in the present study. The prevalence was 2.5% (95% CI: 0.9 – 5.4), 8.7% (95% CI: 4.4-14.9) and 2.3% (95% CI: 0.8- 5.3) in cattle, beef and diarrheic patients, respectively. There was a significant difference between the prevalence in beef and in cattle and humans ($Chi^2 = 10.69$, $P = 0.005$). There was no significant difference in the prevalence of *Salmonella* between cattle at slaughterhouses and diarrheic patients at hospital ($P > 0.05$). *Salmonella* was recovered from 3.4% (95% CI: 0.9-8.5) and 1.6% (95% CI: 0.2-5.7) of cattle rectal content sampled at the municipal and the private slaughterhouse, respectively. The difference was not significant ($Chi^2 = 0.79$, $P = 0.37$). Among the diarrheic patients, 57% (n=216) of them were males. The mean age of the diarrheic patients was 27.5 years (range: 1 to 82 years). *Salmonella* was detected from diarrheic patients of 22, 23, 31, 35 and 52 years old; three females and two males.

The *Salmonella* isolates belonged to four serotypes: *S.* Typhimurium (eight isolates), *S.* Eastbourne *(nine isolates), S.* Saintpaul (four isolates) and *S.* Cotham (one isolate) (Table 4.1). *S.* Typhimurium and *S.* Eastbourne were isolated from cattle and beef while *S.* Saintpaul and *S.* Cotham were isolated from diarrheic patients.

Table 4.1. Prevalence and serotypes of *Salmonella* in cattle, beef and diarrheic patients in Bishoftu, Ethiopia.

Source	No. Examined	No. Positive	Prevalence	Serotypes (Number)
Cattle	240	6	2.5	*S*. Typhimurium (2), *S*. Eastbourne *(4)*
Beef	127	11	8.7	*S*. Typhimurium (6), *S*. Eastbourne *(5)*
Humans	216	5	2.3	*S*. Saintpaul (4), *S*. Cotham (1)

The isolates were grouped into 5 pulsotypes (A-E) (Figure 4.2). Both serotypes *S.* Typhimurium and *S*. Eastbourne were further divided into two pulsotypes. Two pulsotypes (B and C) contained isolates from cattle and beef. Pulsotype A and D contained isolates only from beef and cattle, respectively. Pulsotype E contained an isolate from human and the four *S*. Saintpaul isolates originating from diarrheic patients were non-typable by PFGE.

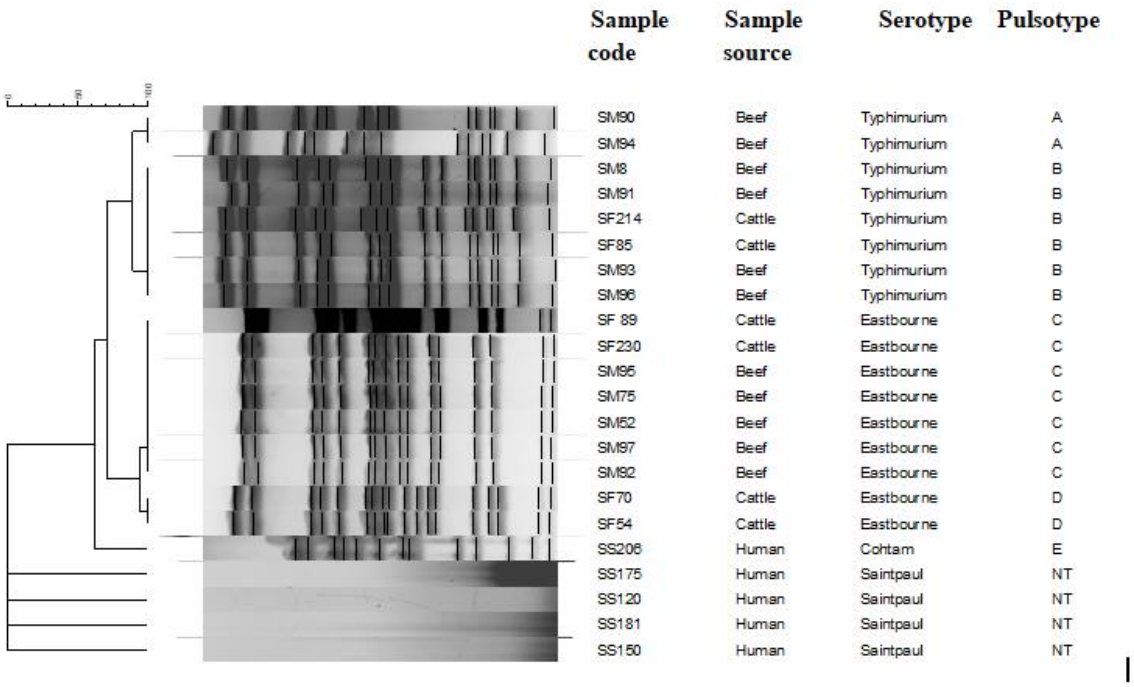
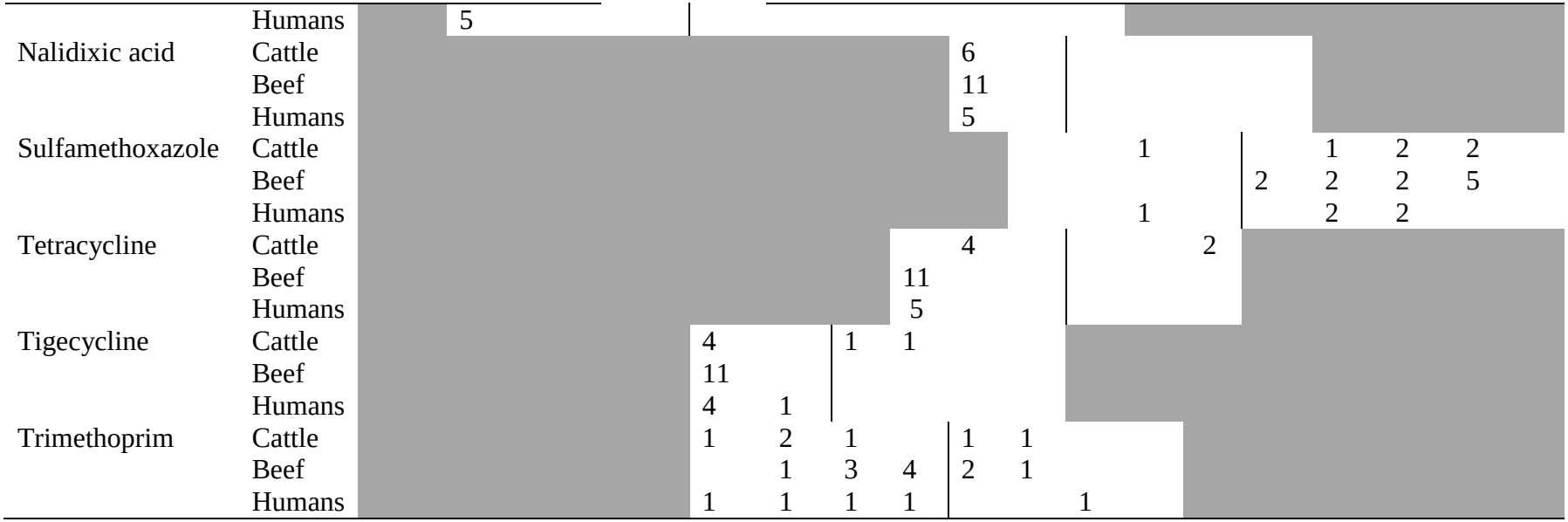


Figure 4.2. Pulsed-field gel electrophoresis patterns of *Salmonella* isolates from cattle, beef and humans in Bishoftu, Ethiopia.

Table 4.2 shows MIC distributions of the *Salmonella* isolates. Among the 22 isolates, 21 (95.5%) were resistant to at least one of the 14 antimicrobials tested. One *S.* Saintpaul isolate from a human sample was susceptible to all antimicrobials. Sulfamethoxazole (20/22, 90.9%) and trimethoprim (5/22, 22.7%) resistance were the most frequently observed in the *Salmonella* isolates regardless of sample source. Cattle isolates were also resistant to up to three other antimicrobials, namely ampicillin (3 isolates), tetracycline (2 isolates) and tigecycline (2 isolates). All *Salmonella* isolates were susceptible to the remaining 9 antimicrobials. Three of the 6 isolates from cattle were multi-drug resistant: one *S.* Typhimurium and two *S.* Eastbourne isolates were resistant to 4 and 3 antimicrobials, respectively (Table 4.3).

Table 4.2. Distribution of the minimum inhibitory concentrations (MICs) of *Salmonella* isolates obtained from cattle (n=6), beef (n=11) and humans (n=5) in Bishoftu town, Ethiopia.

Antimicrobial	Source	Number of isolates with minimal inhibitory concentrations (µg/ml)																
		0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
Ampicillin	Cattle							3						3				
	Beef							11										
	Humans							5										
Azithromycin	Cattle								2	4								
	Beef								7	4								
	Humans								3	1	1							
Cefotaxime	Cattle					6												
	Beef					10	1											
	Humans					5												
Ceftazidime	Cattle						6											
	Beef						11											
	Humans						5											
Chloramphenicol	Cattle										6							
	Beef										11							
	Humans										5							
Ciprofloxacin	Cattle	6																
	Beef	11																
	Humans	2	3															
Colistin	Cattle							6										
	Beef							11										
	Humans							5										
Gentamicin	Cattle						6											
	Beef						11											
	Humans						5											
Meropenem	Cattle		6															
	Beef		10	1														



The unshaded areas indicate the range of concentrations tested for each antimicrobial. The vertical bars indicate the epidemiological breakpoints for resistance by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019). The MICs values of *E. coli* were used for sulfamethoxazole, tigecycline and colistin. Numbers listed within the lowest tested concentration represent the number of isolates with MICs ≤ this concentration. Numbers listed within the highest tested concentration represent the number of isolates with MICs ≥ this concentration.

Table 4.3. Antimicrobial resistance patterns of *Salmonella* from cattle, beef and diarrheic patients in Bishoftu, Ethiopia.

Pattern	Resistance pattern	No. Antimicrobials	Origin	Number of resistant serotypes			
				S. Typhimurium	*S.* Eastbourne	*S.* Saintpaul	*S.* Cotham
1	AMP*SMX*TET*TGC	4	Cattle	1			
2	AMP*TET*TGC	3	Cattle		1		
3	AMP*SMX*TMP	3	Cattle		1		
4	SMX*TMP	2	Cattle	1			
			Beef	2	1		
			Humans			1	
5	SMX	1	Cattle		2		
			Beef	4	4		
			Humans			2	1

AMP-Ampicillin; SMX-Sulfamethoxazole; TMP-Trimethoprim; TET-Tetracycline; TGC- Tigecycline

4.4. Discussion

The 2.5% prevalence of *Salmonella* we observed in cattle at slaughterhouses is lower compared to the pooled prevalence estimate of 7.1% for cattle at slaughterhouses in Ethiopia with a variation from 2.1% to 16.2% (Tadesse and Tessema, 2014). At the global level, a recent study by Gutema et al (2019) indicated summary estimate of *Salmonella* prevalence in healthy cattle at 9%. Also, in that study a high variation in prevalence (from 0% up to 95%) was observed between studies, especially in those from countries located in North America and Africa.

The observed 8.7% prevalence of *Salmonella* in retail beef is comparable to the estimated prevalence of 10% with a range from 6% to 12% in beef in Ethiopia (Zelalem et al., 2019). Other recent studies reported *Salmonella* prevalence from 3.3% in Thailand (Prasertsee et al., 2019) up to 30% in Ghana (Ekli et al., 2019).

The 2.3% prevalence of *Salmonella* in diarrheic patients is comparable with the prevalence reported by another study from Ethiopia (Teshome et al., 2019) but much lower than the national estimated summary prevalence in children (8.7%) and adults (5.7%) with diarrhea (Tadesse, 2014). According to recent studies from other countries, prevalence of *Salmonella* between 0.4% in Guatemala (Arvelo et al., 2019) and 18.8% in Iraq (Kaabi and AL-Yassari, 2019) were reported in diarrheic patients. In the present study, *Salmonella* was isolated from adult patients. The five diarrheic patients who were positive for *Salmonella* reported raw beef consumption behavior and four of them had a history of raw beef consumption within 14 days prior to the onset of diarrhea.

In the present study, *Salmonella* was detected in only 22 samples of a total of 583 collected samples. From each positive sample, one isolate was further characterized. The *Salmonella* isolates belonged to 4 serotypes. Out of the four serotypes identified, *S*. Eastbourne *and S*. Typhimurium were isolated from both cattle and beef. Even within each serotype a common pulsotype was detected in isolates from cattle and beef. The observed genetic similarity within

each serotype of *S.* Eastbourne and *S.* Typhimurium isolated from cattle and beef suggests the possible transfer from cattle to beef from which humans can acquire infection.

Moreover, the prevalence of *Salmonella* on beef at retail was remarkably higher than in cattle. This demonstrates that a cross-contamination of carcasses during slaughter and transport to and in retail shops may occur. *S.* Saintpaul and *S.* Cotham were the two *Salmonella* serotypes isolated only from diarrheic patients. Based on these limited findings, a link between cattle and beef on one hand and human illness on the other hand could not be established.

Several studies from Ethiopia demonstrated that *Salmonella* serotypes causing diarrhea in humans were also present in cattle and beef as reviewed by Tadesse and Tessema (2014) and Zelalem et al (2019), respectively. More specifically, studies in the country reported *S.* Eastbourne and *S.* Typhimurium from cattle (Tadesse and Tessema, 2014), *S.* Typhimurium and *S.* Saintpaul from beef (Ketema et al., 2018; Tadesse and Gebremedhin, 2015) and *S.* Typhimurium and *S.* Saintpaul from diarrheic patients (Eguale et al., 2015). No citable information is available for *S.* Cotham from cattle, beef and humans in Ethiopia.

Eguale et al (2018) found that similar genotypes were present within different serotypes isolated from humans and cattle. Moreover, they showed that also same serotypes and genotypes were present in other food producing animals. Their findings indicate that different animal species can be the source for *Salmonella* infection in humans in Ethiopia.

Resistance to at least one antimicrobial substance was observed in 95.5% (21/22) of the *Salmonella* isolates with multidrug resistance in three isolates from cattle. High frequency of resistance to sulfamethoxazole (90.9%) was found, followed by trimethoprim (22.7%) and was present in isolates originating from cattle, beef and humans and in all 4 serotypes. Resistance to 3 other antimicrobials was found in only 3 isolates for cattle. In comparison to the present data, within the EU the resistance in *Salmonella* from animals, foods and humans to sulfamethoxazole was much lower (varying from 30% to 60%) while resistance to trimethoprim was rather similar (varying from 8% to 21%) (EFSA and ECDC, 2019b). Moreover, the latter report listed higher

resistance level for ampicillin and tetracycline and indicated that multidrug resistance differs considerably between EU countries.

Different studies were performed in Ethiopia testing the resistance to different antimicrobials of *Salmonella* isolated from humans and cattle and meat thereof. These studies were based on the disk diffusion method and clinical resistant values, making a relevant comparison with the present result difficult. Nevertheless, data from those studies testing the same antimicrobials and *Salmonella* serotypes indicate that the resistant level was in most cases higher than in the present study (Ketema et al., 2018).

The combination of sulfamethoxazole and trimethoprim, known as co-trimoxazole, is the most commonly prescribed drug (58.7%) for the treatment of acute diarrhea at the Bishoftu hospital, Ethiopia (Tulu et al., 2018). The resistance of the *Salmonella* isolates to co-trimoxazole was not tested in the present study so that no conclusion about the resistance of the isolates to this product can be directly made. However, based on data mentioned in the EFSA and ECDC report (2019), it can be hypothesized that most of the isolates resistant to trimethoprim may also be resistant to co-trimoxazole. This means that the four isolates originating from cattle (1), beef (2) and humans (1) were potentially resistant to co-trimoxazole.

The study has some limitations. One limitation of this study is the lack of linkage among the sample sources due to sampling of cattle at slaughterhouses and beef at retail shops and stool from patients with diarrhea at a hospital at different time periods. Secondly, rectal contents, beef and stool samples were collected in only one town within Ethiopia which may not represent the situation in the whole country. Lastly, due to limited number of *Salmonella* isolates identified in humans (only five isolates), inferences on the presence or absence of genetic relatedness between the isolates from cattle and beef and humans could not be made.

4.5. Conclusions

Our study showed a prevalence of *Salmonella* of 2.5% in cattle at slaughterhouses, 8.7% in beef, and 2.3% in diarrheic patients and genetic similarity between *Salmonella* isolates from cattle and beef. There was no correlation between cattle or beef isolates and human isolates suggesting other sources may be involved in human infections. It also revealed a high resistance to sulfamethoxazole and to a much lesser extent to trimethoprim, ampicillin, tigecycline and tetracycline. In this study, *S.* Typhimurium and *S.* Eastbourne were isolated from cattle and beef while *S.* Saintpaul and *S.* Cotham were isolated from diarrheic patients. The presence of *Salmonella* in cattle, the potential transfer of *Salmonella* from cattle to beef and the common habit to consume raw or undercooked beef in Ethiopia can be a risk for humans. Further robust studies are needed to establish the epidemiological link and to identify the sources of infection in humans.

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Chapter 5

5. Occurrence, Molecular Characteristics and Antimicrobial Resistance of *Escherichia coli* O157 in Cattle, Beef and Humans in Bishoftu, Central Ethiopia.

