MOLECULAR IDENTIFICATION OF VERTEBRATE SOURCES AND POTENTIAL ZOONOTIC PATHOGENS IN THE MEAT VALUE CHAIN FROM SELECTED VENDORS IN NAIROBI, KENYA.

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DECLARATION

This is to testify that this thesis is my original work and has not been presented for award of degree in any other University.

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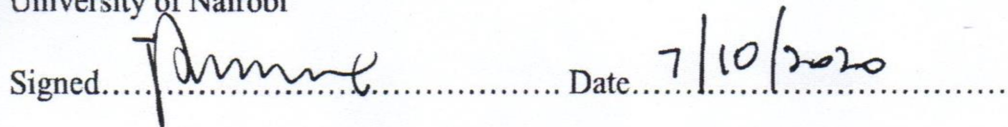

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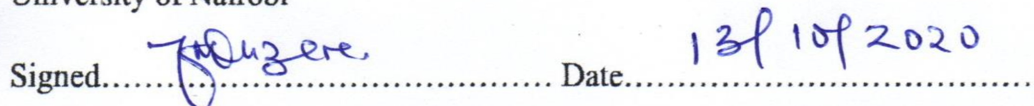

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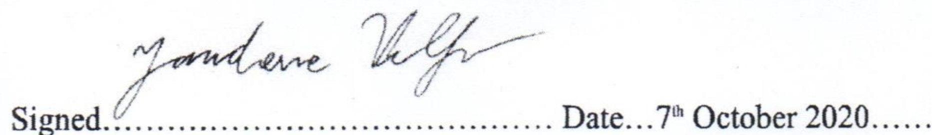

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This work is dedicated to my mother Elmina Saru Mghalu, whose support and guidance have been instrumental in driving me towards academic excellence. May she live to see all her children do great things.

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LIST OF ABBREVIATIONS AND ACRONYMS

ANOVA -Analysis of Variance

ASAL- Arid and semi-arid lands

BLAST- Basic Local Alignment Search Tool

BSE- Bovine Spongiform encephalopathy

CAAT- cross agglutination absorption test

CO1- Cytochrome oxidase subunit1

Cyt b- Cytochrome B

DEG- diethylene glycol

DNA- Deoxyribonucleic acid

EPZA- Export Processing Zonal Authority

EU- European Union

FAO- Food and Agriculture Organization

FBAT- febrile antigen Brucella agglutination test

GC content - guanine-cytosine content

GOK – Government of Kenya

HRM – High-resolution melting

HRMA- High-resolution melting analysis

ITS- internal transcribed spacer region

KEBS- Kenya Bureau of Standards

KNBS- Kenya National Bureau of Statistics

KShs- Kenya Shillings

KWS- Kenya Wildlife Services

LTM- Local terminal market

LPC- Large Processing Companies

MAT- Microscopic agglutination test

MM- meat markets

mtDNA- mitochondrial Deoxyribonucleic acid

NCBI- National Center for Biotechnology Information

NTC- Non- template control

PCR- Polymerase Chain Reaction

Q fever- query fever

RAPD- randomly amplified polymorphic DNA

RASFF- Rapid Alert System for Food and Feed

RFLP- restriction fragment length polymorphism

SMAC- sorbitol MacConkey agar culture

Tm- Melting temperature

UK- United Kingdom

USAID- United States Agency for International Development

USD- United States Dollars

vCJD- variant Creudzfeldt Jacob disease

WHO- World Health Organization

ABSTRACT

The steady increase in the utilization of meat products has led to a rise in demand as well as its market price. Substitution of meat from animal species of higher commercial value with those from cheaper or undesirable species occurs often, posing ethical, religious, and dietary concerns. In Nairobi and Naivasha, there have been reports of bushmeat and undeclared species sold to unsuspecting customers. The aim of the study was to identify the vertebrate sources of meat sold to consumers in Nairobi hence determining if there was any species substitution in the meat value chain; and also, to detect potential pathogens in the meat sold to consumers.

A total of 115 meat samples were obtained randomly from butcheries in Burma Market, Nairobi, and its surrounding areas. Additionally, seven goat samples were obtained from Kiamaiko as controls. Extraction of DNA from these samples was done using the ISOLATE II Genomic DNA extraction kit (Bioline, UK) and determination of species and bacterial screening carried out. Furthermore, ninety-nine meat samples similarly obtained from Naivasha butcheries and stored at -80°C were also analyzed for presence of bacterial pathogens, making the total number of samples 221. Species identification was done using high-resolution melting (HRM) analysis of the PCR products. Three mitochondrial DNA markers, cytochrome oxidase subunit 1 (CO1), cytochrome b (cyt b), and 16S rRNA genes were targeted to identify vertebrate species. Presence of selected bacterial zoonotic pathogens was evaluated through analysis of HRM of PCR products targeting the genes *bcsp31*, *secYIV*, *PL3* and *IS1111* for *Brucella* spp., pathogenic *Leptospira* spp., *Bacillus anthracis* and *Coxiella burnetii* respectively. Additionally, sequencing of PCR amplified products using universal 16S rRNA was utilized to screen for more bacterial contaminants.