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5.1. Abstract

Shiga toxin-producing *E. coli* O157 causes disease in humans. Cattle are the primary reservoir of the pathogen. Information regarding the contribution of cattle to diarrheal illnesses in humans through consumption of contaminated beef is scarce in Ethiopia. We collected samples from 240 cattle, 127 beef and 216 diarrheic patients in Bishoftu town in Ethiopia to assess the occurrence and determine the virulence genes, genetic relatedness and antimicrobial resistance of *E. coli* O157. *E. coli* O157 was detected in 7.1% of the rectal content samples from cattle in slaughterhouses, in 6.3% of the beef samples, and in 2.8% of the diarrheic patients' stool samples. All isolates were positive for *eae* gene, 77% of them were positive for *stx*2 gene (21 *stx*2c and 3 *stx*2a), while *stx*1 gene was not detected. Molecular typing grouped the isolates into eight pulsed-field gel electrophoresis pulsotypes with three pulsotypes containing isolates from all three sources, one pulsotype containing one isolate from human origin and one isolate from beef. The remaining four pulsotypes contained isolates unique either to beef or humans. With the exception of one multidrug resistant isolate from beef, which was resistant to eight antimicrobial drugs, the remaining 30 isolates were susceptible to the 14 antimicrobials tested. In conclusion, the finding of genetically similar isolates in cattle, beef and humans may indicate a potential transmission of *E. coli* O157 from cattle to humans through beef. However, more robust studies are required to confirm this epidemiological link.

5.2. Introduction

Diarrheal disease is one of the leading causes of morbidity and mortality globally (Abubakar et al., 2015) accounting for an estimated 1.6 million deaths annually with most occurring in resource limited countries and in young children (Troeger et al., 2018). Over 90% of the diarrheal deaths occurred in sub-Saharan Africa and South Asian countries (Troeger et al., 2018; Vos et al., 2016). In Ethiopia, diarrhea is among the top five leading causes of mortality. In 2015, the death rate in the country due to diarrhea was estimated at 88.6 per 100,000 people (Misganaw et al., 2017).

E. coli belongs to the normal intestinal micro flora of warm-blooded animals and humans (Croxen et al., 2013). However, some *E. coli* strains can cause infection such as diarrheal diseases in humans (Nataro and Kaper, 1998). Diarrheagenic *E. coli* strains are divided into 7 pathotypes (Croxen et al., 2013). A subset of pathotype Shiga toxin-producing *E. coli* (STEC), called enterohemorrhagic *E. coli* (EHEC), are associated with bloody diarrhea, hemorrhagic colitis, and life-threatening conditions including hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in humans (Croxen et al., 2013). *E. coli* O157:H7 is the most widely known STEC serotype (Lim et al., 2010). It was estimated that STEC causes 2,801,000 acute illnesses, 3,890 cases of HUS and 230 deaths in humans annually across the world (Majowicz et al., 2014). Among those, a total of 10,200 acute illnesses of STEC infections occur in Africa with an incidence rate of 1.4 cases per 100,000 persons/year. *E. coli* O157 was estimated to contribute 10% to these cases in Africa (Majowicz et al., 2014).

Cattle are the primary reservoir of *E. coli* O157 (Gyles, 2007). They play an important role in the epidemiology of diarrheal illness in humans, serving as an important source of (Griffin and Tauxe, 1991). The most frequent mode of transmission of *E. coli* O157 to humans is the consumption of contaminated meat and meat products (Croxen et al., 2013). Beef is one of the most important food sources attributed to STEC infection (Pires *et al*., 2019). Studies about the occurrence of *E. coli* O157 in cattle (Abdissa et al., 2017; Atnafie et al., 2017; Haile et al., 2017) and in beef (Assefa, 2019) in Ethiopia are available.

With the exception of one study about *E. coli* in children with diarrhea (Adugna et al., 2015), information on the occurrence of *E. coli* O157 in diarrheic patients is lacking for Ethiopia. Moreover, there is no information concerning the genetic relatedness and antimicrobial resistance profile of *E. coli* O157 in cattle and beef and its potential association with human diarrheal illness. Therefore, the objective of this study was to investigate the occurrence and antimicrobial resistance of *E. coli* O157 in slaughter cattle, beef at retail shops and diarrheic patients in humans in Bishoftu town, Ethiopia. The genetic relatedness of the isolates from cattle, beef and human sources was compared to establish any potential transmission of *E. coli* O157 from cattle-to-beef-to-humans.

5.3. Materials and methods

5.3.1. Settings and sample collection

A cross-sectional study was conducted at two slaughterhouses (one municipal and one private), 127 retail shops and Bishoftu hospital in Bishoftu town located in East Shewa Zone of Oromia regional state of Ethiopia from June 2017 to May 2018. Both slaughterhouses were small in capacity where the municipal slaughterhouse and the private slaughterhouse usually slaughtered 5-15 and 15-30 cattle per day, respectively. Rectal content samples (at least 25g) were collected from the available number of cattle at the moment of sampling on 14 occasions at the municipal slaughterhouse (June to September, 2017) and on nine occasions at the private slaughterhouse (October, 2017 to January, 2018). A total of 240 rectal content samples, 120 from each slaughterhouse, were collected. The samples were collected directly from the rectum using rectal gloves in the lairage prior to slaughter. Meat samples were collected from all (n=127) retail shops in the city. On average 10 meat samples were collected per week. From each retail shop, one pooled sample of beef cuts (at least 25 g) from the exterior of the carcass (fat tissue) and surface of lean beef available at the time of collection were collected into a sterile polyethylene bag. Diarrheic patients of one year or older with a history of passing at least three loose or liquid stools per day visiting Bishoftu hospital were included in the study. Samples were collected from consecutive diarrheic patients identified during each visit at outpatient wards at the hospital.

From the stool samples submitted to the clinical laboratory of the hospital for routine testing from qualifying patients, a 1g stool sample was collected into 9 ml buffered peptone water (BPW) and stored at 4°C until transport. In total, 216 stool samples were collected. All collected samples were transported in an icebox to the laboratory and stored at $+4^{o}$C until processed within 24h.

5.3.2. Detection and characterization of *E. coli* O157

Twenty five gram of each rectal content and beef cut sample was transferred into a stomacher bag containing 225 ml of modified tryptone soya broth (Oxoid, Basingstoke, UK) supplemented with 20 mg/l novobiocin (Sigma Aldrich, MO; USA) (mTSBn), homogenized using a stomacher blender for 1 minute at normal speed (200 rpm) and incubated at 41.5 °C for 6 h. The enriched samples were subjected to immunomagnetic separation (IMS) using Dynabeads anti-*E. coli* O157 (Thermo Fisher Scientific, West Palm Beach, FL, USA) according to the manufacturers' instruction. The final washed bead-bacteria complexes were spread onto cefixime tellurite sorbitol MacConkey agar (Oxoid) containing 0.05 mg/l cefixime and 2.5 mg /l potassium tellurite (Oxoid) (CT-SMAC). For stool samples, after incubating the BPW media containing the sample at 37 °C for 24 h, a loopful was streaked plated onto CT-SMAC. After incubating all CT-SMAC plates at 37 °C for 24 h, the agar plates were examined for the presence of suspect colonies.

From each selective agar plate up to three suspect colonies were subjected to indole, Kligler Iron agar and *E. coli* O157 latex agglutination (Oxoid) tests. From each positive sample, one isolate was further tested for the presence of the gene defining the somatic antigen O157 (Wang et al., 2002) and virulence genes coding for Shiga-toxins (*stx*1 and *stx*2) and intimin (*eae*) by the multiplex PCR protocol described by Botteldoorn et al (2003). The isolates positive for the gene *stx2* were further subtyped using the PCR method described by Scheutz et al (2012). Moreover, all the *E. coli* O157 isolates were genotyped by Pulsed Field Gel Electrophoresis (PFGE) after digestion with *Xba*I enzyme according to the standardized PulseNet international protocol (CDC, 2017). The obtained fingerprints were grouped according to their similarity with Bionumerics 7.6

software (Applied Maths, Biomérieux, Sint-Martens-Latem, Belgium) using the Pearson coefficient and Unweighted-Pair Group Method using Arithmetic averages (UPGMA) with an optimization of 2%. Pulsotypes were assigned based on their polymorphisms, namely the difference in the presence of at least one band in the fingerprint (Cobbaut et al., 2009).

5.3.3. Antimicrobial susceptibility testing

The antimicrobial resistance of the isolates was evaluated by determining the minimum inhibitory concentration (MIC) using EUVSEC Sensititre plates (Thermo Fisher Scientific). The tests were performed according to the manufacturer's instructions. The following 14 antimicrobial agents were evaluated: ampicillin, azithromycin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, meropenem, nalidixic acid, sulfamethoxazole, tetracycline, tigecycline and trimethoprim. The standard reference strain *E. coli* ATCC 25922 was used as quality control. European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological breakpoint values were used to categorize the isolates as resistant or susceptible (EUCAST, 2019).

5.3.4. Data management and statistical analysis

Data were entered into Microsoft Excel spread sheets (Microsoft Corp., Redmond, Washington, USA), imported to and analysed using STATA version 15.1 (STATA corp., College Station, TX, USA). The occurrence of *E. coli* O157 was derived as the percentage of culture positive samples from the total samples tested from each source. The difference in the occurrence of *E. coli* O157 between the two slaughterhouses was tested using Fischer's exact test. Antimicrobial resistance and molecular profiles of *E. coli* O157 isolates were expressed using frequency and percentage.

5.3.5. Ethics statement

Ethical clearance was obtained from Addis Ababa University VM/ERC/06/05/09/2017), Ministry of Science and Technology of Ethiopia (3/10/006/2018) and University Hospital Gent, Belgium (2017/0612). During sample collection, all diarrheic patients were informed about the purpose of

the study and samples were collected after obtaining written consent, for minors assent was requested from the children and written consent was obtained from their parents or guardians.

5.4. Results

E. coli O157 was detected in 17 (7.1%) out of the 240 rectal content samples from cattle. *E. coli* O157 occurrence was significantly higher (Fischer's exact $P < 0.001$) in cattle sampled at the municipal slaughterhouse (13.3%, 16/120) than in cattle at the private slaughterhouse (0.8%, 1/120). Eight (6.3%) of the 127 beef cut samples collected at retail shops and six (2.8%) of the diarrheic patients were positive for *E. coli* O157. Over a half (57.0%, 123/216) of the diarrheic patients were males. The mean age of the diarrheic patients was 27.5 years (range: 1 to 82 years). Of the *E. coli* O157 positive patients, four had watery diarrhea while the other two had mixed (mucoid and bloody) diarrhea. All positive patients were males; in the group of $\leq$ 5 years (n=22) there was one positive child of 4 years old; in the group of 5-64 years (n=188) there were four people testing positive aged 26, 29, 35 and 52 years; and in the group of $\geq$ 65 years old (n=6) there was one person of 78 years old. All *E. coli* O157 positive adult diarrheic patients had a history of raw beef consumption and three of them had consumed raw beef within 14 days prior to diarrheal onset.

All 31 *E. coli* O157 isolates were tested for the presence of virulence genes, antimicrobial susceptibility and genotyping by PFGE. The *eae* gene was detected in all isolates, and the *stx*2 gene in 24 (77%) isolates (cattle=14, beef=6 and humans=4), while seven isolates (3 from cattle and 2 from each beef and humans) were negative for the *stx*2 gene. The *stx*1 gene was not detected in any of the isolates.

Among the 24 *stx*2 positive isolates, 21 were positive for the subtype *stx*2c and the other three were positive for the subtype *stx*2a (2 from beef and 1 from humans). Based on the PFGE genotyping results, the 31 *E. coli* O157 isolates were grouped into 8 pulsotypes (A-H) (Figure 5.1). Three pulsotypes (D, E, F) contained isolates from the three sources (cattle, beef and humans), pulsotype A contained one isolate from 2 sources (human and beef) and the remaining

four pulsotype groups (B, C, G and H) representing isolate(s) only from one source. One third (32%) of the isolates shared the predominant pulsotype F and originated from cattle (eight isolates), beef (one isolate) and human (one isolate). The second common (23% of all isolates) pulsotype D was shared among cattle (three isolates), beef (two isolates) and humans (two isolates). All the 7 *stx*2 gene negative isolates were grouped into the pulsotype D. Of the 31 *E. coli* O157 isolates, only one isolate from beef showed resistance, exhibiting multidrug resistance being resistant to eight antimicrobials representing five antimicrobial classes: ampicillin, azithromycin, cefotaxime, ceftazidime, colistin, sulfamethoxazole, tetracycline and tigecycline. This isolate belonged to pulsotype D and carried only the *eae* gene.

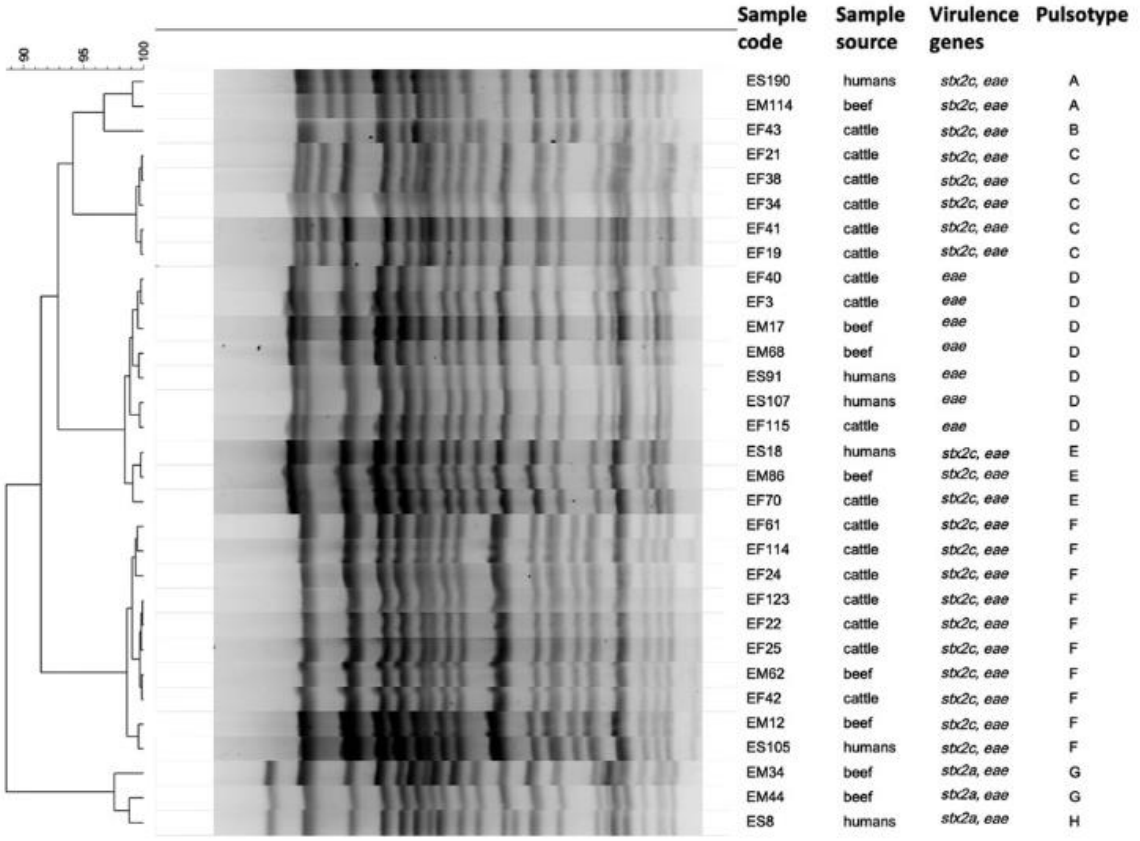

Figure 5.1. Pulsed-field gel electrophoresis patterns and virulence genes of *E. coli* O157 isolates from cattle, beef and humans in Ethiopia.

5.5. Discussion

In the present study, the occurrence of *E. coli* O157 in cattle was 7.1%. *E. coli* O157 occurrence was significantly higher in cattle sampled at the municipal slaughterhouse (13.3%) than in cattle at the private slaughterhouse (0.8%). The season of sampling, origin of cattle and transportation of cattle from origin to the market and then to the slaughterhouse might have contributed to this difference (Barham et al., 2002; Hussein and Bollinger, 2005). The overall occurrence was comparable with the global prevalence estimate of 5.7% that ranges from 0.1% to 61.8% (Islam et al., 2014). The difference in the occurrence of *E. coli* O157 in cattle could be attributed to several factors like seasonal variation, age, type of cattle, diet and differences in detection methods (Meyer-Broseta et al., 2001).

The *E. coli* O157 occurrence of 6.3% in beef was comparable with the national prevalence estimate (6%) in Ethiopia (Zelalem et al., 2019).This percentage was higher compared to studies in other countries which reported 2.2% in Nigeria (Tafida et al., 2014), 0.3% in European Union (EFSA and ECDC, 2013), 0.8% in USA (Hill et al., 2011), 1.7% in Australia (Kiermeier et al., 2011). The difference in the occurrence of *E. coli* O157 in beef can be due to differences in the hygienic handling practices during slaughter, transport of beef and handling and storage of beef at retail shops (Callaway et al., 2009). Beef contaminated with *E. coli* O157 can potentially lead to diarrheal illness in humans (Heredia and García, 2018), particularly in countries where consumption of raw or undercooked beef is common like in Ethiopia (Avery, 2014).

E. coli O157 was detected in the stool from 2.8% of patients with diarrhea. Previous studies reported lower prevalence of *E. coli* O157 from patients with diarrhea like 0.5% in Spain (Blanco et al., 2004), 0.5% in Tunisia (Al-Gallas et al., 2006) and 1.2% in Bangladesh (Islam et al., 2007). Although the number of patients of $\leq$ 5 years (n=22) and $\geq$ 65 years (n=6) was low, one *E. coli* O157 was isolated from these age categories. These age groups are at higher risk of experiencing illness caused by *E. coli* O157 and may have more-serious complications from the infection (Smith, 1998; Vally et al., 2012).

All 31 isolates were positive for eae gene, while *stx*2 gene was detected in 24 isolates. The predominant occurrence of *stx*2 genes as the most virulence factor and predictor of infection in humans were reported by previous studies (Bai et al., 2018; Chapman et al., 2001; Kawano et al., 2008). Of the *stx*2 positive isolates, 13% harbored the *stx*2a gene, while 87% carried the *stx*2c gene. The *stx*2a gene has formerly been identified as an independent risk factor for the development of HUS (Brandal et al., 2015; Dallman et al., 2015; De Rauw et al., 2019; Naseer et al., 2017).

According to the classification proposed by De Rauw et al (2019), STEC isolates carrying the *stx*2a gene and the *stx*2c gene have a high and a medium risk for HUS development in patients, respectively. This suggests that patients positive for *E. coli* O157 carrying such *stx*2 genes, may be more at risk to develop HUS. However, no data about HUS cases in Ethiopia are available up to now. Future study on the ability to produce stx2 and the presence of defective *stx* phages would elucidate the role of *E. coli* O157 in HUS infection in the country (Rahman et al., 2018). Among the isolates, 7 were negative for *stx* genes. It was hypothesized that the absence of such virulence genes may be due to the spontaneous loss of *stx* genes during multiple sub-culturing of isolates (Joris et al., 2011; Vaishnavi et al., 2010) or the occurrence of inherently *stx*-negative isolates (Cobbaut et al., 2009; Trabulsi et al., 2002). *E. coli* O157 strains missing stx genes clustered phylogenetically with *E. coli* O157 carrying *stx* gene (Ferdous, et al., 2015) and were considered to be atypical enteropathogenic *E. coli* (aEPEC) (Hu and Torres, 2015; Trabulsi et al., 2002).

Within the isolates from cattle, 5 pulsotypes were detected of which pulsotypes C and F were dominant in cattle, suggesting that certain pulsotypes may be widely spread in cattle reared in Ethiopia. This is in contrast to other studies showing that no dominant genetic types of *E. coli* O157 were present in the cattle population (Cobbaut et al., 2009). The presence of cattle and/or beef and human isolates within the same pulsotype (A, D, E and F) suggests the occurrence of circulating isolates and a possible epidemiological link between *E. coli* O157 in cattle/beef and human infections. Indeed, carcass contamination can occur at the abattoir during the slaughter

process (Elder et al., 2000) as well as cross contamination at the retail shops, especially when no hygienic handling practices are applied, leading to human infection when beef is not well cooked (Maruzumi et al., 2005). Given the significance of few cells of *E. coli* O157 (Caprioli et al., 2005), the carriage of this pathogen by cattle is critically important due to the substandard hygienic practices at the slaughter houses in Ethiopia (Eshetie et al., 2018) and the risk of carcass contamination during slaughter (Callaway et al., 2009).

Our findings indicated that only one isolate from beef showed resistance (and even multidrug resistance) to the tested antimicrobials. This result is in contrast with published data, indicating that *E. coli* O157 showed in general a variable resistance against limited antimicrobials such as sulfonamides, streptomycin, tetracycline and ampicillin (Mir and Kudva, 2019).

The study has some limitations. Firstly, the study lack directionality since we used a cross-sectional study design for cattle in slaughterhouses, potentially originated from different parts of the country, for the beef sampling at retail shops and for the patients with diarrhea at the hospital. Secondly, fecal, beef and stool samples were collected in only one city within Ethiopia which may not represent the situation in the whole country. Lastly, due to limited number of *E. coli* O157 isolates recovered from diarrheic patients (only six isolates), the possible risk factors and their causal relationship of cattle isolates with human diarrhea could not be assessed.

5.6. Conclusions

E. coli O157 was observed in 7.1%, 6.3%, and 2.8% in rectal content from cattle, beef at retail shops, and human stools from diarrheic patients, respectively. Genetic similarities were observed for a number of *E. coli* O157 isolates detected in cattle, beef and humans, suggesting a potential role of cattle in the development of diarrheal illnesses due to *E. coli* O157 in humans. In Ethiopia consumption of raw beef in the form of steak ("*kurt*") dipped in plant-based spices or beef tartare ("*kitfo*") made from raw minced beef are very common (Avery, 2004; Seleshe *et al*., 2014). Consumption of these raw beef products may be an important source for *E. coli* O157 infections in the country.

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Chapter 6

6. Assessment of Hygienic Practices in Beef cattle Slaughterhouses and Retail Shops in Bishoftu, Ethiopia: Implication for Public Health.

Based on: Gutema, F.D., Agga, G.E., Abdi, R.D., Alemnesh, J. Duchateau, L. De Zutter, L. Gabriël, S. Assessment of Hygienic Practices in Beef Slaughterhouses and Retail Shops in Bishoftu, Ethiopia: Implication for Public Health. International Journal of Environmental Health and Public Health, 2021, 18, 1-13. https://doi.org/10.3390/ijerph18052729.

6.1. Abstract

Understanding the potential drivers of microbial meat contamination along the entire meat supply chain is needed to identify targets for interventions to reduce the number of meatborne bacterial outbreaks. We assessed the hygienic practices in cattle slaughterhouses (28 employees) and retail shops (127 employees) through face-to-face interviews and direct personal observations. At the slaughterhouses, stunning, de-hiding and evisceration in vertical position, carcass washing and separate storage of offals were the identified good practices. Lack of hot water baths, absence of a chilling room, infrequent hand washing, insufficiently trained staff and irregular medical check-up were practices that lead to unhygienic handling of carcasses. At the retail shops, cleaning equipment using soap and hot water (81%), storing unsold meat in refrigerator (92%), concrete floors and white painted walls and ceilings were the good practices. Adjacently displaying offal and meat (39%), lack of cold chain, wrapping meat with plastic bags and newspapers, using plastic or wooden cutting board (57%), infrequent washing of equipment and floors, and inadequately trained employees were practices that could result in unhygienic handling of beef. Our study identified unhygienic practices both at the slaughterhouses and retail shops that can predispose the public to meatborne infections, which could be improved through training and implementation of quality control systems.

6.2. Introduction

The global increase in human population is associated with an increased demand for foods of animal origin (Thornton, 2010). Consequently, ensuring security, quality, and safety of food is a worldwide concern (OIE, 2018). It is particularly a significant problem in developing countries as animals and products thereof are often produced under sub-optimal hygienic conditions (Bello et al., 2015; Grace, 2015).

Most of the meatborne bacterial outbreaks are usually attributed to contamination along the supply chain due to poor handling practices (Chepkemoi et al., 2015). Food producing animals are the major sources of many foodborne pathogens and can lead to meat contamination, which may result in a widespread occurrence of foodborne diarrheal illnesses in humans (Heredia and García, 2018; Uche et al., 2017). Cattle slaughterhouses are one of the critical units in the supply chain from which foodborne pathogens can disseminate along the processing and distribution continuum including retail shops subsequently reaching the consumers. As a result, good hygienic practices at slaughterhouses and during distribution to and storage at retail shops and during sales are key points in ensuring the quality and safety of meat to safeguard public health (Lues and Van Tonder, 2007; Rani et al., 2017). Inadequate facilities and improper handling of the animals at the slaughterhouses further aggravate the microbial contamination of beef which can result in the transmission of foodborne pathogens to humans (Cook et al., 2017; Komba et al., 2012).

Meat hygiene and safety is usually less controlled in many developing countries where meat for human consumption is approved based on visual inspection, if at all, without routine microbiological testing (Cook et al., 2017). Several studies investigated the occurrence of pathogens along the entire beef supply chain (Abayneh et al., 2014; Islam et al., 2014; Silva et al., 2011; Tadesse and Gebremedhin, 2015; Tadesse and Tessema, 2014), while others identified contamination at specific levels such as at slaughterhouses (Bakhtiary et al., 2016; Bogere and Baluka, 2014; Kariuki et al., 2013; Niyonzima et al., 2013) and in retail shops (Bogere and Baluka, 2014; Gormley et al., 2010; Yang et al., 2010) in different countries including Ethiopia.

Contamination and cross-contamination from raw meat is a major cause of foodborne diseases particularly in developing countries (Ansari-Lari et al., 2010; Adesokan et al., 2014). According to world health organization estimation, FBD resulted in 600 million cases and 420,000 deaths resulting in nearly 33 million disability adjusted life years globally with the highest mortality burden in Africa in 2010 (Havelaar et al.,2010). Foods of animal origin such as beef are major contributors to the burden. The global burden of foodborne diseases due to all animal source foods and beef was estimated at 168 and 10 Disability Adjusted Life Years per 100,000 population, respectively (Li et al., 2010). However, information on the burden of foodborne diseases due to poor meat handling practices is limited. Improving hygienic handling practices by meat handlers during meat production, distribution, storage and sales at retail shops prevent or reduce microbial contamination (Lues and Van Tonder, 2007). It is very evident that food safety problems require intervention measures along the entire beef supply chain. To identify specific targets for intervention in specified settings, a clear understanding of local drivers for microbial meat contamination along the meat production, processing, and distribution chain is needed.

In Ethiopia, there are over 300 local slaughterhouses that supply meat for local consumption with different capacities and facilities, however all with low basic hygienic standards (Eshetie et al., 2018). Although foodborne bacteria have been reported from cattle at slaughterhouses and beef in the retail shops as reviewed by Abayneh et al., 2014, little information is available concerning beef hygienic handling practices along the beef production and distribution continuum in Ethiopia. Therefore, the objective of this study was to assess beef hygienic handling practices at cattle slaughterhouses and retail shops to contribute to the identification of intervention targets.

6.3. Materials and methods

6.3.1. Study settings

This study was conducted from June 2017 to May 2018 at the two local cattle slaughterhouses (one municipal and one privately-owned) found in Bishoftu, and all 127 retail shops selling beef in Bishoftu town. The town is located in East Shoa Zone of Oromia region, Ethiopia. According

to the 2007 Ethiopian census report (CSA, 2007), the total human population of Bishoftu town was estimated at 100,114. The slaughterhouses slaughtered cattle brought directly from open markets by retail shop owners. Both slaughterhouses were small in capacity where the municipal slaughterhouse and the private slaughterhouse usually slaughtered 5-15 and 15-30 cattle per day, respectively. The retail shop owners buy cattle from markets and bring them to the slaughterhouse for slaughter service. The retail shops store meat in open display rooms with no cooling for sale to the local consumers.

6.3.2. Study design and data collection

Data were collected through face-to-face interviews and direct personal observation using pre-tested semi-structured questionnaires and check lists to assess the beef hygienic handling practices at slaughterhouses and beef retail shops (supplementary file). The questionnaires and check-list were adapted from similar previous studies in Ethiopia (Haileselassie et al., 2013; Tegegne et al., 2017) and structured into i) sociodemographic characteristics of the respondents, ii) check list for direct observations and, iii) questions for face-to-face interviews. The questionnaires were first prepared in English and then translated into Afaan Oromo and Amharic, the commonly spoken local languages in the study area. Data were collected by three trained data collectors. All employees in the two slaughterhouses (municipal =16 and private =12) and one employee from each of the retail shops (n=127) engaged in beef handling activities were included in the survey. The purpose of the study was explained to the study participants and data were collected after obtaining full written consent from the participants. At the end of each interview, completeness and accuracy of the data were checked and ensured by the principal investigator. Ethical clearance was obtained from College of Veterinary Medicine and Agriculture of Addis Ababa University, VM/ERC/06/05/09/2017), Ministry of Science and Technology of Ethiopia (Ref no.3/10/006/2018) and the University Hospital Gent, Belgium (Ref no. 2017/0612).

6.3.3. Data management and analysis

The collected data were entered to Microsoft Excel spread sheet (Microsoft Corp., Redmond, Washington, USA) and analysed using STATA version 15.1 (STATA corp. College Station, TX, USA). Descriptive statistics such as frequency and percentage are used to summarize the data. Fisher's exact test was used to assess the difference in the sociodemographic characteristics and hygienic handling practices of the employees between the municipal and private slaughterhouses. A *p*-value of less than 0.05 was set as a significance level. The hygienic handling practices at the beef retail shops were described descriptively.

6.4. Results

6.4.1. Hygienic practices at cattle slaughterhouses

6.4.1.1. Sociodemographic characteristics

Table 6.1 summarizes the sociodemographic characteristics of the employees at the municipal (n=16) and private (n=12) slaughterhouses. The private and the municipal slaughterhouses did not significantly differ based on the sex, age, level of education and main duty of their employees (Fisher's exact test $P > 0.05$). However, there was a significant difference between the slaughterhouses with respect to years of experience of the employees (Fisher's exact test $P < 0.05$). Employees at the municipal slaughterhouse had more years of work experience (mean = 9.8 years, standard deviation [SD = 5.2]) than those working in the private one (mean = 2.4 years, (SD =1.4)). The combined mean age of the employees from the two slaughterhouses was 32.3 years (SD = 8.1) ranging from 19-50 years.

Table 6.1. Sociodemographic characteristics of the slaughterhouses' employees in Bishoftu town, Ethiopia.

Variables		Number (%) of respondents (n=28)
Sex	Male	25 (89.3)
	Female	3 (10.7)
Age	15-24	4 (14.3)
	25-54	24 (85.7)
Educational status	Informal	2 (7.1)
	Primary	12 (42.9)
	Secondary	10 (35.7)
	Higher education	4 (14.3)
Service duration in years	1-5	16 (57.1)
	>5	12 (42.9)
Main duty at the slaughterhouse	Stunning and bleeding	2 (7.0)
	De-hiding	18 (65.0)
	Evisceration	6 (21.0)
	Meat inspector	2 (7.0)

Figure 6.1 summarizes the identified beef processing and handling practices in the two slaughterhouses and the beef retail shops evaluated in the study.

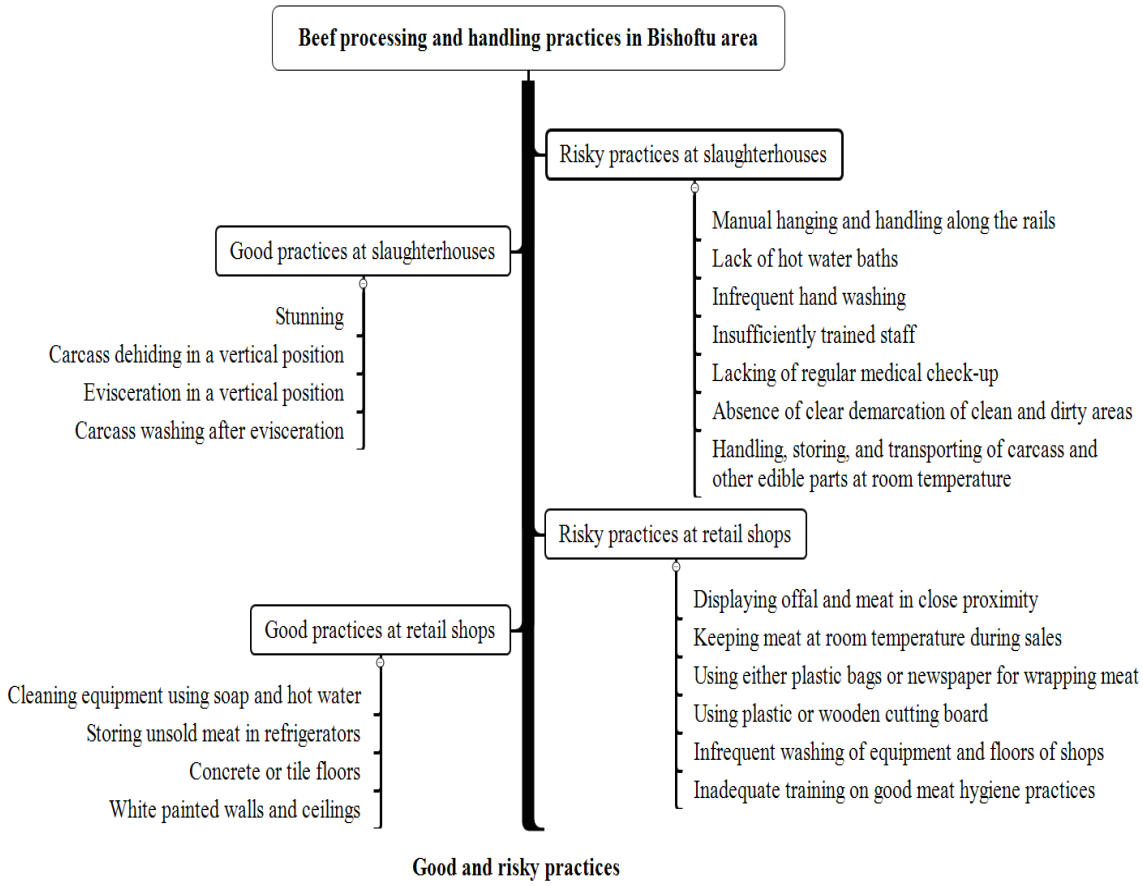

Figure 6.1.1. Beef processing and handling practices in the studied slaughterhouses and retail shops in Bishoftu town, Ethiopia.

6.4.1.2. Slaughter process

Both slaughterhouses had their own veterinarian who was in charge of the supervision of slaughter process and meat inspection. Overall, the slaughter steps are similar at both slaughterhouses (Table 6.2). The slaughtering started with the stunning of the animals by stabbing at the atlanto-occipital region using a sharp edge of knife, immediately followed by bleeding in a horizontal position on the floor. Following removal of head and feet, the remaining slaughter steps are performed in vertical position after manually hanging the carcass fitted with

hooks and sliding it over the rail system. Finally, the carcasses are stored and transported at room temperature. A description of the steps of the slaughter process at the small slaughterhouses serving the local community is indicated in Table 6.2.