The study detected incidence of species substitution in the meat value chain, with 15/115 samples collected in Nairobi found to be substituted. Presence of zoonotic bacteria that pose a risk to individuals involved in the meat value chain was also detected with 27/221 of the samples being positive for pathogenic *Brucella,* and 1/221 of the samples being positive for *Leptospira interrogans.* None of the samples were positive for *Bacillus anthracis* or *Coxiella burnetii.* Using the universal 16S rRNA gene primers allowed the amplification of food-borne pathogens including *Lactococcus garvieae*, *Clostridium* spp. and *Aeromonas caviae* in 3% of the samples, where 7/221 samples were found to have bacteria that are enteropathogenic or enterotoxin producing. These findings reveal that there may be loopholes in the meat value chain that could place consumers at risk of allergic reactions due to undeclared meat species, and to public health because of the zoonotic and food-borne bacterial pathogens present. However, no correlation could be found between species substitution and pathogens.

CHAPTER ONE

1.0. INTRODUCTION

1.1 Background to the study

Meat consumption has steadily risen because of the increasing demand for animal products such as meat in most parts of the world (Cawthorn *et al.*, 2013). This demand is mostly driven by the growth in population, elevation of incomes, urbanization, and changing consumer preferences (Delgado, 2003; Gamba, 2005). Based on research carried out by the Food and Agriculture Organization (FAO) of the United Nations, the global meat output stood at 336.4 million tonnes by the year 2018, a 1.2% increase from 2017 (FAO, 2019). This increase is also reflected in countries in sub-Saharan African like Kenya, where the recorded purchase and slaughter of livestock by licensed abattoirs between 2010 and 2016 rose with a collective count of over 2.6 million livestock (Kenya National Bureau of Statistics, 2018).

This growth in demand has in turn increased the total value of meat products. For instance, the value of products from cattle and calves grew from KShs 8,886.4 million to KShs 11,476.1 million between the years 1999 and 2003 (Export Processing Zonal Authority, 2005; Shibia *et al.*, 2017). Higher market value of meat and meat products makes this product more susceptible to food fraud (misrepresentation, adulteration with undesirables, and switching of species (Cawthorn *et al*., 2013)). Meat adulteration carried out intentionally usually entails

the addition or substitution of animal proteins or meat with other cheaper or undesirable alternatives such as plant proteins (soya and gluten) (Spink & Moyer, 2011; Cawthorn *et al.*, 2013).

Although the incentive for food fraud is often financial or economic, its result is a tangible vulnerability to public health, because even if a public health event does not ensue, the adulteration or misrepresentation could create the likelihood for harm (Spink & Moyer, 2011). Owing to the different motivations driving food fraud, simple food safety methods may not be the most efficient at identifying and deterring it.

Food fraud instances have been described in many countries resulting in public health problems and even mortality (Bouzembrak *et al.*, 2018). In the past decade, food fraud has become a major threat in several countries due to the direct implications on public health as well as international livestock trade (Cartín-Rojas, 2018). It is estimated that in 2014, the global cost of fighting food fraud and adulteration was more than USD 79 billion.

The present age of food legislations started with the U.S. Federal Food, Drug and Cosmetic (FD&C) Act in 1938 (Spink & Moyer, 2011). This law was put in effect due to the poisoning of numerous patients by medicine that had diethylene glycol (DEG). In the EU, the body of food law was developed to facilitate the production and sale of safe food (McEvoy, 2016).

In the horsemeat scandal that occurred in the UK and Ireland in 2013, the Rapid Alert System for Food and Feed (RASFF) was used for the interchange of pertinent data during this time, as well as to protect consumers (Bouzembrak *et al.*, 2018). There was also an immediate implementation of traceability checks and an action plan by the European Commission to battle against food fraud. This was to reinforce the European Union system as well as to restore the confidence of consumers. Several countries are coming up with regulations and frameworks to tackle this problem. The enactment of the European Parliament Resolution of 2014 on Fraud in the Food Chain (Acutis *et al.*, 2019) kick started frameworks to monitor such fraud in the regional and national level. These are being enforced to address the traceability and labeling of food products through general laws and regulations (Kets, 2016). Various reports already depict active monitoring of fraud in meat value chains. For instance, all of the member states of the EU are expected to convey the results of their inspection to the Commission every year, with the Commission publishing the overall report throughout the EU (McEvoy, 2016).

This move has been seen in Africa as well, with regulatory bodies, such as those in South Africa, publishing new regulations that encourage transparency as well as the appropriate description of food items (Cawthorn *et al.*, 2013). The Kenya Food and Drugs Authority Bill 2019 also prohibits the sale of unsafe, toxic, or adulterated food (Kenya National Assembly, 2019). The country has a monitoring system to control food safety, that spans various sectors and involves several Ministries as well as regulatory bodies. These bodies are mandated to carry out their tasks by

legislation or Acts of Parliament e.g. Department of Public Health ensures consumer protection based on laws such as the Meat Control Act Cap 356 (Government of Kenya, 2012), Public Health Act Cap 242 (Rev. 2002) and the Food Drugs Chemical Substances Act Cap 254 (Rev 2002)

Such practices raise ethical, religious, dietary, and moral concerns where consumers end up buying meat from species they would not normally eat (Chuah *et al.*, 2016; Abbas *et al.*, 2018). The presence of undeclared animal species in the meat also raises food safety issues where it can be a health liability to those with allergies (Di Pinto *et al.*, 2015), as well as bring about public health risks, e.g. food-borne and zoonotic diseases in cases where the substituting species are obtained from unconventional sources such as bushmeat (wildlife), subjected to unhygienic handling, or may not have undergone quality checks such as meat inspection (Ouso et al., 2020).

Some common food-borne bacterial pathogens in Kenya that are zoonotic in nature include *Brucella* sp., *Bacillus anthracis*, pathogenic: *Leptospira* sp.; *Salmonella* sp., and enterotoxigenic *Escherichia coli*. Livestock have large populations of microbiota, including fungi, archaea, bacteria and protozoa (Jami & Mizrahi, 2012), especially in their digestive tract. Several studies have disclosed the presence of pathogenic pathogens such as Brucella, Salmonella, Escherichia coli O157:H7, Campylobacter, and Listeria (Morales-Estrada *et al*., 2016) in animal manure, which could be transmitted to other animals through direct contact, inhalation and

consumption of contaminated water. Although some of these pathogens could be transmitted from animals to humans through arthropods like ticks, stable flies, and lice (Wang *et al.*, 2018), they are also transferred to people through ingestion or direct contact with contaminated animal tissue. Such zoonotic diseases affect both wild animals and economically important domestic animals like cattle and small ruminants (Alexander *et al.*, 2012).

Although there are protocols put in place by legislation to ensure meat quality and protection of consumer health (Kenya Bureau of Standards, 2017), there are still reported incidences of species substitution in the country. This includes the economically motivated cases of cat and dog meat (Otieno, 2018) as well as game meat such as zebra and giraffe being found in major meat markets (Wa Maina, 2018). This poses a health risk as these animals are often acquired illegally or unethically and have not gone through inspection leading to the sale of contaminated meat (Garba *et al*., 2013). Additionally, there have been fatalities in Kenya of individuals who ate infected meat and succumbed to bacterial and viral diseases like Rift-valley fever , brucellosis and anthrax (Munyua *et al.*, 2016). Such outbreaks affect not only the public health of our country, but also have a socio-economic impact and can cause great economic losses.

In light of the global trends that depict a steady increase in the demand for meat, monitoring food fraud in the meat supply chain is important, not just to protect consumers financially, but from possible contaminants that could pose a threat to

their health. In this study, the novel technique, polymerase chain reaction followed by high-resolution melting analysis (PCR-HRM) was used to establish the occurrence of species substitution in the meat value chain in Nairobi, and screen for possible zoonotic pathogens in meat sold to consumers in both Nairobi and Naivasha.

1.2. Statement of the Problem

Meat species substitution, along with other forms of fraud in the supply of meat pose a risk to public health. This is especially true because the undeclared species are mainly obtained through irregular channels (McEvoy, 2016). For instance, in the horsemeat scandal of 2013 in the UK and Ireland, beef was substituted with horse meat (O'Mahony, 2013; Di Pinto *et al.*, 2015) and in the country of China, mutton was substituted with murine meat (Fang & Zhang, 2016) i.e. meat from mice and related rodents. Although rodents are considered delicacies in some parts of Kenya such as Kilifi (Lwanga & Atieno, 2019), there have been no recorded cases of these species being sold to unknowing customers. However, there is inadequate information concerning this phenomenon in the country's meat value chain.