Table 6.2. Description of the slaughter process steps at the two small slaughterhouses supplying beef to local consumers in Bishoftu town, Ethiopia

Processing step	**Description**
Stunning	Stabbing at the atlanto-occipital region with a sharp edge of knife
Bleeding	Cutting arteries and veins in the throat region in a horizontal position on the floor using a knife
Removal of head and feet	Performed in horizontal position on the floor
Hanging of carcass	Manually de-hiding the upper part of both hind legs and hanging of the carcasses on slide rail conveyor by hooks through the hind legs
De-hiding	Removal of the hide manually starting for the hind leg down to the forelegs
Evisceration	Removal of the visceral organs
Carcass washing	Washing the carcass manually using municipal tap water
Post-mortem inspection	Inspection of the carcass and organs for any pathological conditions
Carcass labelling	Applying the identification number using food grade coloring agent
Storage	Storing the carcass and other edible parts at room temperature for 1-6 hours until transport
Transport to retail shops	Transport and distribution to retail shops using closed vehicle devoid of a cooling facility

6.4.1.3. Beef handling practices

Both slaughterhouses reported the use of water from the municipal city supply. Hand washing was not a frequent practice during slaughter operations according to 53.6% of the respondents (Table 6.3, Figure 6.1). There was no significant difference between the municipal and private slaughterhouse based on hand washing practice, perceived sources of carcass contamination, training on meat hygiene and frequency of medical check-up of the employees (Fisher's exact test $P > 0.05$). The use of aprons, white coats, boots and hair covering, as well as the presence of sinks for hand washing were good practices observed at both slaughterhouses. However, none of the employees wore hand gloves during operations. We also observed lack of hot water for hand washing and dipping of knives.

Table 6.3. Beef handling practices at slaughterhouses in Bishoftu town, Ethiopia.

Variables		**Number (%) of respondents (n=28)**
Hand washing between activities during work	Yes	13 (46.4)
	No	15 (53.6)
Perceived major sources of carcass contamination	Fecesduring evisceration	10 (36.0)
	Hides	10 (36.0)
	Handler's hand	2 (7.0)
	Knife	6 (21.0)
Received on the job training on meat hygiene practices	Yes	17 (60.7)
	No	11 (39.3)
Frequency of medical checkup	Every three months	14 (50.0)
	Every six months	14 (50.0)

6.4.2. Beef handling practices in retail shops

6.4.2.1. Sociodemographic characteristics

The sociodemographic characteristics of the study participants from the retail shops are indicated in Table 6.4. All respondents (n=127) were males with a mean age of 25.3 years (SD=5.9) ranging from 18 to 56 years. Most (70.1%) respondents at retail shops attended only up to primary school and 85.8% of them did not receive training on the best practices of handling meat.

Table 6.4. Sociodemographic characteristics of employees at retail shops in Bishoftu town, Ethiopia.

Variables		Number (%) of respondents (n=127)
Age	18-24	65 (51.2)
	25-56	62 (48.8)
Education level	Informal	9 (7.1)
	Primary	89 (70.1)
	Secondary	29 (22.8)
Ethnicity	Gurage	52 (40.9)
	Hadiya	28 (22.0)
	Oromo	21 (16.5)
	Amhara	18 (14.2)
	Tigire	8 (6.3)
Religion	Orthodox	82 (64.6)
	Protestant	45 (35.4)
Experiences in years	< 5	88 (69.3)
	> 5	39 (30.7)

6.4.2.2. Beef handling practices

According to the respondents, carcasses are transported from the slaughterhouses to the retail shops using closed vehicles without a cooling facility. The municipal water supply was the source of water for all retail shops. Of the retail shops, 39.4% displayed offal (heart, kidneys, liver, and stomach) and meat next to each other on the same display cabinet, 4.7% used the same knife for cutting offal and meat. Among the respondents, 85.0% of them used the same coat for the entire day; 9.0% did not wash hands before touching meat; 11.8% did not use soap for hand washing, and 2.4% collected money while handling meat. Ninety-two percent had a refrigerator (1-5^{o}C) for overnight storage of leftover meat (Table 6.5).

Table 6.5. Respondent's response on beef handling practices at retail shops in Bishoftu town, Ethiopia.

Variables		Number (%) of respondents (n=127)
Use of a clean white coat	Two per day	17 (13.4)
	One per day	108 (85.0)
	One every two days	2 (1.6)
Washing hands before touching meat	Yes	115 (90.6)
	No	12 (9.4)
Using of soap for hand washing	Yes	112 (88.2)
	No	15 (11.8)
Received training	Yes	18 (14.2)
	No	109 (85.8)
Medical checkup	Yes	125 (98.4)
	No	2 (1.6)
Frequency of medical checkups	Every three months	91 (71.6)
	Every six months	27 (21.3)
	Once per year	9 (7.1)
Fly control methods	Horsetail fly swatter	86 (68.0)
	Roach killer	4 (3.1)
	Fumigation	4 (3.1)
	Fumigation and roach killer	3 (2.4)
	Horsetail fly swatter and fumigation	3 (2.4)
	No control	27 (21.0)
Maximum duration of meat storage before sale	Two days	15 (11.8)
	One day	93 (73.2)
	12 hours	19 (15.0)
Having refrigerator for storage	Yes	117 (92.1)
	No	10 (7.9)
Money collection from buyers by person handling the meat	Yes	3 (2.4)
	No	124 (97.6)
Storage of offal and meat on the same display cabinet	Yes	50 (39.4)
	No	77 (60.6)
Use of the same knife for offal and meat	Yes	6 (4.7)
	No	121 (95.3)
Is there a need for quality improvement?	Yes	3 (2.4)
	No	124 (97.6)
Complaint from consumers about the quality of meat	Yes	10 (7.9)
	No	117 (92.1)

A variable frequency of washing equipment, display cabinet, and floor was reported. In most of the retail shops (>70%) equipment, floors and the display cabinet were cleaned once per day. The majority (81.1%) of retail shops reported cleaning their equipment with soap and hot water, chemical disinfection was not done in any of the retail shops (Table 6.6).

Table 6.6. Equipment and floor washing practices at beef retail shops in Bishoftu town, Ethiopia.

Variables		Number (%) of respondents (n=127)
Frequency of washing equipment and floor		
Knife	More than twice per day	9 (7.1)
	Twice per day	9 (7.1)
	Once per day	109 (85.8)
Cutting board	More than twice per day	2 (1.6)
	Twice per day	12 (9.4)
	Once per day	113 (89.0)
Saw/axes	Twice per day	4 (3.1)
	Once per day	104 (81.9)
	Once in every two days	19 (15.0)
Display cabinet	Once per day	93 (73.2)
	Every two days	34 (26.8)
Hooks	Once per day	102 (80.3)
	Every two days	25 (19.7)
Floor	Once per day	89 (70.1)
	Every Two days	38 (29.9)
Use of soap and hot water to clean equipment		
	Yes	103 (81.1)
	No	24 (18.9)

All respondents wore a white coat, but none of them put on gloves. In all retail shops, there were light bulbs, either concrete or tile floors and white painted walls and ceilings. However, in all shops meat was displayed at room temperature, with no covering, being exposed to dust particles and domestic flies. All shops use either plastic bags or newspapers for wrapping the meat (Figure 6.1). Among the retail shops, 85% had no hand wash sink at the display room. Standby hot water baths were not available for dipping knives. Unclean retail shop ceilings and white walls with

observable dirty spots were noticed in about 79% of the shops. Table 6.7 summarizes the observational assessments on the hygienic status of the beef retail shops.

Table 6.7. Summary of the observational assessment of outcome of the hygienic status of the beef retail shops in Bishoftu town, Ethiopia.

Variables		Number (%) of retailshops (n=127)
Floor-type	Tile	37 (29.1)
	Concrete	90 (70.9)
Clean wall and ceiling	Yes	27 (21.3)
	No	100 (78.7)
Presence of a sink for hand washing at the display	Yes	19 (15.0)
	No	108 (85.0)
Type of cutting board	Wood	33 (26.0)
	Marble	42 (33.1)
	Plastic	40 (31.5)
	Marble and plastic	8 (6.3)
	Marble and wood	4 (3.1)
Materials used for meat wrapping	Plastic bags	100 (79.0)
	News paper	7 (5.0)
	Plastic bags and news paper	20 (16.0)
Use of a head cover	Yes	51 (40.2)
	No	76 (59.8)

6.5. Discussion

Proper meat handling practices play a significant role in ensuring meat quality and safety (Rani et al., 2017). Knowledge on meat hygienic handling practices during beef production, processing and distribution is essential to formulate preventive measures to mitigate the contribution of meat to FBD (Havelaar et al., 2018). We investigated the status of beef hygienic handling practices in cattle slaughterhouses and retail shops in Bishoftu town, Ethiopia. Our study revealed both good and unhygienic handling practices at the slaughterhouses and retail shops. The discussion below focuses on the main meat handling practices identified with their potential implication for public health. Moreover, the practices are evaluated in view of the requirements of the Ethiopian

proclamations: Meat inspection proclamation (No. 274/1970) (EFNG, 1970), Public health Proclamation (No. 200/2000) (EFNG, 2000) and Food, Medicine and Health Care Administration and Control Proclamation (No. 661/2009) (EFNG, 2010) and the Codex Alimentarius commission (CAC) on general principles of food hygiene (CAC, 2003) and code of hygienic practice for meat (CAC, 2005) that have been formulated to ensure the production and marketing of sound, wholesome and quality meat and meat products for consumer's protection. Ethiopia is a member of codex Alimentarius commission and the Codex standards are the basic reference materials for standard settings and serve as enforcing tools for food safety where there are no developed Ethiopian standards (FAO/WHO,2005; Temesgen et al., 2015).

In the present study, lack of hot water baths for hand washing and dipping of knives, infrequent hand washing, insufficiently trained operational employees, lack of regular medical check-up and lack of cooling facilities (for storage in slaughterhouses and display in retail shops) were bad practices identified both at the slaughterhouses and retail shops. Hot water, which is essential for hand and knife washing to remove the potential surface contaminants and to prevent further cross contamination of meat, was lacking at washing basins of both at slaughterhouses and retail shops (Van Zyl, 1995). Even though Ethiopia is a member of CAC, the present finding indicated lack of adherence to the requirements of CAC that demands the presence of an adequate and easily accessible supply of hot and cold potable water at all times during handling meat for effective sanitizing of equipment and hand washing (CAC, 2005).

According to the 53.6% of the respondents at slaughterhouses hand washing was not a frequent practice during slaughter operations, and few (9.4%) employees at retail shops did not wash their hands before touching meat. This practice is not consistent with the requirements of the CAC which recommends that food handlers should wash their hands at every stage of food production to safeguard the consumer from FBD (CAC, 2003).

About 40% of slaughterhouses and 85.8% of retails shops employees did not receive training on hygienic handlings of meat. Previous studies also reported that a considerable proportion of meat processing employees (Haileselassie et al., 2013; Little et al., 1999; Wassie et al., 2017) and meat retail shops employees (Haileselassie et al., 2013; Wassie et al., 2017) did not receive basic training on hygienic handling of meat. This is contrary to the basic requirements for personnel working in the food industry. Employees working in food establishments such as slaughterhouses and retail shops should be trained on food safety issues (Sun and Ockerman, 2005). According to Food, Medicine and Health Care Administration and Control Proclamation (No.661/2009) of Ethiopia, a certificate of competence from the appropriate organ is required for any person working in food catering (EFNG, 2010). FAO also recommends the provision of food safety training to food handlers as an important intervention to improve their knowledge and skills (FAO, 2019).

All employees at the slaughterhouses and 98% of the respondents at retail shops confirmed having had a medical check-up. However, when asked about the frequency of the check-up, answers were variable and not in line with the actual requirement by the Ethiopian regulatory body. Having a periodic medical check-up would partly limit the transmission of pathogens from sick or potentially carrier employees (Gopinath et al., 2012). In addition, strict regulation in the uniformity of the frequency of the check-up as mentioned by the requirements of the Oromia Health Bureau - recommending the need for medical check-up of all employees in food establishments every three months is essential. More importantly, it is required that sick employees should seek medical attention and refrain themselves from handling foods.

Carcasses were stored at room temperature at the slaughterhouses and transported to beef retail shops using vehicles without cooling facility. However, in 92% of the retailshops a refrigerator was used for overnight storage of leftover meat from the sales of the day to avoid meat loss due to spoilage. At all retail shops, meat was displayed openly with no cooling and no cover, being exposed to dust particles and domestic flies. The meat could remain as such for hours until sold. The mean annual temperature of the study area is estimated at 20.2°C (range: 10.91-29.45°C)

(Abebe, 2017), which is the ideal temperature suitable for the growth of a wide range of spoilage and pathogenic organisms to potentially unsafe levels. Cold chain management in meat storing and supplying is an exceedingly important requirement to ensure the quality and safety of meat and meat products (Nastasijević et al., 2017; Sani and Siow, 2014).

None of the employees in slaughterhouses and retailshops wore hand gloves during handling of meat. The use of gloves may protect the meat against contamination and the hands against knife cuts (Alhaji and Baiwa, 2015). In countries like in Ethiopia, where the frequent change of used gloves is economically not feasible, frequent hand washing is an effective measure to prevent cross contamination of meat.

At the slaughterhouses, the use of aprons, white coats, boots and hair covering, as well as the presence of sinks for hand washing were good practices observed at both slaughterhouses. These practices are important to protect both the personnel and the meat from exposure to pathogens (Nel et al., 2004).

Stunning of the animals, hanging of carcass over the rail system for dehiding and eviscerations and carcass washing after eviscerations were the good practices identified at the slaughterhouses. These practices are essential to ensure production of quality and safe meat and needs to be maintained at all times (CAC, 2005, 2003; EFNG, 2010, 2000, 1970). However, we observed that bleeding was carried out on ground, and the hanging and de-hiding of the carcass were done manually. These operations can lead to carcass contamination from the ground, workers' hands and cross contamination from carcass to carcass contact (FAO, 2019). Automatic carcass hoisting, hide removal and sliding of carcasses reduces the risk of carcass contamination (Bakhtiary et al., 2016). Establishing slaughterhouses equipped with the necessary facilities and basic infrastructures would improve the hygienic production in slaughterhouses particularly in government based municipal slaughterhouses in Ethiopia.

According to the respondent's perception, feces during evisceration, hides, handler's hands and knifes were the potential sources of carcass contamination at the slaughterhouses with 36% of them reporting feces and hides as the major sources. This was consistent with previous reports (Gill, 2004; Sheridan, 1998). Previous studies reported the occurrence of foodborne pathogens such as *E. coli* O157 and *Salmonella* in cattle feces and on hides and the possibility of their transfer to carcass during slaughter operations (Arthur et al., 2011; Brichta-Harhay et al., 2008; El-Gamal and EL-Bahi, 2016; Gutema et al., 2021a; Gutema et al., 2021). Further studies to identify all possible sources for carcass contamination and designing effective intervention measures are needed in these slaughterhouses. This would help to improve handling practices (Koohmaraie et al., 2005).

At retail shops, the use of soap and water for hand and equipment washing, storing leftover meat in refrigerators, concrete/tile made floors, and white painted walls and ceilings were the identified good practices. These were in line with the basic requirements of Ethiopian proclamations and can contribute to hygienic handling of meat (CAC, 2005; EFNG, 2010).

However, displaying offal and meat in close proximity (39.4%), use of either plastic bags or newspapers for wrapping meat (53.5%), use of plastic or wooden cutting boards, use of one coat for the entire day (85%) and infrequent washing of equipment and floors were sub-standard practices that can lead to carcass contamination (CAC, 2005; EFNG, 2010).

The use of plastic bags or newspapers were in contrary to the requirements of the Ethiopian Food, Medicine and Healthcare Administration and Control Authority Proclamation (No. 661/2009) that require packaging material to be made out of substances, which are safe and suitable for their intended use, and the product to be packed in container which will safeguard its hygienic, safety, quality and food grade. Further, the proclamation states that "no packaging material shall be put into use unless it complies with the international and national safety and quality standards", which was lacking in the beef retails shops in Bishoftu town (EFNG, 2010).

In most of the retail shops (>70%) equipment, floors and the display cabinet were cleaned once per day. Unclean retail shops ceilings and white walls with observable dirty spots were noticed in 79% of the shops. Frequent and scheduled cleaning of equipment and working environments at food establishments are the basic essential requirements to ensure the continuing effective control of food hazards likely to contaminate food (CAC, 2003).

Only about 8% of the retail shops reported having received complaints from consumers regarding the quality of their beef, with most of the complaints covering the non-tenderness of beef and mischief on the actual weight of the beef to be sold. The majority (92.1%) of the respondents believed that their retail shop was in good condition to provide the desired quality of the meat. This was contrary to our observations that identified various unhygienic practices indicating the significance of an observational study component in minimizing bias.

In general, the observed unhygienic practices at the slaughterhouses and retail shops can be linked with lack or inadequate knowledge of basic hygienic practices (Haileselassie et al., 2013; Jianu and Goleț, 2014; Kago et al., 2014; Niyonzima et al., 2018), lack of infrastructure or facilities (Kibret and Abera, 2012) and poor compliance to standards of good handling practices of food (Kago et al., 2014). Moreover, the insufficient implementation of the government control systems and ensuing timely corrective actions by the food regulatory bodies, which is common in most developing countries including Ethiopia, might contribute to sustaining such unhygienic practices leading to a higher risk for human infection necessitating urgent interventions (Grace, 2015; Temesgen and Abdisa, 2015).

The study has some limitations. The study used questionnaires as a data collection tool, which totally relies on the answers of the respondents that might not necessarily correspond to the actual situation. For examples, 91% of the employees at the retail shops and 46% of employees at slaughterhouses responded washing their hands before touching the meat and between activities during work, which was contrary to our observations; based on observation, 85% of the retailshops had no sink for hand washing at the display but 91% answered that they washed their hands before touching meat indicating the significance of an observational study component in

minimizing bias. Almost all the respondents confirmed having had a medical check-up. However, when asked about the frequency of the check-up, answers were variable and not in line with the actual requirement by the regulatory body. Combining questionnaires with personal observations reduced the study limitations in part, while of course, the presence of the study team might have induced practice changes.

6.6. Conclusions

The study showed a combination of good and unhygienic meat handling practices in slaughterhouses and retail shops. The unhygienic handling practices potentially lead to a higher possibility for contamination and cross-contamination of the meat and may have serious public health implications. Observational study in combination with questionnaire survey can minimize personal bias and can be used as an important data collection tool in assessing hygienic practices particularly at retailshops.The unhygienic handling practices coupled with consumption of raw or under cooked meat which is a common habit in Ethiopia (Avery, 2008; Seleshe et al., 2014) could serve as suitable pathways for meatborne pathogens to enter the food chain. Our findings suggest the need for interventions through provision of food safety training to improve the hygienic meat handling practices along beef supply chain. Improving the infrastructure of the slaughterhouses and retail shops and strengthening food quality control systems by the government regulatory authorities to verify the hygienic meat production and marketing at all stages needs more attention. Moreover, educational sessions such as information campaign to raise food handlers' and consumers' awareness on adequate cooking practices, kitchen hygiene, and personal hygiene are important intervention areas to ensure beef safety.