This raises concerns of presence of zoonotic pathogens, or pharmacologically active substances or toxins that are a risk for public health. One such case was the link made between meat from animals affected by bovine spongiform encephalopathy (BSE) and the incidence of the new fatal variant Creutzfeldt Jacob disease (vCJD), which was found in people in the European Union (McEvoy, 2016). According to

the World Health Organization (WHO), there were 224 recorded cases of vCJD globally, with 175 of these occurring in the UK between 1996 and 2011 (McEvoy, 2016). This was due to the consumption of beef from cattle that had acquired the causative agent through their own feed.

The problem in Kenya is that the vertebrate sources of illicit meats often found in urban markets are not confirmed. Research carried out by Alarcon *et al*., 2017a, indicated that dead animals including cattle, sheep, pigs and goats are often sold in slums, and through slaughterhouses where vets lives are threatened. This exposes consumers to food safety risks such as exposure to the pathogens that led to animal mortality. Previous publications have shown animals such as camels to transmit brucellosis, Rift Valley Fever, *Coxiella burnetti*, Crimean-Congo hemorrhagic fever, among others. With increase in prolonged drought experienced in sub-Saharan Africa as well as diversifying of food sources, camels are gaining popularity for their products such as meat and milk (Zhu *et al.*, 2019). This could place individuals throughout the food chain at risk of infection or spill-over into epidemics.

Therefore, it is imperative to have increased measures put in place, not just the visual inspection in slaughter houses (Kenya Bureau of Standards, 2017), but also additional consistent monitoring to ensure the safety of consumers (McEvoy, 2016). Due to the laid-out regulations and guidelines detailing the process of meat inspection (GOK, 2012) and the reports of coercion (where the lives of vets are

threatened by farmers to ensure sale of dead animals through abattoirs (Alarcon *et al.*, 2017a)) a survey of meat inspection practices was considered too complex. However, determining the application of rapid and sensitive techniques such as PCR-HRM, to determine vertebrate species and detect bacterial contaminants would be advantageous in monitoring of the meat quality.

1.3 Justification

The slaughter and supply of meat in Kenya is carried out by a network of individuals and organizations who are guided through the Meat Control Act, defined in Chapter 356 of the Laws of Kenya (GOK, 2012). This law requires that all animals to be slaughtered undergo ante- and post-mortem inspection by qualified meat inspection officers (GOK, 2012) before being sold for human consumption. The act also stipulates which animal species are permitted for human consumption, as well as the slaughter processes, which should be done humanely, under hygienic conditions in slaughter houses or slaughter slabs. It also regulates how meat should be stored, transported, and handled by vendors. The occurrence of undisclosed meat species (Barnes, 2018; Mutahi, 2017; Ureport, 2018) in the meat sold to consumers reveals a surveillance deficiency in this control system. This deficit could not only allow unapproved meat species such as bushmeat (Kimwele *et al.*, 2012; Ouso *et al.*, 2020), but could also introduce zoonotic pathogens into the food chain, that can cause millions of cases of sporadic illnesses, challenging outbreaks (Munyua *et al.*, 2016) or chronic complications (Heredia & García, 2018).

The reports of the sale of bushmeat (Kimwele *et al*., 2012; Ouso *et al*., 2020) or even dead animals (Alarcon *et al.*, 2017a) raises the concern of public health safety as it creates a possible mode of entry of dangerous zoonoses into the food chain. The presence of bushmeat sold in the meat markets could also indicate a weakness in the enforcement of wildlife conservation efforts. Use of a novel high-resolution melting (HRM) analysis technique allows for quick and accurate distinction of vertebrate sources of meat. Furthermore, the survey of possible pathogens present in the meat could reveal if handling the meat available in the market puts humans at risk.

1.4 Research Objectives

1.4.1 Overall Objective

To investigate vertebrate meat sources sold in Nairobi and compare potential bacterial pathogens in the meat value chain Nairobi and Naivasha, Kenya.

1.4.2 Specific objectives

1. To identify vertebrate sources of meat sold for human consumption by vendors in Nairobi using PCR-HRM.
2. To investigate presence of zoonotic bacterial pathogens including *Leptospira*, *Bacillus*, and *Brucella* species in meat sold for human consumption in Naivasha and Nairobi.

1.5 Hypothesis

i) There are cases of species substitution in the meat value chain in Nairobi.

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 Food fraud in the food value chain

The fraudulent misrepresentation of food to consumers is a global phenomenon, especially with products of higher economic value such as virgin olive oil, Basmati rice, meat, and other products (Woolfe & Primrose, 2004; Silvis *et al.*, 2017). Currently, consumers are more conscious of the significance of food origin, ingredients, and labeling, which guides them when making purchases (Vlachos *et al.*, 2016). However, there are several ways that food products can be improperly described. These include: substitution of one ingredient by one of less economic value; sullying of food with a core ingredient; for instance, water, non-declaration of a process used in the making of the food product, and over-declaring the quantity of an ingredient (Woolfe & Primrose, 2004).

This kind of fraudulent activity has taken place since antiquity, as illustrated by evidence of regulations in times of ancient Rome concerning the adulteration of wine by adding colouring agents, sweeteners or watering it down (Handford *et al.*, 2016). Several high-value products including Basmati rice (which is valued for its taste and distinctive aroma), premium varieties of tuna, virgin olive oil, milk (Handford *et al.*, 2016), vegetables (Woolfe & Primrose, 2004; Panghal *et al.*, 2018), spices (Silvis *et al.*, 2017), as well as meat (Fang & Zhang, 2016; Nešić *et al.*, 2017; Rastogi *et al.*, 2007) have been subjected to substitution.

Food fraud has grown as a concern in global trade, not only as a crime against consumer rights, but also because of the inherent risks posed to human health. This is especially due to the insertion of any other food component that could be unsafe to the health of consumers, e.g. toxic compounds, potential allergens, or those that may be problematic for the diets of some consumers (Di Pinto *et al.*, 2015).

Animal products are extremely vulnerable to food fraud and adulteration due to various reasons. One is that they have high economic value, hence tampering or adulteration would be highly profitable. Post-slaughter processing changes their characteristic taste, texture, colour or flavor making it hard to discern any substitutions. Additionally, they are sources of essential iron and proteins which makes them a food type that is highly sought after by consumers (Cartín-Rojas, 2018).

2.2. Food fraud in the meat industry

Meat contains high amounts of biologically relevant proteins and is a vital food for human beings (Cetin *et al.*, 2016; Rahmati *et al.*, 2016). The consumption of meat goods has been driven up by the growth in population, urbanization, elevation in income, and changing consumer preferences (Delgado, 2003; Gamba, 2005). Modern manufacturing techniques have also increased the consumption of meat (Cetin *et al.*, 2016). According to research carried out by the FAO, the output of meat world-wide stood at 336.4 million tonnes by the year 2018, which was a 1.2% increase from 2017 (FAO, 2019). This increased demand has also caused the rise in

meat market value (Cawthorn *et al.*, 2013), which has led to economically motivated fraud (Spink & Moyer, 2011).

Although many countries have made legislative regulations concerning how meat products are labeled for the protection of consumers (Ren *et al.*, 2017) , post-slaughter processing may make meat indistinguishable (Perestam *et al.*, 2017). This increases the opportunity for species mislabeling since processing techniques and conditions could alter the texture, colour, and flavor of meat. Meat obtained from different species often has dissimilar prices. Consumer preferences will also direct the purchasing trends. In addition to economic reasons, lifestyle preferences such as vegetarianism, religious and moral reasons (Cai *et al.*, 2017) as well as health reasons, such as allergic reactions to certain animal proteins will be key in the consumers decision to purchase the product (Di Pinto *et al.*, 2015; Cai *et al.*, 2017).

Major fraudulent activities in the meat industry include: replacing the species, breeds or tissues with different animal species; falsifying the animal feeding routine or meat origin; alterations in the processing procedures; or the addition of ingredients other than meat such as water (Abbas *et al.*, 2018). Although this is a financial crime against the consumers, there is a risk of the spread of zoonoses to those handling and consuming the meat.

2.3 Meat fraud and public health

Undeclared meat species may pose public health safety risks, such as foodborne and zoonotic diseases, since the substituting species utilized may not have undergone quality checks like meat inspection, may have been acquired from unconventional sources such as wildlife (bushmeat), or may have been subjected to unhygienic handling (Spink & Moyer, 2011; Ouso *et al.*, 2020).

In general, food-producing mammals like cattle, camels, goats, and sheep are major reservoirs of many food-borne pathogens such as *Listeria monocytogenes*, *Escherichia coli*, non-*Typhi* serotypes of *Salmonella enterica*, *Brucella* species (Probert *et al.*, 2004), pathogenic *Leptospira* (Jobbins & Alexander, 2015), and *Bacillus anthracis* among many more (Heredia & García, 2018; Omer *et al.*, 2018).