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Chapter 7

7. Assessment of Beef Carcass Contamination with *Salmonella* and *E. coli* O 157 in Slaughterhouses in Bishoftu, Ethiopia

Based on: Gutema, F.D., Abdi, R.D., Agga, G. E., Seyoum, F., Rasschaert,G., Mattheus,W., Crombe, F, Duchateau, L., Gabriël, S., De Zutter, L. Assessment of Beef Carcass Contamination with *Salmonella* and *E. coli* O 157 in Slaughterhouses in Bishoftu, Ethiopia. International Journal of Food Contamination (Submitted).

7.1. Abstract

The aim of this study was to assess the sources of beef carcass contamination with *Salmonella* and *E. coli* O157 during slaughter. Rectal contents and hide- and carcass-swabs (from three sites: foreleg, brisket and hind leg) were collected from 70 beef cattle at two small scale slaughterhouses. Isolates were genotyped by the Pulsed Field Gel Electrophoresis method and tested for resistance against 14 microbial drugs. *Salmonella* was detected at equal proportions (7.1%) in rectal content samples and hide swabs. *E. coli* O157 was detected in 8.6% of the rectal contents and 4.3% of the hide swabs. The proportion of contaminated carcasses was 8.6% for *Salmonella* and 7.1% for *E. coli* O157. Genetic linkage between the *Salmonella* and *E. coli* O157 isolates from the rectal contents and/or hides and carcasses were observed only in a few cases (2 and 1 carcasses, respectively) indicating the limited direct transfer of the pathogens from the feces and/or hide to the carcass during slaughter. Most carcasses became positive by cross-contamination. All the *S.* Typhimurium isolates (n=8) were multidrug resistant being resistant to ampicillin, chloramphenicol, sulfamethoxazole and tetracycline. The two *S.* Dublin isolates were resistant to colistin. All *E. coli* O157 isolates were susceptible to the antimicrobials tested. The results indicated that cross contamination may be an important source for carcass contamination.

7.2. Introduction

Foodborne diseases (FBD) are a worldwide problem. Consumption of contaminated food of animal origin is associated with potential food safety risks and a major source of FBD. *Salmonella* and Shiga toxin-producing *E. coli* are major causes of FBD (Havelaar et al., 2015). Ruminants, particularly cattle, are reservoirs and asymptomatic carriers of *Salmonella* (Cummings et al., 2010; Gutema et al., 2019) and *E. coli* O157 (Gyles, 2007). Studies reported the occurrence of these pathogens in the feces and on the hides of cattle on farms and in slaughterhouses in developed countries (Arthur et al., 2010; Cobbaut et al., 2008; Essendoubi et al., 2019; Madoroba et al., 2016). The presence of *Salmonella* and *E. coli* O157 in the feces and on the hides of cattle may lead to their transfer to carcasses during hide removal and evisceration (Croxen et al., 2013; Cummings et al., 2010; Gutema et al. 2021a).

Consumption of contaminated beef and beef products is one of the transmission routes of *Salmonella* and *E. coli* O157 to humans (EFSA and ECDC, 2018; Pires et al., 2019) and has been implicated in many foodborne outbreaks (CDC, 2016; Plumb et al., 2019). This is particularly important in countries like Ethiopia where consumption of raw or under-cooked beef in the form of steak ("diimina") or beef tartare ("kitfoo") made from raw minced beef, is common (Avery, 2014; Seleshe et al., 2014). Consumption of raw beef products can be a source of *Salmonella* and *E. coli* O157 infections in Ethiopia (Gutema et al., 2021 b,c).

In Ethiopia, few studies have reported the prevalence of *Salmonella* in cattle feces (Gutema et al., 2021c; Takele et al., 2018), on hides (Sibhat et al., 2011) and on carcasses (Atsbha et al., 2018; Takele et al., 2018). Similarly, *E. coli* O157 was reported in cattle feces (Abdissa et al., 2017; Gutema et al., 2021c; Haile et al., 2017), on hides (Abdissa et al., 2017) and on carcasses (Atnafie et al., 2017) at the slaughterhouse level. We previously identified dehiding and evisceration as two major potential sources of carcass contamination at slaughterhouses in Ethiopia (Gutema et al., 2021a). However, there is currently no data confirming the potential association between the presence of these pathogens in the rectal content and/or on the hide of cattle and their presence on the carcass.

Determining the genetic relatedness of *Salmonella* and *E. coli* O157 in cattle feces, on the hide and on the carcass is essential to investigate the potential transfer to carcasses. This will also contribute to the identification of critical control points and the development of mitigation strategies to ensure beef safety. The objective of this study was to investigate the occurrence and the genetic relatedness for both *Salmonella* and *E. coli* O157 isolated from the rectal content and hide, and the carcass at slaughterhouses. Antimicrobial resistance of *Salmonella* and *E. coli* O157 isolates obtained from rectal contents, hides and carcass was further assessed.

7.3. Materials and methods

7.3.1. Slaughterhouses

The study was conducted from November 2018 to May 2019 at two slaughterhouses in Bishoftu town located in East Shoa Zone, Oromia, Ethiopia. Both slaughterhouses were small in capacity processing where the municipal slaughterhouse and the private slaughterhouse usually slaughtered 5-15 and 15-30 cattle per day, respectively. The slaughter process at both slaughterhouses was rather similar. Briefly, the manual slaughter process involved stunning with a sharp knife, bleeding, removal of head and feet and de-hiding the upper part of the hind legs on the floor followed by hanging of the carcass, manual dehiding, evisceration, carcass washing, post-mortem inspection, carcass labeling and storage at environmental temperature until distribution to retail shops. The slaughterhouses did not have a stand-by pressurized water supply and hot water for hand and equipment, including knives, washing. Moreover, slaughterhouse workers were involved in different slaughter steps and received no or limited hygienic training (Gutema et al., 2021a).

7.3.2. Sample collection

Samples were collected from 70 animals (35 in each slaughterhouse). Seven visits per slaughterhouse were organized whereby each time, 5 carcasses were sampled during slaughter. Due to the presence of relatively many cattle in the lairage of the private slaughterhouse, animals were selected using systematic random sampling before slaughter whereas at the municipal

slaughterhouse due to the limited number of animals present in the lairage, five consecutively slaughtered animals slaughtered the day of sampling were sampled. The following samples were collected from each carcass: one rectal content (50 grams), one hide and three carcass swab samples. The hide swab was taken from the medial side of the foreleg and hind leg and the brisket from one half of the carcass immediately after stunning. From each hide swabbing site, an area of 20x20 cm was swabbed using the same sterile cotton swab pre-moistened in 10 ml buffered peptone water (BPW; Difco, BD, Sparks, MD, USA). Separate carcass swabs (20 x 20 cm) per site were obtained after evisceration and before washing from the same sites as the hide swabs, but on the other half of the carcass. Samples were transported in an icebox to the laboratory and stored at +4^{o}C until processing within 24 hours.

7.3.3. Detection of *Salmonella* and *E. coli* O157

For processing of the hide and carcass swabs, each swab in 10 ml BPW was transferred into a stomacher bag containing another 30 ml BPW to make a final volume of 40 ml and homogenized for 2 minutes using a stomacher. From the final volume of homogenized solution, 20 ml was transferred into another stomacher bag.

Salmonella detection was based on the International Organization for Standardization guideline ISO 6579-1: 2017 (ISO, 2017). Briefly, 25 g of rectal content was transferred into a sterile stomacher bag, 225 ml of BPW was added and the mixture was homogenized using a stomacher blender for 1 minute at 200 rpm. Homogenized rectal content, hide and carcass swabs were incubated at 37°C for 18 h.

After the incubation of the pre-enrichment broths, 0.1 ml of each culture medium was spotted in 3 drops onto a modified semi solid Rappaport-Vassiliadis medium (MSRV; Oxoid, Basingstoke, UK) and incubated at 41.5°C for 24 h. After incubation, plates were examined for the presence of migration zones. A loopful from the edge of a migration zone was streaked onto xylose lysine deoxycholate (XLD, Difco) agar plates and incubated at 37ºC for 24 h. Plates were examined for the presence of suspect *Salmonella* colonies. Suspected colonies were biochemically tested using

triple sugar iron agar slants (Difco, BD), lysine decarboxylase test (BBL, BD), and indole test (BBL, BD). One confirmed isolate per sample was stored at -18°C for further characterization. Collected *Salmonella* isolates were subjected to a *S.* Typhimurium PCR using the primers described by Lin et al (1999). All isolates negative for this PCR were then clustered using enterobacterial repetitive intergenic consensus (ERIC) PCR as described by Rasschaert et al (2005). Based on the data obtained from each ERIC profile at least one isolate was selected for serotyping according to the Kauffmann-White scheme (Grimont and Weill, 2007) at Belgian National Reference Laboratory for *Salmonella*.

E. coli O157 detection was based on International Organization for Standardization, horizontal method for the detection of *E. coli* O157-ISO 16654: 2001 (ISO, 2001). Twenty-five gram of each rectal content sample was transferred into a stomacher bag containing 225 ml of modified tryptone soya broth (Oxoid) supplemented with 20 mg/l novobiocin (Sigma Aldrich, MO; USA) (mTSBn), homogenized using a stomacher blender for 1 minute at 200 rpm. For the detection of *E. coli* O157 from the swab samples, 20 ml double concentrated mTSBn was added to stomacher bags containing 20 ml of the sample homogenate. After the incubation of the enrichment broths at 41.5 °C for 6 h, 1 ml of each broth was subjected to immunomagnetic separation (IMS) using Dynabeads anti-*E.coli* O157 (ThermoFisher Scientific, West Palm Beach, FL, USA) according to the manufacturers' instruction. The final washed bead-bacteria complexes were spread onto cefixime tellurite sorbitol MacConkey agar plates (Oxoid) containing 0.05 mg/l cefixime and 2.5 mg /l potassium tellurite (Oxoid) (CT-SMAC).

After incubation at 37 °C for 24 h, the plates were examined for the presence of suspect colonies. From each selective agar plate, up to three suspect colonies were subjected to Kligler Iron agar, indole and *E. coli* O157 latex agglutination (Oxoid) tests. In the frame of another research project, one isolate per positive sample was further analyzed using whole genome sequencing at Belgian National Reference center for STEC. Data on the presence of *stx* genes, *eae* gene, and *ehxA* gene in those isolates was obtained from this analysis.

7.3.4. Pulsed field gel electrophoresis

Both *Salmonella* and *E. coli* O157 isolates (one isolate per positive sample) were genotyped by PFGE after digestion with *Xba*I enzyme (CDC, 2017). The fingerprints were grouped according to their similarity with Bionumerics 7.6 software (Applied Maths, Biomérieux, Sint-Martens-Latem, Belgium) using the band-based dice coefficient with a 2% position tolerance and unweighted-pair group method using arithmetic averages (UPGMA). Pulsotypes were assigned based on the difference of at least one band in the fingerprints and indicated by capital letter.

7.3.5. Antimicrobial susceptibility testing

All *Salmonella* and *E. coli* O157 isolates were tested for their antimicrobial resistance to the following 14 antimicrobial drugs with tested concentration range (µg/ml) in brackets: ampicillin (1-64), azithromycin (2-64), cefotaxime (0.25-4), ceftazidime (0.5-8), chloramphenicol (8-128), ciprofloxacin (0.015-8), colistin (1-16), gentamicin (0.5-32), meropenem (0.03-16), nalidixic acid (4-128), sulfamethoxazole (8-1024), tetracycline (2-64), tigecycline (0.25-8) and trimethoprim (0.25-32). The resistance profiling was evaluated based on the minimum inhibitory concentration (MIC) using Sensititre EU surveillance *Salmonella*/*E. coli* (EUVSEC) plates (Thermo Fisher Scientific, Merelbeke, Belgium). The tests were performed according to the manufacturer's instructions. The standard reference strain *E. coli* ATCC 25922 was used as quality control. European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological breakpoint values were used to categorize the isolates as resistant or susceptible. In case of *Salmonella*, for sulfamethoxazole, tigecycline and colistin the epidemiological breakpoints for *E. coli* were used (EUCAST, 2019).

7.4. Results

From the 70 cattle examined, 23 (32.9%) were positive for *Salmonella* and/or *E. coli* O157 in at least one sample. Specifically, 14 (20.0%) animals were positive for *Salmonella*, and 11 (15.7%) for *E. coli* O157 (Table 7.1). Two animals were positive for both *Salmonella* and *E. coli* O157 (Table 7. 2).

Table 7.1. Proportion of *Salmonella* and *E. coli* O157 in the rectal content, hide and carcass swabs obtained from 70 beef cattle in Bishoftu, Ethiopia.

Source	**Number of sample**	***Salmonella***		***E. coli* O157**
		Number (%)	**Serotypes**	**Number (%)**
Rectal content	70	5 (7.1)	Typhimurium (5)	6 (8.6)
Hide	70	5 (7.1)	Typhimurium (1), Dublin (1), Chailey (2), Muenchen (1)	3 (4.3)
Carcass	210	6 (8.6)		5 (7.1)
Fore leg	70	1 (1.4)	Dublin (1)	1 (1.4)
Hind leg	70	3 (4.3)	Typhimurium (1), Chailey (1), Muenchen (1)	2 (2.8)
Brisket	70	2 (2.8)	Typhimurium (1), Muenchen (1)	2 (2.8)
Total	**350**	**16**		14
Animal		**14(20.0)**		**11 (15.7)**

Table 7.2. Distribution of *Salmonella* and *E. coli* O157 isolates among the positive cattle identified at two slaughterhouses in Bishoftu, Ethiopia.

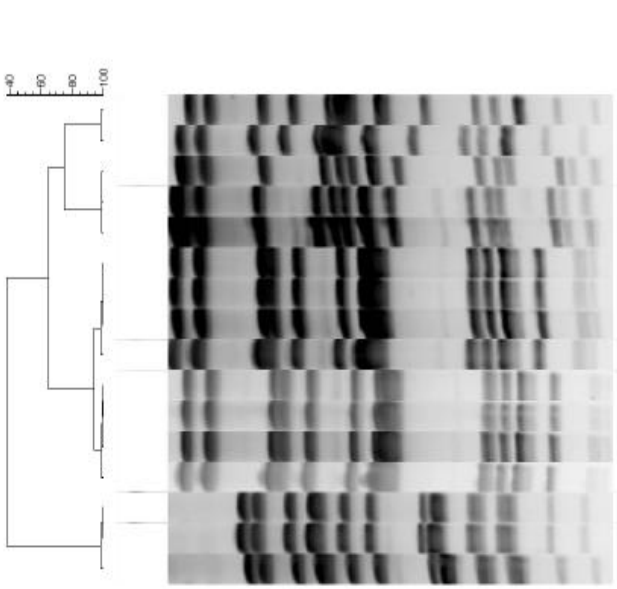
Slaughterhouse	Visit	Animal ID	Sample type				
			Rectal content	Hide	Carcass swabs		
					Foreleg	Brisket	Hind leg
Municipal	B	1	+	+	-	+	+
	B	5	+	-	-	-	-
	C	3	+	-	-	-	-
	D	3	-	+	-	-	-
	D	4	+	-	-	-	+
	E	2	-	+	-	-	-
	E	5	+	-	-	-	-
	F	3	+	-	-	-	-
	F	4	-	-	-	-	+
	G	3	-	-	-	+	-
Private	A	2	+	+	-	-	-
	A	3	-	-	+	-	-
	C	4	+	-	-	+	-
	D	1	-	-	-	-	+
	D	2	+	-	-	-	-
	D	3	-	+	-	-	-
	D	4	-	+	-	-	-
	D	5	-	+	-	-	-
	E	1	+	-	-	-	-
	E	5	+	-	-	-	-
	F	2	-	-	-	+	-
	F	5	-	-	-	-	+
	G	3	-	+	+	-	-

+ Sample positive for *Salmonella;* + Sample positive for *E. coli* O157; **ID animal identification number**

Salmonella

From the 14 *Salmonella* positive carcasses, the following 16 samples were positive: 5 rectal contents (7.1%), 5 hides (7.1%) and 6 carcasses (1 foreleg, 2 briskets and 3 hind legs) (8.6%).

Only in two cases, two samples of the same animal were positive: the rectal content and the carcass (brisket) from one animal, and the rectal content and the carcass (hind leg) from the other animal. The 16 *Salmonella* isolates were identified as *S.* Chailey, *S.* Dublin, *S.* Muenchen and *S.* Typhimurium. All isolates within a serotype belonged to a single pulsotype (Figure 7. 1). The isolates from the animals with two positive samples were identified as *S.* Typhimurium. All *S.* Typhimurium isolates showed the same resistance profile, namely resistant to ampicillin, chloramphenicol, sulfamethoxazole and tetracycline, while the two *S.* Dublin isolates were only resistant to colistin. *Salmonella* Chailey and *S.* Muenchen were sensitive to all 14 antimicrobial drugs tested.



Sample code[o]	Serotype	Pulsotype	Resistance Profile[oo]
PD3H	S. Dublin	A	COL
PA3Fo	S. Dublin	A	COL
PD4H	S. Chailey	B	S
PD1HI	S. Chailey	B	S
PD5H	S. Chailey	B	S
MB1Br *	S. Typhimurium	C	AMP-CHL-SMX-TET
MD4HI **	S. Typhimurium	C	AMP-CHL-SMX-TET
PE5R	S. Typhimurium	C	AMP-CHL-SMX-TET
PG3H	S. Typhimurium	C	AMP-CHL-SMX-TET
MC3R	S. Typhimurium	C	AMP-CHL-SMX-TET
MD4R **	S. Typhimurium	C	AMP-CHL-SMX-TET
ME5R	S. Typhimurium	C	AMP-CHL-SMX-TET
MB1R *	S. Typhimurium	C	AMP-CHL-SMX-TET
PF5HI	S. Muenchen	D	S
MG3Br	S. Muenchen	D	S
ME2H	S. Muenchen	D	S

Figure7.1. Pulsed-field gel electrophoresis patterns and resistance profiles of *Salmonella* isolates from rectal contents, hides and beef carcasses in Bishoftu, Ethiopia. [o] 1st letter - slaughterhouse (M: Municipal, P: Private); 2nd letter - sampling visit (A to G); number - carcass within each visit (1 to 5), last letter(s) - sample sources (R: rectal content; H: hide, Br: brisket carcass, Hl: hind leg carcass, Fo: foreleg carcass); oo S-susceptible; AMP-ampicillin; CHL-chloramphenicol; COL-colistin; SMX- sulfamethoxazole; TET-tetracycline; * Isolates from a same carcass; **Isolates from a same carcass.

***E. coli* O157**

Of the 11 *E. coli* O157 positive carcasses, the following 14 samples were positive: 6 rectal contents (8.6%), 3 hides (4.3%) and 5 carcasses (1 foreleg, 2 briskets and 2 hind legs) (7.1%). In three cases, two samples of the same animal were positive for *E. coli* O157: hide and carcass (hind leg), rectal content and hide, and rectal content and carcass (hind leg). *E. coli* O157 isolates were grouped into eight pulsotypes (A-I) (Figure 7. 2). Among the isolates obtained from the same animals (n=3), genetic relatedness was observed only between isolates obtained from a hide and a carcass (hind leg) swab of one animal sampled at the municipal slaughterhouse. All the *E. coli* O157 isolates carried the *eae* and the *ehxA* gene; the *stx*2 gene (10 and 2 stx2a) was detected in 85.7% (12/14) of the isolates while the *stx*1 gene was not detected in any of the isolates. The stx2a subtypes were detected in isolates from a brisket and a hide swab. All *E. coli* O157 isolates were sensitive to the 14 antimicrobial drugs tested.

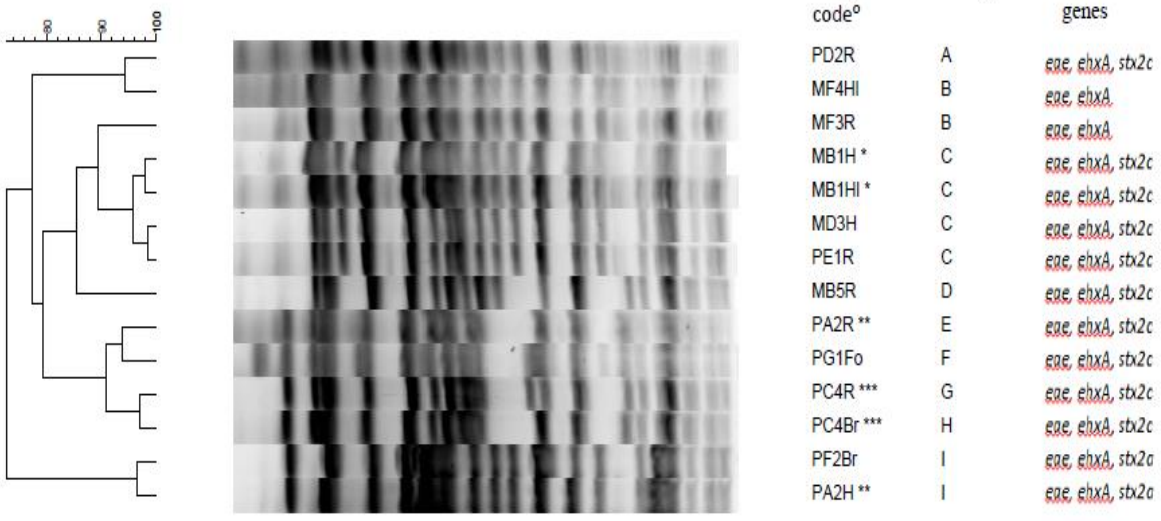


Figure 7.2. Pulsed-field gel electrophoresis patterns and virulence factors of *E. coli* O157 isolates obtained from rectal contents, hides and beef carcasses in Bishoftu, Ethiopia. °1st letter - slaughterhouse (M-Municipal, P-Private); 2nd letter - sampling visit (A to G); number - carcass within each visit (1 to 5), last letter(s) - sample sources (R: rectal content; H: hide, Br: brisket carcass, Hl: hind leg carcass, Fo: foreleg carcass); * Isolates from a same carcass;** Isolates from a same carcass;*** Isolates from a same carcass.

7.5. Discussion

The present study detected for the first time in Ethiopia *Salmonella* and *E. coli* O157 in the rectal content, on the hide and on cattle carcass at slaughterhouses. Although *Salmonella* was detected at the same proportion (7.1%) of the feces and the hide swabs, it was not simultaneously detected from the same carcasses. The proportion of positive rectal contents was comparable with the national prevalence estimate of 7.1% (variation from 2.1% to 16.2%) in Ethiopia (Tadesse and Tessema, 2014) but lower than the adjusted prevalence estimate of 15.4% with a variation from 11.7 to 20% in Africa (Thomas et al., 2020). The proportion of *Salmonella* positive hide samples was lower compared to a study by Sibhat et al (2011) who reported a prevalence of 31% in Ethiopia. Studies that have been conducted elsewhere indicated the occurrence of *Salmonella* on hides of cattle at slaughterhouses with a variable prevalence ranging from 17.7% in England (Reid et al., 2002) and up to 94% in USA (Brichta-Harhay et al., 2008).

The proportion of *E. coli* O157 in rectal contents and on hides was 8.6% and 4.3%, respectively. The proportion of positive rectal contents was slightly higher compared to global prevalence estimate of 5.7% that ranges from 0.1% to 61.8% (Islam et al., 2014). A recent study by Gutema et al (2021c) reported 7.1% positive rectal contents collected from cattle in the lairage at the same slaughterhouses. The 4.3% positive hides was lower compared to the global prevalence estimate of 44% with a variation from 7.3 to 76% (Rhoades et al., 2009). Only from one carcass, *E. coli* O157 was detected from the rectal content and the hide concomitantly. However, genetic typing showed that the isolates from both samples were not identical, indicating that the feces of the animal was not the source of the hide contamination.

In the present study, we observed a carcass contamination rate of 8.6% and 7.1% for *Salmonella* and *E. coli* O157, respectively. The carcass contamination with *Salmonella* was comparable to the prevalence of 7.6% (Muluneh and Kibret, 2015), 12.5% (Wabeto et al., 2017) and 11.3% (Takele et al., 2018) in Ethiopia. For *E. coli* O157 variable proportions of carcass contamination were reported: from 0.54% by Abdissa et al (2017) up to 13.3% by Bekele et al (2014) for Ethiopia. For both pathogens, positive carcasses were only found positive on one carcass

site,indicating that the carcass contamination is in most cases not widespread over the positive carcasses. As a consequence, collecting swab samples from multiple sites on a carcass may increase the number of positive carcasses. According to the EU regulation 2073/2005 beef carcasses sampled for bacteriological analysis, four carcass sites have to be swabbed (European Commission, 2005). However, such regulation is not available for Ethiopia.

Only in a few cases *Salmonella* or *E. coli* O157 were detected simultaneously in the rectal content or on the hide, and on the corresponding carcasses. For the two *Salmonella* isolates and one of the three *E. coli* O157 isolates, from the rectal content and hide, and the carcass swabs were genetically similar, respectively indicating a possible direct transfer of the pathogen to the carcass surface. In all other cases, no genetic link was stated between isolates from the rectal content and/or hide isolates and the carcass swabs. The observed low level of linkage of *Salmonella* and *E. coli* O157 isolates on carcass with those from rectal contents and/ or hide indicates that other sources may be involved in the carcass contamination during slaughter. This could be due to cross contamination caused by unhygienic handling practices such as infrequent washing of knifes and hands (Gutema et al., 2021a). Contamination and cross contamination of hides during cattle transport or in the lairage could increase the risk of carcass contamination.

Salmonella Dublin was isolated from the hide of one carcass and from the foreleg of another carcass in this study and it was also previously reported from retail beef in Ethiopia (Ejeta et al., 2004) indicating that this serotype is present in the cattle population and can be a source for human infections. *S.* Dublin is known to cause invasive infections and fatalities in humans (Harvey et al., 2017; Mattheus et al., 2018). In Ethiopia, *S.* Dublin was sporadically reported from stool of diarrheic patients (Tadesse, 2014).

All *S.*Typhimurium isolates were multidrug resistant being resistant to ampicillin, chloramphenicol, sulfamethoxazole and tetracycline. Except for chloramphenicol, similar resistance profile was observed for *S.* Typhimurium isolates from cattle before slaughter at the same slaughterhouses (Gutema et al., 2021b). This suggests the widespread occurrence of ampicillin, sulfonamides and tetracycline resistance in *S.* Typhimurium isolated from cattle in

Ethiopia and may be related to the long-time marketing and accessibility of these drugs. The two *S.* Dublin isolates were resistant to colistin. Resistance to this antibiotic seems to be common in *S.* Dublin isolates (Agerso et al., 2012, EFSA and ECDC, 2020). The finding that all 14 *E. coli* O157 isolates being susceptible to the 14 antimicrobials tested was in agreement with our previous study that have reported susceptibility of *E. coli* O157 isolates except one isolate obtained from beef in the study area (Gutema et al., 2021c).

7.6. Conclusions

During slaughter, beef carcasses can become contaminated with *Salmonella* and/or *E. coli* 0157. The contamination was not widespread over positive carcasses. The study indicated the frequent occurrence of cross contamination besides the direct contamination of carcasses by feces and hide of positive animals. More studies are needed to unravel the sources for this cross contamination and to develop efficient preventive measures to ensure beef safety.

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Chapter 8

8. General Discussion

8.1. Introduction

Salmonella and *E. coli* O157 are among the most common bacterial causes of foodborne diseases. Consumption of meat and meat products is a major source of foodborne diseases and meat and meat products are implicated in several foodborne outbreaks. In Ethiopia, beef is commonly consumed and eaten frequently raw "foon dheedhii" or undercooked in the form of steak ("diimina") or beef tartare ("kitfoo") made from raw minced beef. Information on the potential association of cattle with the occurrence of human diarrheal illness due to *Salmonella* and *E. coli* O157 through consumption of beef is lacking in Ethiopia.

The studies presented in this thesis attempted to address the question of linkage between *Salmonella* and *E. coli* O157 in cattle and/or beef and in diarrheic patients. The linkage between consumption of beef contaminated with *Salmonella* and *E. coli* O157 and potential development of diarrheal illness in humans depends on the carriage of the pathogens in cattle feces and on hides, hygienic measures applied during beef production, transportation and processing at retail shops, the level of beef contamination and exposures to contaminated beef such as via consumption of raw or undercooked meat. To that end, the following major three parts were examined: First, the research investigated the occurrence and the genetic relatedness of *Salmonella* and *E. coli* O157 in cattle, beef and in diarrheic patients. Second, the research assessed the hygienic practices in slaughterhouses and in beef retail shops. Third, the research investigated the sources (feces and hides) of carcass contamination with *Salmonella* and *E. coli* O157 in slaughterhouses. Furthermore, this research investigated the antimicrobial resistance profile of the identified *Salmonella* and *E. coli* O157 strains.

In this research, the systematic review and meta-analysis study estimated the prevalence of *Salmonella* at 9% in cattle. The microbiological investigation estimated the prevalence of *Salmonella* at 2.5% and 7.1% and for *E. coli* O157, 7.1% and 8.9% in cattle before and after slaughter, respectively. Slaughtered cattle also harbored *Salmonella* (7.1%) and *E. coli* O157 (4.3%) on their hides. Comparable proportions of beef contamination with *Salmonella* (8.6% vs.

8.7%) and *E. coli* O157 (7.1% vs. 6.3%) were observed both at slaughterhouses and retail shops, respectively.

As presented in chapter 6 of this thesis, there are various unhygienic practices in slaughterhouses and retail shops that can contribute to contamination of beef *with Salmonella* and *E. coli* O157. The study presented a prevalence of 2.3% of *Salmonella* and 2.8% of *E. coli* O157 in diarrheic patients and all the patients' positive for these pathogens had raw beef consumption behavior. The antimicrobial resistance study revealed a considerable proportion of resistant *Salmonella* and only one resistant *E. coli* O157 strain.

Taken together, the results of the studies presented in this thesis show the potential transfer of *Salmonella* and *E. coli* O157 from cattle via beef to humans which can lead to diarrheal illness in susceptible people. This research also suggests that the occurrence of these pathogens in beef largely depends on the overall hygienic practices in slaughterhouses and retail shops. Moreover, the results of the research indicated occurrence of high resistant *Salmonella* and very low resistant *E. coli* O157 strains circulating in the study area. Further investigation is needed to explain why most *Salmonella* serotypes are resistant while most *E. coli* O157 are not. Details of the key findings presented in this thesis are discussed in the following sections.

8.2. *Salmonella* and *E. coli* O157 in beef supply chain

The prevalence of *Salmonella* in the rectal content samples of cattle before and after slaughter (Chapter 4 and chapter 7) was higher after slaughter than before slaughter. This may be due to the difference in the sample size. Previously a prevalence of *Salmonella* in cattle ranging from 0.62% (Alemayehu et al., 2003) to 23.5% (Hiko et al., 2016) was reported in Ethiopia. The wide variation in the prevalence between both studies might be attributed to the difference in the detection method and sample size: in the study by Alemayehu et al. (2003) the used method was not optimal for the detection of *Salmonella*, while in the study by Hiko et al. (2016) the sample size (n=34) was small. The 7.1% prevalence of *E. coli* O157 in cattle is comparable with the report of 7.3% in Jimma (Haile et al., 2017) and higher compared to the prevalence of 1.9% in

Addis Ababa and in Debre Berhan (Abdissa et al., 2017) and 4.7% in Hawassa (Atnafie et al., 2017) in Ethiopia.

The other important part of findings in the present study is the contamination of the hides with *Salmonella* and *E. coli* O157 (chapter 7). *Salmonella* was detected at the same proportion (7.1%) from the rectal contents and the hide swabs. *Salmonella* was not detected from rectal content and hide of the same animal, while *E. coli* O157 was detected from the rectal content and the hide of one animal concomitantly. However, genetic typing of the *E. coli* O157 strains showed, that the isolates from both samples were not identical, indicating that the feces of the animal was not the source of the hide contamination. The study therefore suggested hide contamination from other sources, such as contamination either from the environment or fecal contamination at farms, cattle markets, during transport, in the lairage and in slaughterhouses, seem to be more important than the spreading of own feces over their hides by the animals themselves.

The 8.6% prevalence of *Salmonella* on carcasses is comparable with other reports from Ethiopia (Muluneh and Kibret, 2015, Wabeto et al., 2017 and Takele et al., 2018). The 7.1% of *E. coli* O157 on carcases is somewhat higher than the 4.7% prevalence reported by Bekele et al (2014). In the latter case, the detection method used for *E. coli* O157 was sub-optimal. Given the unhygienic practices at the slaughterhouses (chapter 6), it is likely that the carcass contamination with *Salmonella* and *E. coli* O157 could occur from rectal contents and hides. However, only in a few cases *Salmonella* and *E. coli* O157 detected in the rectal content and/or on hides were related to the corresponding isolates on the carcasses. *Salmonella* was simultaneously detected from rectal content and carcass only in two animals and were serotyped as *S.* Typhimurium and shared the same pulsotype. Similarly in the case of *E. coli* O157, genetic relatedness was observed only between isolates obtained from a hide and carcass of one animal (chapter 7). This indicates minimal direct transfer of the pathogens from rectal contents or hides onto the carcass during slaughter operation. This could be due to the few animals being slaughtered on each day especially at the municipal slaughterhouse that enables the employees to carefully operate at a slow pace. The small number of positive samples from each sample sources can be another explanation.

In all other cases, no genetic link was observed between isolates from the rectal content and/or hide isolates and the carcass. The absence of linkage of the two pathogens on carcass with those from rectal contents and/or hide indicates that other sources are involved in the carcass contamination during slaughter.

This could be due to cross contamination from unhygienic handling practices such as infrequent washing of knife and hands at the slaughterhouses (chapter 6). A study by Hiko et al (2016) also reported a higher prevalence of *Salmonella* from the slaughter line environment than animal related samples, indicating the significance of other sources of carcass contamination.

Results indicate a prevalence of 8.6% of *Salmonella* (Chapter 4) and 6.3% of *E. coli* O157 (chapter 5) in beef cut samples collected from beef retail shops. In Ethiopia, variable proportions of *Salmonella* were reported in different forms of beef (raw vs. minced) and from different sources (abattoir, retail shops, supermarket and food establishments such as hotels) of beef samples up to 35.6% prevalence (Garedew et al., 2015). The 6.3% of *E. coli* O157 in beef is comparable to the 6% pooled prevalence estimate in beef at retailshops in Ethiopia (Zelalem et al., 2019).

In addition to the contamination of carcasses at slaughterhouses, storage conditions during transportation and distribution, the level of *Salmonella* and *E. coli* O157 on beef depends also on the hygienic conditions at retail shops (Garedew et al., 2015, chapter 6). Within each serotype of *S.* Eastbourne and *S.* Typhimurium isolated from cattle and beef, genetic similarity was observed, suggesting the possible transfer from cattle to beef. Similarly, genotyping of *E. coli* O157 isolates from cattle and beef revealed genetic similarity demonstrating possible linkage between *E. coli* O157 in cattle and beef (chapter 5). However, based on the results, a direct link between *Salmonella* and *E. coli* O157 in cattle and beef cannot be established due to sampling of cattle and beef at a different time. However, the results indicate the circulation of genetically similar *Salmonella* and *E. coli* O157 strains in the study area.