The zoonotic nature of these pathogens, together with their capability to release toxins and cause diseases or mortality, makes preventing their transmission of vital importance (Heredia & García, 2018). They can cause disease through contact with or ingesting of contaminated products of animal origin, including meat, milk, eggs, etc. (Jobbins & Alexander, 2015; Heredia & García, 2018). This contamination can occur from animal handling such as slaughtering and processing exercises or environmental causes like water from different sources and associated fauna.

Although the meat value chain has several meat hygiene protocols to ensure the quality of meat being sold, those who choose to defraud their consumers may opt to

by-pass some measures, which could lead to the existence of these zoonotic microbes in the food chain, and thus exposure of consumers.

2.4. Meat consumption

In Africa, the consumption of meat is predicted to surge from 5.5 million tonnes (metric) in 1997 to 13.3 million tonnes in 2025 (Bett *et al.*, 2012). Like many African countries located in sub-Sahara, the supply of animal products in Kenya has steadily grown since the early 1900s (Cornelsen *et al.*, 2016). This is especially due to the growing population size and urbanization. According to the FAO, consumption of beef in Kenya is projected to grow from 467 thousand tonnes to 1277 thousand tonnes between 2010 and 2050 (FAO; USAID, 2017), with consumption of the other animal products also increasing over the years.

The demand for meat is affected by several factors such as income levels, prices, and even health considerations (Gamba, 2005). Generally, families that have a higher income tend to eat more meat than those at a lower tercile (Gamba, 2005; Alarcon *et al.*, 2017a; Alarcon *et al.*, 2017b), making meat a luxury product whose use increases with increase in revenue.

The consumption of meat in Kenya is mostly urban and stratified according to income, with the middle class being the highest consumers (Farmer & Mbwika, 2012). In urban areas, most of the meat purchased is in the form of a) boiled, roasted or grilled (*nyama choma*), or fried meat consumed at the point of sale or b) raw

meat bought in butcheries or supermarkets to be cooked at home (Farmer & Mbwika, 2012; Alarcon *et al.*, 2017b).

2.4.1 The meat value chain in Kenya

More than 80% of all the meat consumed locally is made up of red-meat sources including cattle, sheep, goat, and camel meat (EPZA, 2005; Juma *et al.*, 2010). White meat, mainly poultry and pork, consist of 19% of meat sold on the Kenyan market. Close to sixty-seven percent of the red meat comes from arid and semi-arid lands (ASALs) whereas ranches and sustenance farmers produce some of the meat consumed in the country (EPZA, 2005). Some meat is also imported from neighboring countries (Bergevoet & Van Engelen, 2014) such as Tanzania, Ethiopia, and Somalia (Farmer & Mbwika, 2012).

The meat- producing animals are taken to licensed slaughterhouses where they are then inspected and slaughtered for sale, processing, or export. Later, the meat is transported to abattoirs, supermarkets, local butchers, or hotels (Farmer & Mbwika, 2012). However, recent incidences of meat adulteration (Barnes, 2018; Mutahi, 2017; Ureport, 2018; Ouso *et al*., 2020) indicate that this stipulated chain is not always followed.

2.4.2 Sources of meat in Kenya

Official slaughters make up two-thirds of meat processed in the country, while the rest accounts for the amount that is slaughtered informally (in backyards and/or not formally inspected) (Farmer & Mbwika, 2012). The contribution of game meat,

such as zebra, and other meats, such as dog and cat meat, are negligible, amounting to less than 1% of the total meat consumed in the country (EPZA, 2005). This is especially true because consumption of bushmeat is prohibited by law (GOK, 2012).

2.4.3 The Nairobi meat market

In Kenya, Nairobi city is a major center for the consumption of meat obtained from ruminants, making up 14% of the national consumption (Alarcon, *et al.*, 2017b), with an average beef consumption of 19.1 kg per capita in 2014. As Kenya's population rises to the predicted 97.2 million by 2050, a majority of this growth in consumption is expected to be concentrated around the urban centers such as Nairobi, hence increasing the demand for food items (Alarcon *et al.*, 2017a).

The Nairobi meat market is made up of three major food segment categories, large processing companies (LPCs), meat markets (MMs) including Shauri Moyo (Burma) Market and City Market, and local terminal markets (LTMs) such as Kiserian, Njiru, Kiamaiko, and Dagoretti markets (Alarcon *et al.*, 2017b). There are two large processing companies, which are concerned with the value of meat expected, as they depend on specific standards for beef cattle traded. Meat that goes through these companies is sold to high-end markets.