The results in chapter 6 of this thesis indicate storage of carcasses at room temperature at the slaughterhouses and transportation to beef retail shops using vehicles without cooling facility. In addition, at all retail shops, beef was displayed openly with no cooling and no cover, being exposed to dust particles and domestic flies. Moreover, there are various unhygienic practices at retail shops. All the factors together can lead to contamination of beef with *Salmonella* and *E. coli* O157 and their potential proliferation (Heredia and García, 2018).

8.3. *Salmonella* and *E. coli* O157 in diarrheic patients

As discussed in the previous sections, results in this thesis indicate the occurrence of *Salmonella* and *E. coli* O157 in cattle and beef with the potential to reach beef consumers. In Ethiopia, no information is available on the incidence of *Salmonella* and *E. coli* O157 infections in humans. Only data on the prevalence of *Salmonella* in stool samples from diarrheic patients are available. The prevalence of *E. coli* O157 in such samples is very limitted. The 2.3% prevalence of *Salmonella* is low compared to the meta analysis study that estimated a prevalence of *Salmonella* in children and adults with diarrhea at 8.7% and 5.7%, respectively (Tadesse, 2014). According to Tadesse (2014), *S.* Concord (34%), *S.* Typhi (32.5%), *S.* Typhimurium (9.4%) and *S.* Paratyphi (6.1%) were the dominant serotypes in Ethiopia. However, the present study is comparable with the 2.5% prevalence of *Salmonella* recently reported from diarrheic patients in Ethiopia (Teshome et al., 2019). Although the present study did not investigate typhoidal *Salmonella*, the typhoidal serotypes seem important in human infections in Ethiopia.

Despite the identified unhygienic practices at slaughterhouses and retail shops (chapter 6) and the common habit of raw or undercooked beef consumption, the observed occurrence of *Salmonella* and *E. coli* O157 in diarrheic patients is low compared to the occurrence on carcasses at slaughterhouses and beef at retail shops. This could be due to the absence of direct exposure of diarrheic patients to the pathogens in cattle and beef. It might also indicate the importance of other pathogens in causing diarrheal illness. Some studies in Ethiopia indicated that parasites are more important causes of diarrhea in humans indicating that *Salmonella* and *E. coli O157* can only explain a small proportion of diarrheal illness (Ayenew et al., 2019; Mekonnen et al., 2019).

As presented in chapter 4 and chapter 7 of this thesis, serotypes in cattle, beef in retailshops, hide and carcasses were different from humans. Of all serotypes identified, only *S.* Eastbourne and *S.* Typhimurium were isolated in two or more than two sample sources (chapter 4, 7). *S.* Eastbourne and *S.* Typhimurium identified from cattle and beef shared similar genetic profiles. However, the *S.* Typhimurium identified from slaughtered cattle, hide and carcasses were genetically different from those strains identified in cattle before slaughter, beef and humans. Other studies showed that different serotypes in cattle and beef were isolated from humans and vice versa (Tadesse and Tessema, 2014; Tadesse, 2014; Zelalem et al., 2019). One study in Ethiopia reported genetic similarity within serotypes of *Salmonella* isolated from humans and cattle (Eguale et al., 2018). The present research was not designed to prove explicitly a direct linkage of *Salmonella* in cattle and beef with isolates from humans. Nonetheless, the observed genetic similarity between *Salmonella* serotypes from cattle and beef and the overall identified serotypes in this research implicitly suggests the importance of *Salmonella* in humans in Bishoftu town.

All *Salmonella* serotypes (*S.* Typhimurium , *S.* Eastbourne, *S.* Saintpaul, *S.* Chailey and *S.* Munchen, S. Dublin) identified in this present study except *S.* Cotham were reported form apparently healthy cattle (Chapter 3). These *Salmonella* serotypes can potentially enter into food chain particularly during de-hiding and evisceration (Chapter 7). Previous studies elsewhere indicated the importance of these serotypes in human infections. For instance, S. Typhimurium in European union countries (EFSA and ECDC, 2021), *S.* Cotham in USA (CDC, 2014), *S.* Saintpaul in Australia (Ford et al., 2018), *S.* Dublin in Europe (Wesley et al., 2018) and *S.* Eastbourne in Canada (Craven et al., 1975) were reported from human patients. *Salmonella* Chailey and *S.* Munchen are rarely causing outbreaks in humans (CDC, 2016b; Luna et al., 2018).

For *E. coli* O157, some strains obtained from cattle and beef were genetically related to those isolates from diarrheic patients suggesting a possible linkage. However, *E.* coli O157 identified from slaughtered cattle, hide and carcasses were genetically different from those strains identified in cattle before slaughter, beef and humans (chapter 5, 7). Further, this study reported for first time the virulence factors *sxt*1, *stx*2, *eae* and *ehxA* in *E. coli* O157 strains obtained from

cattle, beef, hide and humans in Ethiopia. All the *E. coli* O157 isolates identified in this research carried the *eae*, most carried stx2 majorly stx2c subtypes and none of the isolates carried *stx*1. Except from hide swabs, stx2 negative strains were detected from cattle, beef/carcass and humans (chapter 5, 7). These virulence factors are important factors for development of *E. coli* O157 infections in humans.

Given the common practice of consumption of raw or undercooked beef and beef products (Avery, 2014; Seleshe et al., 2014), the contamination of beef with the pathogens represents an important food safety risk in Ethiopia. However, it is also important to note that *Salmonella* and *E. coli* O157 are also present in other animal species such as *Salmonella* in poultry (Eguale et al 2018) and *E. coli* O157 in sheep and goats (Abreham et al., 2019) that can also contribute to the infection in humans.

8.4. **Antimicrobial resistance of *Salmonella* and *E.coli* O157**

Of the *Salmonella* isolates identified from cattle, beef at retail shops and diarrheic patients and from rectal contents, hides and carcass of slaughtered cattle, 95.5% (n=22) and 62.5% (n=16) showed resistance at least to one of the 14 antimicrobials tested, respectively. Specifically, *Salmonella* resistant to ampicillin, chloramphenicol, sulfamethoxazole, tetracycline, tigecycline and trimethoprim with high frequency to sulfamethoxazole was observed. More importantly, a multidrug resistant *Salmonella* obtained from cattle was observed. *Salmonella* obtained from beef and humans were resistant only to sulfamethoxazole and trimethoprim which were also the case for isolates from cattle suggesting the widespread resistance of *Salmonella* to these antimicrobials (Chapter 4, 7). More specifically, multidrug resistance was observed among *S.* Typhimurium isolates from cattle before slaughter and after slaughter on hide, carcass and rectal content at the slaughterhouses, which also indicates the widespread of resistant *S.* Typhimurium strains in the study area. Except one isolate from beef, almost all *E. coli* O157 identified in this research were susceptible to antimicrobials tested (chapter 5, chapter 7). Due to the small number of isolates from each sample source and observed variable resistance patterns, assessment for the

relationship was not made based on the observed antimicrobial resistance patterns between isolates from humans and other sources based.

The observed antimicrobial resistance and even the MDR in *Salmonella* obtained in the study may be related to the use of the antimicrobials in animals and humans in the study area. Antimicrobials such as oxytetracycline, the combination penicillin/streptomycin and sulfa drugs are commonly used antimicrobials for treatment of clinical cases related to digestive systems and systemic diseases merely based on clinical signs and physical examination of animals (Tufa et al., 2018). Regulations on antimicrobial use in livestock are poorly enforced and antimicrobials are sold without prescription, farmers have free access to buy and treat their animals in Ethiopia (Gemeda et al., 2020). This misuse may contribute to development of antimicrobial resistance bacteria like *Salmonella* and is a high risk for humans due to the potential transfer of resistant *Salmonella* strain from animals via consumption of foods of animal origin. As presented in Chapter 4, except one *S.* Saintpaul serotype isolate obtained from humans, all the other resistant *Salmonella* isolates (n=22) from cattle, beef and humans were resistant to sulfamethoxazole. In contrast to *Salmonella*, *E. coli* O157 antimicrobial resistance was much less observed, although one isolate was multidrug resistant. Overall, the findings indicate considerable resistant *Salmonella* and negligible resistant *E. coli* O157 strains circulating in the study area.

8.5. **Conclusions and future persepectives**

The results presented in this thesis provide information on the occurrence of *Salmonella* and *E. coli* O157 in cattle, hides, beef and humans, genetic similarity and antimicrobial susceptibility of the isolates that have not previously been available for Bishoftu town in Ethiopia. The study also assessed the hygienic practices in slaughterhouses and meat retail shops. The PFGE patterns of some of the *Salmonella* and *E. coli* O157 isolates from cattle, beef, hides and humans have similar genetic patterns suggesting that the pathogens circulating in the cattle-beef-human continuum may have epidemiological inter-linkage. Observational study in combination with questionnaire survey can minimize personal bias and can be used as an important data collection tool in assessing hygienic practices particularly at retailshops. The study showed unhygienic

handling practices in slaughterhouses and retail shops that can further intensify the transmission of *Salmonella* and *E. coli* O157. The knowledge obtained from this study will be used as baseline information to design tailored intervention measures to improve beef safety and thereby preventing diarrheal illness linked to beef consumption in Ethiopia.

To reduce the risk of meat contamination, implementation and monitoring of the Codex general principles of food hygiene and code of hygienic practice for meat and local food safety proclamations are required. Capacity building and awareness raising of all actors such as meat inspectors, employees at slaughterhouses and retail shops, and health extension workers through provision of continuous professional development and educational sessions such as information campaign (e.g use of TV and radio, billboard etc) on food safety is therefore needed.

Further studies are required to better understand the epidemiological link among cattle production, beef consumption and foodborne diseases in humans:

- Mapping of the environmental contamination in the meat supply chain to identify all possible sources for the contamination of meat should be performed.
- Future, more in depth studies are required to identify and characterize *Salmonella* and *E. coli* O157 using both primary and secondary restriction enzymes from a large number of positive samples to clearly establish the role of cattle production and beef consumption in the development of diarrheal illness in humans in Ethiopia.
- An in-depth study is needed on the practice of antimicrobial usage in cattle production and in humans, as well as an investigation of the drivers and magnitude of the antimicrobial resistance.
- Further studies are also needed to characterize antimicrobial resistance patterns and resistance genes as additional information to investigate the relationship between *Salmonella* and *E. coli* O157 isolates along beef supply chain human isolates.
- A risk assessment of *Salmonella* and *E. coli* O157 linked with consumption of raw or under-cooked beef and kitifo-minced beef should be conducted.

- ❖ Additional study on the status of *Salmonella* and *E. coli* O157 in other food animals (e.g. sheep, goat, poultry) and food matrices (e.g. mutton, poultry) should be implemented, and exposure assessment conducted in human.

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Chapter 9

9. Summary

Chapter 1 of this thesis presents the current knowledge on the burden of foodborne diseases with particular emphasis to *Salmonella* and *E. coli* O157 infections associated with consumption of beef and beef products. Additionally, the occurrence of *Salmonella* in humans and cattle and the role of cattle in human infections are described. Furthermore, the chapter presents the taxonomy and characteristics of *E. coli* O157, *E. coli* O157 in humans, in cattle and the role of cattle in human infections, where after the detection methods, molecular characterization and antimicrobial resistance of *Salmonella* and *E. coli* O157 are described. Last, it presents the current status of *Salmonella* and *E. coli* O157, the beef production systems and beef consumption practices in Ethiopia.

Chapter 2 presents the aim and the objectives of this study. The overall aim of the study was to assess the potential association of cattle with the occurrence of human diarrheal illness due to *Salmonella* and *E. coli* O157 via consumption of beef.

In chapter 3, the global prevalence of *Salmonella* in apparently healthy cattle and the serotype diversity identified through a systematic review and meta-analysis are presented. Pooled prevalence of *Salmonella* in cattle was estimated at 9% (95% CI: 7-11%) with significantly high heterogeneity (I^2=98.7%, $P < 0.01$) among studies. Prevalence varied from 2% (Europe) to 16% (North America). Overall, 143 different serotypes were reported with the most diverse serotypes being reported from Africa (76 different serotypes) followed by North America (49 serotypes). *Salmonella* Montevideo and *S.* Dublin were the most frequently reported serotypes in North America and Europe, respectively, while *S.* Typhimurium was the most frequent in Africa, Asia and Australasia. Five of the top 10 serotypes (*S.* Montevideo, *S.* Typhimurium, *S.* Anatum, *S.* Mbandaka, and *S.* Newport) identified in this study are among the serotypes most commonly associated with clinical illnesses in humans. Variability both in the prevalence and serotype diversity of *Salmonella* in cattle across continents was observed.

Chapter 4 presents the prevalence of *Salmonella* in samples collected from 240 cattle (rectal content), 127 retail shops (beef), and 216 diarrheic patients (stool samples). *Salmonella* isolates were serotyped and then genotyped by pulsed field gel electrophoresis (PFGE). Antimicrobial resistance was assessed by MIC determination using EUVSEC sensititre plates. *Salmonella* was detected in 2.5% of cattle, from 8.7% of beef samples, and in 2.3% of the diarrheic patients. *S.* Typhimurium and *S.* E*astbourne* were isolated from cattle and beef while *S.* Saintpaul and *S.* Cotham were isolated only from diarrheic patients. Except for serotype *S.* Saintpaul, all isolates were grouped into five pulsotypes of which two pulsotypes contained isolates from cattle and beef. Isolates from humans represent unique pulsotypes. Among the 22 *Salmonella* isolates tested, 95.5 % were resistant to at least one of the 14 antimicrobials tested. Three isolates originating from cattle were multidrug resistant. One human isolate was susceptible to all antimicrobials tested. The most frequently observed resistance was to sulfamethoxazole (90.9 %, 20/22) followed by trimethoprim (22.7%, 5/22).

Chapter 5 focuses on the occurrence, virulence genes, genetic relatedness, and antimicrobial resistance of *E. coli* O157 assessed in the same samples presented in Chapter 4. From each positive sample, one isolate was further tested for the presence of the gene defining the somatic antigen O157, virulence genes coding for Shiga-toxins (*stx*1 and *stx*2) and intimin (*eae*). The isolates positive for the gene *stx2* were further subtyped. All the isolates were genotyped by pulsed field gel electrophoresis (PFGE). Antimicrobial resistance was assessed by MIC determination using EUVSEC sensititre plates. *E. coli* O157 was detected in 7.1% of the rectal content samples from cattle at slaughterhouses, in 6.3% of the beef samples, and in 2.8% of the diarrheic patients' stool samples. All isolates were positive for eae gene, 24 (77%) of them were positive for stx2 gene (21 stx2c and 3 stx2a), whereas stx1 gene was not detected. The isolates were grouped into eight PFGE pulsotypes with three pulsotypes containing isolates from all three sources, one pulsotype containing one isolate from human origin and one isolate from beef. With the exception of one multidrug resistant isolate from beef (resistant to 8 antimicrobial drugs), the remaining 30 isolates were susceptible to the 14 antimicrobials tested.

Chapter 6 describes the hygienic practices in cattle slaughterhouses and retail shops. Data were collected through questionnaire surveys and observations. Twenty eight employees from 2 cattle slaughterhouses and 127 employees from all meat retail shops (one employee from each) participated in the study. At the slaughterhouses, vertical dehiding and evisceration, carcass washing and separate storage of offals were identified as good practices. Manual hanging and handling, lack of hot water baths, no demarcation between clean and dirty areas, absence of a chilling room, infrequent hand washing, insufficiently trained staff and irregular medical check-up were identified as risky practices. At the retail shops, cleaning equipment using soap and hot water (81%), storing unsold meat in refrigerator (92%), concrete floors and white painted walls and ceilings were the good practices. Adjacently displaying offal and meat (39%), lack of cold chain, wrapping meat with plastic bags and newspapers, using plastic or wooden cutting boards (57%), infrequent washing of equipment and floors, and inadequately trained employees were risky practices.

Chapter 7 presents the potential sources of carcass contamination with *Salmonella* and *E. coli* O157 at cattle slaughterhouses. From 70 carcasses following samples were collected: rectal content, pooled hide swab sample and carcass sample. *Salmonella* was recovered from 7.1% of rectal content and hide samples and 2.8% of carcass samples, while *E. coli* O157 was detected in 8.9% of feces, 4.3% of hide and 2.4 % of carcass samples. Two animals harbored both bacteria concomitantly. *Salmonella* was detected simultaneously from feces and carcass samples in two animals identified as genetically similar *S.* Typhimurium serotypes. *E. coli* O157 was detected from two samples concomitantly in four animals: hide and carcass (n=1), feces and hide (n=1), and feces and carcass (n=2). Only the two isolates from the first animal were genetically identical. All 14 *E. coli* O*157* isolates were positive for *eae* and *ehxA* genes and negative for *stx*1; *stx*2 (10 stx2c and 2 stx2a) was detected in 85.7% (12/14) of the isolates. Antimicrobial resistance to at least one of the tested antimicrobials and multi-drug resistance were observed in 62.5% and 50% of *Salmonella* isolates, respectively. *Salmonella* resistant to ampicillin, chloramphenicol, sulfamethoxazole, tetracycline and colistin were observed. *E. coli* O*157* isolates were susceptible to all tested antimicrobials.

A general discussion is found in **chapter 8**. Overall, the research shows the potential transfer of *Salmonella* and *E. coli* O157 from cattle-to-beef-humans that can lead to diarrheal illness in susceptible people. The study revealed some genetic similarities among *E. coli* O157 in cattle, beef and humans and between *Salmonella* in cattle and beef. Unique *Salmonella* serotypes were identified from diarrheic patients. A considerable number of resistant *Salmonella* to commonly antimicrobials used in humans was observed. The study indicated the importance of frequent occurrence of cross contamination besides the direct contamination of carcasses by feces and hide of animals. However, more robust studies are required to establish the direct epidemiological link.

10. **Samenvatting**

Hoofdstuk 1 van dit proefschrift wordt een overzicht gegeven van de huidige kennis omtrent voedselgebonden ziekten met bijzondere aandacht voor *Salmonella* en *E. coli* O157 infecties geassocieerd met de consumptie van rundvlees en rundvleesproducten. Bovendien wordt het voorkomen van *Salmonella* bij mensen en runderen en de rol van runderen voor humane infecties beschreven. Verder wordt ingegaan op de taxonomie en de karakteristieken van *E. coli* O157, *E. coli* O157 bij mensen, runderen en de rol van runderen voor humane infecties. Verder wordt ingegaan op de detectiemethoden, moleculaire karakterisering en antimicrobiële resistentie van *Salmonella* en *E. coli* O157. Ten slotte wordt de huidige *Salmonella* en *E. coli* O157 status, de rundveeproductiesystemen en de rundvleesconsumptiepraktijken in Ethiopië besproken.

Hoofdstuk 2 beschrijft de doelstellingen van deze studie. De studie had tot doel om de mogelijke associatie van rundvee met het voorkomen van diarree bij de mens, veroorzaakt *Salmonella* en *E. coli* O157 via de consumptie van rundvlees, te onderzoeken.

In een eerste studie werd een inschatting gemaakt van de *Salmonella* prevalentie en de serotype-diversiteit bij gezonde runderen aan de hand van een systematische literatuurstudie en meta-analyse (**hoofdstuk 3)**. De wereldwijde *Salmonella* prevalentie bij runderen werd geschat op 9% (95% BI: 7-11%) met een significant hoge heterogeniteit (I^2 = 98,7%, P <0,01) tussen de onderzoeken. De gemiddelde prevalentie varieerde van 2% in Europa tot 16% in Noord-Amerika. In totaal werden 143 verschillende serotypes gerapporteerd, waarbij het grootst aantal serotypen in Afrika (76 serotypes) gevolgd door Noord-Amerika (49 serotypes). *Salmonella* Montevideo en *S.* Dublin waren de meest frequent gemelde serotypen in respectievelijk Noord-Amerika en Europa, terwijl *S.* Typhimurium het meest frequent voorkwam in Afrika, Azië en Australazië. Vijf van de top 10 serotypen (*S.* Montevideo, *S.* Typhimurium, *S.* Anatum, *S.* Mbandaka en *S.* Newport), die in deze analyse werden geïdentificeerd, behoren tot de serotypes die frequent worden geassocieerd met infecties bij mensen. Variabiliteit in zowel de prevalentie als de diversiteit van *Salmonella* serotypes bij runderen over de continenten werd waargenomen.

Vervolgens werd het voorkomen van *Salmonella* in monsters verzameld bij 240 runderen (rectuminhoud), 127 vleeswinkels (rundvlees) en 216 patiënten met diarree (stoelgang) bestudeerd

(**hoofdstuk 4)**. *Salmonella* isolaten werden geserotypeerd en vervolgens genetisch getypeerd door middel van pulsed field gel elektroforese (PFGE). Antimicrobiële resistentie werd opgespoord met behulp van de MIC methode. *Salmonella* werd gedetecteerd bij 2,5% van de runderen, bij 8,7% van de rundvleesmonsters en bij 2,3% van de diarree-patiënten. *S.* Typhimurium en *S.* Eastbourne werden geïsoleerd bij runderen en rundvlees, terwijl *S.* Saintpaul en *S.* Cotham alleen werden geïsoleerd bij patiënten met diarree. Met uitzondering van *S.* Saintpaul, werden alle isolaten gegroepeerd in vijf pulsotypen, waarvan twee pulsotypes isolaten van zowel runderen als rundvlees bevatten. Isolaten van mensen behoorden tot unieke pulsotypes. Van de 22 geteste *Salmonella* isolaten was 95,5% resistent tegen tenminste één van de 14 geteste antimicrobiële producten. Drie isolaten afkomstig van runderen waren multiresistent. Eén humane isolaat was gevoelig voor alle geteste antimicrobiële producten. De meest frequent waargenomen resistentie was tegen sulfamethoxazol (90,9%, 20/22), gevolgd door trimethoprim (22,7%, 5/22).

Hoofdstuk 5 concentreert zich op het voorkomen, virulentiegenen, genetische verwantschap en antimicrobiële resistentie van *E. coli* O157 aanwezig in dezelfde monsters in hoofdstuk 4 onderzocht. Van elk positief monster werd één isolaat getest op de aanwezigheid van het gen verantwoordelijk voor de aanmaak van de somatische antigeen O157 en de virulentiegenen die coderen voor Shiga-toxines (*stx*1 en *stx2*) en intimine (*eae*). Van de isolaten, positief voor het gen *stx2*, werden ook het subtype bepaald. Alle isolaten werden genetisch getypeerd met behulp van pulsed field gel elektroforese (PFGE). Antimicrobiële resistentie werd beoordeeld door MIC-bepalingen met behulp van EUVSEC-gevoelige platen. *E. coli* O157 werd gedetecteerd in 7,1% van de rectuminhoudmonsters van runderen in slachthuizen, in 6,3% van de rundvleesmonsters en in 2,8% van de ontlastingsmonsters van diarree-patiënten. Alle isolaten waren positief voor het *eae*-gen, 24 (77%) van hen waren positief voor het *stx2*-gen (21 *stx2c* en 3 *stx2a*), terwijl het *stx1*-gen niet werd gedetecteerd. De isolaten werden gegroepeerd in acht PFGE-pulsotypen, waarvan drie pulsotypen die isolaten uit de drie bronnen bevatten en een pulsotype met één isolaat van humane oorsprong en één isolaat van rundvlees. Met uitzondering van één multiresistente isolaat uit rundvlees (resistent tegen 8 antimicrobiële geneesmiddelen), waren de overige 30 isolaten gevoelig aan de 14 geteste antimicrobiële stoffen.

Hoofdstuk 6 beschrijft de hygiënepraktijken in rundveeslachthuizen en vleeswinkels. De gegevens werden verzameld door middel van vragenlijsten en observaties. Achtentwintig medewerkers van 2 rundveeslachthuizen en 127 medewerkers van alle vleeswinkels (één medewerker per winkel) namen deel aan het onderzoek. In de slachthuizen werden de onthuiding en de verwijdering van de ingewanden in verticale positie van de karkassen, het wassen van de karkassen en de gescheiden opslag van slachtafval als goede praktijken beschouwd. Handmatig ophangen en hanteren, gebrek aan warmwaterbaden, geen afbakening tussen schone en vuile ruimtes, afwezigheid van een koelruimte, weinig handen wassen, onvoldoende opgeleid personeel en onregelmatige medische controle werden als risicovolle praktijken aangemerkt. In de winkels waren het schoonmaken van apparatuur met zeep en warm water (81%), het opslaan van onverkocht vlees in koelkasten (92%), betonnen vloeren en wit geschilderde muren en plafonds de goede praktijken. Het tentoonstellen van slachtafval en vlees (39%) naast elkaar, het ontbreken van een koelketen, het verpakken van vlees in plastic zakken en kranten, het gebruik van plastic of houten snijplanken (57%), het onregelmatig wassen van apparatuur en vloeren en onvoldoende opgeleide werknemers waren risicovolle praktijken.

In een laatste studie (**hoofdstuk 6)** werden mogelijke bronnen voor contaminatie van karkassen met *Salmonella* en *E. coli* O157 in rundveeslachthuizen onderzocht. Van 70 karkassen werden de volgende monsters verzameld: rectuminhoud, gepoold huidswab en karkasswabs. *Salmonella* werd geïsoleerd uit 7,1% van de rectuminhoud- en huidmonsters en 2,8% van de karkasmonsters, terwijl *E. coli* O157 werd aangetroffen in 8,9% van de rectuminhoud- , 4,3% van de huid- en 2,4% van de karkasmonsters. Van 2 dieren was de rectuminhoud en huid positief voor beide bacteriën. *Salmonella* werd gedetecteerd uit de rectuminhoud en één karkasmonster bij twee dieren en de isolaten waren genetisch identiek. *E. coli* O157 werd gedetecteerd in twee monsters van 3 dieren: huid en karkas (n = 1), rectuminhoud en huid (n = 1), en rectuminhoud en karkas (n = 2). Alleen de twee isolaten van het eerste dier waren genetisch identiek. Alle 14 *E. coli* O157 isolaten waren positief voor het *eae* en *ehxA* gen en negatief voor *stx1*; *stx2* (10 *stx2c* en 2 *stx2a*) werd gedetecteerd in 85,7% (12/14) van de isolaten. Antimicrobiële resistentie tegen ten minste één van de geteste antimicrobiële producten en resistentie tegen meerdere producten werd waargenomen bij respectievelijk 62,5% en 50% van de *Salmonella* isolaten. Er werd bij *Salmonella* resistentie

waargenomen tegen ampicilline, chlooramfenicol, sulfamethoxazole, tetracycline en colistine. *E. coli* O157 isolaten waren gevoelig voor alle geteste antimicrobiële producten.

De algemene discussie is te vinden in **hoofdstuk 8**. Over het geheel genomen, toont het onderzoek de mogelijke overdracht aan van *Salmonella* en *E. coli* O157 van runderen via rundvlees op mensen, wat kan leiden tot diarree bij gevoelige mensen. De studie bracht enkele genetische overeenkomsten aan het licht tussen *E. coli* O157 bij runderen, rundvlees en mensen en tussen *Salmonella* bij runderen en rundvlees. Er zijn unieke *Salmonella*-serotypes geïdentificeerd bij diarree-patiënten. Er werd een aanzienlijk aantal resistente *Salmonella* waargenomen tegen veelvoorkomende antimicrobiële producten die bij mensen worden gebruikt. De studie wees op het belang van het frequent voorkomen van kruisbesmetting naast de directe contaminatie van karkassen door feces en huid van dieren. Er zijn echter meer robuuste studies nodig om dit directe epidemiologische verband vast te stellen.

11.**Curriculum vitae**

11.1. Contact address

Fanta Desissa Gutema
Addis Ababa University,
College of Veterinary Medicine and Agriculture
Department of Microbiology, Immunology and Vetrinary Public health
P.O.Box 34, Bishoftu, Ethiopia
E-mail: fanta.desissa@aau.edu.et; fantadesissa@gmail.com
T: +251 911 030 412; Skype: fanta.desissa1

11.2. Academic Qualifications

1. Doctor of Philosophy in Veterinary Science, Gent University, 2021
2. Masters of public health (MPH), Adama General Medical College, 2016
3. Masters in Veterinary public health (MSc), Addis Ababa University, 2010
4. Bachelor degree in Public health officer (BSc), Kea-Med University College, 2012
5. Doctor of Veterinary Medicine (DVM), Addis Ababa University, 2006

11.3. P**ublications**

1. **Gutema FD**, Rasschaert G, Agga GE, Seyoum F, Addisu BD, Abdi RD, Luc Duchateau L, Gabriël S, De Zutter L. Assessment of Beef Carcass Contamination with *Salmonella* and *E. coli* O 157 in Slaughterhouses in Bishoftu, Ethiopia. ***International Journal of Food Contamination*** (**Submitted**).
2. **Gutema FD**, Agga GE , Abdi RD, Alemnesh J, Duchateau L, Gabriël S, De Zutter L. Assessment of Hygienic Practices in Beef Cattle Slaughterhouses and Retail Shops in Bishoftu, Ethiopia: Implications for Public Health. ***International Journal of Enviromental Research and Public Health***, 2021, 18, 1-13.
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12. Supplementary File

I. Questions and check list to assess hygienic handling practices at slaughterhouses

A. Basic information

1. Date _____
2. Code _____

B. Sociodemographic characteristics of the respondents

1. Sex: Male [ ] Female[ ]
2. Age: _____
3. Level of Education: Illiterate [ ] Informal Education [ ] Primary Education [ ] Secondary Education [ ] Other (Specify) _____
4. Duration of working at the slaughter houses (in years): _____
5. Duty at the slaughter houses: Veterinarian/meat inspector [ ] Stunning [ ] De-hiding [ ] Other (specify) _____

C. Hygienic handling practices at slaughterhouses

C-1. Check list for observations

1. Stunning before slaughter: Yes[ ] No [ ]
2. Stunning method: _____
3. Waiting time to start dehiding after stunning and bleeding: _____
4. Method of carcass dressing: Vertical (hanging) Horizontal (on floor):
5. Presence of sink for washing hands in the slaughterhouse: Yes [ ] No [ ]
6. Carcass washing after evisceration: Yes [ ] No [ ]
7. Use of the following protective materials while working in the slaughterhouse:

Protective materials	Response	
	Yes	No
Apron		
white coat		
Head cover		
Gloves		
Boots		

C-2. Questions for face to face interviews

1. Do you wash your hands with soap: Yes [ ] No [ ]
2. Do you wash your hands in between activities: Yes [ ] No [ ]
3. Do you sink the knife in hot water after each activity: Yes [ ] No [ ]
4. What do you think is the major possible sources for carcass contamination: [ ] Feces during evisceration [ ] hides during dehiding [ ] handlers hand [ ] knife [ ] floor [ ] hanging hook [ ] Others(specify)_____
5. What is the source of water used in the slaughterhouse: City/Municipal tap water [ ] borehole [ ] collected rain water [ ] River [ ] others (specify) _____
6. Have you ever received any training on hygienic handling of meat: Yes [ ] No [ ]
7. Have you gone for medical checkups to work at the slaughterhouse: Yes [ ] No [ ]
8. If yes, how frequent you go for medical checkup: every three months [ ] every six months [ ] once per year [ ]
9. Do you think improvements are needed to avoid contamination of carcass at the slaughterhouse:
   Yes [ ] No [ ]
10. If yes, what kind of improvement: _____

II. Questions for face to face interview and check list for direct observation to assess hygienic handling practices at beef retailshops

A. Basic information

1. Date: _____
2. Code: _____

B. Sociodemographic of characteristics

1. Sex: Male [ ] Female[ ]
2. Age: _____
3. Level of Education: Illiterate [ ] Informal Education [ ] Primary Education [ ] Secondary Education [ ] Other (specify)_____
4. Duration of working at meat retail outlet (in years): _____

5. Religion: _____
6. Ethnicity: _____

C. Hygienic handling practices at slaughterhouses

C-1. Check list for observations

1. Presence of any cover on meat display case: Yes [ ] No [ ]
2. Retail shop floor is made of: Concrete [ ] Tile [ ] Wood earthen material [ ]
   others(specify)_____
3. Wall and ceiling are clean or free of dust: Yes [ ] No [ ]
4. Wall painted with white color: Yes [ ] No [ ]
5. Ventilation status of display case and butchery: Good [ ] Fair [ ] Poor [ ]

   Good-ventilation allows air flow into the butchery but sieves off dust and other particles [ ]

   Fair-ventilation allows air flow but do not sieve dust or other particles or allows very little air flow [ ]

   Poor-ventilation does not allow air flow at all [ ]
6. Presence and use of bulbs at the display case: Yes [ ] No [ ]
7. Meat cooling facility (refrigerator) at the display cabinet: Yes [ ] No [ ]
8. Presence of sink for washing hands at beef sale point: Yes [ ] No [ ]
9. Type/kind of cutting board used: Wood [ ] plastic [ ] Metal [ ] concrete [ ] Marble [ ]
10. Presence of hot water baths for dipping knives: Yes [ ] No [ ]
11. Material used to pack or wrap meat for sale: Newspaper[ ] Plastic [ ] Used paper[ ]
12. Use the following protective materials while selling or handling meat:

Protective materials	Response	
	Yes	No
Apron/white coat		
Head cover		
Gloves		

C-2. Questions for face to face interviews

1. What is the means of transporting meat from slaughterhouse to the retail shop: Open vehicle [ ] Closed vehicle [ ] Animal transport (Cart horse) [ ]
2. How frequent do you use washed the protective coat (white coat and apron): Once per day in the
   evening [ ] Twice per day, morning and evening [ ] Once after every two days [ ] Once per week [ ]
   others [ ] _____
3. Do you have a refrigerator for storage of the meat that remains from daily sale: Yes [ ] No [ ]
4. Do you wash your hands before touching meat: Yes [ ] No [ ]
5. Do you wash your hands with soap: Yes [ ] No [ ]
6. What is the source of water for use in the butchery: City/Municipal tap water [ ] borehole [ ] rain collected water [ ] river [ ] others (specify) [ ]
7. How often do you wash the following butchery surfaces and equipment:

Frequency of washing	Equipments /surfaces					
	Knife	Cutting boards	Saw/Axes	Display cabinet	Hooks	Floors
Once per day in the morning						
Once per day in the evening						
Twice per day						
More than twice						
Once in every two days						
Others (specify)						

8. Do you use detergent/disinfectant for cleaning the butchery utensils: Yes [ ] No [ ]

9. If "Yes" what types of detergent/disinfectant:_____
10. What is the way of cleaning butchery equipment: Using cold water only [ ], cold water with soap [ ], hot water only [ ] hot water with soap [ ] wiping with pieces of fabrics [ ] others (specify)_____
11. Is there routine control of flies at the retail shop: Yes [ ] No [ ]
12. If "Yes", what is the method used to control flies: _____
13. How long does the meat stay in your butchery before it is sold: Less than 12 hours [ ] One day [ ] Two days [ ]
14. Do you collect money while handling or selling meat: Yes [ ] No [ ]
15. Have you ever received any training on hygienic handling of meat: Yes [ ] No [ ]
16. Do you ever receive complaints from the consumers on the quality of the meat you sell: Yes [ ] No [ ]
17. If yes, what kind of complaint: Abdominal upsets [ ] Tough meat [ ] Dirty meat [ ] others [ ]_____
18. Have you gone for medical checkups in the last 6 months: Yes [ ] No [ ]
19. How frequent you go for medical checkup: Every three months [ ] very six months [ ] Once per year [ ]
20. Do you have different storage and display cabinets for offal's and meat: Yes [ ] No [ ]
21. Do you use the same equipment's such as knife while handling meat and the offals: Yes [ ] No [ ]
22. Do you believe that the butchery where are you working requires some improvement for better handling of meat: Yes [ ] No [ ]
23. If yes, what kind of improvement:_____

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