In the LTMs, live animals are sold, most of which are slaughtered and traded. Meat markets mostly involve the movement of these animal products and make up to two-thirds of the beef provided to Nairobi (Alarcon *et al.*, 2017b). Kiamaiko is the major market of sheep and goats, slaughtering between 5,000 and 10,000 ruminants per

week. Dagoretti handles a majority of the live animals, i.e. slaughtering 1,200-1,600 cattle per week (Alarcon *et al.*, 2017b). These two major markets are leading in the supply of small ruminants and beef, especially because of their discounted meat prices. The large market share they hold makes these markets vulnerable to prohibited activities and risks to food safety (Nairobi City County Assembly, 2014).

The demand for products from cattle, sheep and goat is projected to be double the current demand by 2030, hence posing a great challenge of supply to the region. The structure of the meat value chain, as well as the presence of both formal and informal channels for the supply of meat, makes determining the quality of meat sold to consumers of great importance.

2.5. Identification of vertebrate sources of meat

Verifying the identity of the vertebrate animal species from whom meat and meat products originate is critical because of the legal, medical cultural ,economic, and religious aspects it raises (Sakaridis *et al.*, 2013; Farag *et al.*, 2015). To help people determine this, various techniques including histological, anatomical, microscopic and chemical methods such as immunological, electrophoretic and chromatographic techniques have been used for identification of species (Sakaridis *et al.*, 2013; Montowska & Spychaj, 2018). Unfortunately, techniques which rely on protein expression patterns such as using agar gel diffusion and immuno-electrophoresis are not reliable in cases where the meat has been cooked (Rastogi *et al.*, 2007; Rahmati *et al.*, 2016). Methods which depend on analysis of nucleic acid,

specifically DNA , especially those relying on PCR (polymerase chain reaction) are acknowledged as the best approaches for identifying species in processed and raw meat (Farag *et al.*, 2015).

2.5.1 Molecular identification

Techniques used in PCR, for instance; restriction fragment length polymorphism (PCR-RFLP), randomly amplified polymorphic DNA (PCR-RAPD), PCR using species-specific primers, real-time PCR, and PCR followed by nucleotide sequencing, enable the distinction of animal sources of meat that have undergone processing conditions (Girish *et al.*, 2013; Farag *et al.*, 2015). A major reason why RAPDS are not used in such analysis, despite being faster and cheaper than most DNA-based methods is that the results found are hard to reproduce (Rastogi *et al.*, 2007).

There have been major endeavors to standardize the approaches used in identification of species through molecular analysis hence making it possible to characterize very many species using similar genetic markers (Lorenz *et al.*, 2005). This resulted in the creation of DNA barcoding, which is a molecular system used globally for identification of living beings. This technique has proven its universality and efficacy in various contexts (Galimberti *et al.*, 2015).

2.5.2. DNA barcoding markers

Barcoding of DNA is a technique that relies on the use of mitochondrial DNA. DNA from this organelle is favored over nuclear DNA because 1) mitochondrial DNA can

be utilized to generate universal primers that can distinguish a large number of species, and 2) it can be used even if found in minute or trace samples such as hair, (Tobe *et al*., 2009). Barcoding primers are used to amplify a part of the gene of interest which is then compared with a sequence which is known (or entries in a database) to confirm the identity of the organism (Tobe *et al*., 2009). Cytochrome Oxidase subunit 1, Cytochrome B and mitochondrial 16S rRNA gene, which is involved in the synthesis of mitochondrial ribosomes, are common mitochondrial markers used in mammalian characterization (Nicolas *et al.,* 2012; Ouso *et al*.,2020; Yang *et al*., 2018). Other regions which have also been used in barcoding animal species include the nuclear ribosomal internal transcribed spacer (ITS) and the 12S ribosomal RNA (12S rRNA) gene (Yang *et al.*, 2018). DNA barcoding is often used in forensics to investigate trafficking of live specimens, poaching or hunting out of season as well as species substitution in food products (Galimberti *et al.*, 2015) .

2.5.2.1. Cytochrome B

The Cytochrome B (Cyt b) gene, is utilized as a valuable marker in forensic identifications. This is due to this genes ability to reliably differentiate species as well as in reconstructing the mammalian phylogeny (Nicolas *et al.*, 2012). Assays targeting this gene usually amplify only a section (usually about 400 bp) of the 1149 bp gene. Additionally, this gene has the useful attribute of being variable in different species but having the same size within species (Tobe *et al*., 2009). This made it ideal for the identification of the vertebrate sources of meat from the samples obtained.

2.5.2.2. Vertebrate Cytochrome c Oxidase subunit 1

The Cytochrome c Oxidase subunit 1 (CO1) is another gene found in the mitochondria. It has been shown to be useful and dependable in classification of organisms and even serves as the backbone of a biological identification system for animals used globally (Hebert *et al.*, 2003). This gene is considered more dependable over the other mitochondrial markers, such as mitochondrial 16S rRNA gene and Cyt b, due to a few features ; one characteristic is that it has a wider array of phylogenetic differentiation than the other mitochondrial genes (Hebert *et al.*, 2003). It also has very active universal primers that allow for the recovery of its 5' end from individuals of approximately all phyla of animals (Hebert *et al.*, 2003). This would also prove useful in the analysis of meat samples.

2.5.2.3. Vertebrate mitochondrial 16S subunit of ribosomal RNA gene

The mitochondrial 16S subunit of ribosomal RNA (16S rRNA) gene is approximately 1500 bps in length and codes for a section of RNA that has enzymatic activity and is one of the components of the 30S ribosomal subunit (Srinivasan *et al.*, 2015). This gene, that codes for mitochondrial ribosomes, allows for species differentiation due to an accelerated evolutionary rate which brings about variations in the sequences. The functional constancy of this gene, and its availability in most living things, makes it a reliable marker and a reliable tool for phylogenetic testing (Srinivasan *et al.*, 2015). Using these three mitochondrial genes simultaneously in an analysis and identification of vertebrate sources can improve the species resolution to allow differentiation between closely related species.

2.5.3. PCR - High Resolution Melting Analysis (HRMA)

Polymerase chain reaction (PCR) is a molecular method that has been used for various applications, including the authentication of food composition (Spychaj *et al.*, 2009). After the amplification of the DNA of interest using PCR, various techniques such as PCR-RFLP and PCR-RAPD can be applied for the detection of the animal meat source (Farag *et al.*, 2015). HRMA is a novel post-PCR technique for DNA analysis that examines genetic discrepancy in DNA sequences based on their dissociation curves (Applied Biosystems, 2009) which are determined by various elements such as the variations in GC content and the length of the amplicon.

Since this technique was introduced in 2002 (Reed *et al.*, 2007), it has increased in popularity because of the availability and accessibility of double-stranded DNA (dsDNA) intercalating dyes together with modern real-time PCR equipment and analysis software (Applied Biosystems, 2009). The melting curve is generated when the amplicon, which contains an intercalating dye is heated over a span of temperatures, followed by measurement of its fluorescence. Due to the nature of the intercalating dye, the DNA will fluoresce brightly at lower temperatures but this fluorescence will decrease as the temperature is increased and the strands dissociate (Reed *et al.*, 2007). The difference in the length ,GC content and sequences of the DNA of different species allows the use of HRM Analysis for efficient discrimination of species (Applied Biosystems, 2009; Reed *et al.*, 2007). This technique has been applied effectively in the establishment of the identity of vertebrate species or blood meal hosts of insect vectors (Peña *et al.*, 2012; Ogola *et*

al., 2017) and in the identification of bushmeat (Ouso et al., 2020). High resolution melting analysis has also proved useful in the investigation of food fraud (Sakaridis *et al.*, 2013; Lopez-Oceja *et al.*, 2017)

2.6 Zoonotic pathogens linked to meat consumption

Zoonoses are those diseases that can be transferred from animals to humans directly, through contact, consumption or through vectors (Cantas & Suer, 2014). Harmful microbes of zoonotic origin make up at least two-thirds of all infectious agents that are transferrable to humans, many of which cause newly emerging infections (Munyua *et al.*, 2016; Asante *et al.*, 2019). These include bacteria, viruses, parasites, and fungi. They present a key threat to public health constituting the majority of the infectious disease burden in low-income countries (Asante *et al.*, 2019). Annually, approximately 2.5 billion cases are reported globally, resulting in 2.7 million deaths (Asante *et al.*, 2019; Grace *et al.*, 2012).

Animals have been linked as vehicles of several food-borne pathogens, including *Brucella*, *Leptospira*, *Bacillus anthracis*, *Salmonella*, *Campylobacter*, and toxigenic *Escherichia coli* among many more (Heredia & García, 2018). The Zoonotic Diseases Unit, set up in Kenya by the Ministry of Livestock Development and Ministry of Public Health and Sanitation, listed several of these diseases as zoonoses of high significance in the country (Munyua *et al.*, 2016).

2.6.1 *Brucella* spp.

Brucellosis, the group of zoonoses transmitted through bacteria of the genus *Brucella* (Ducrotoy *et al.*, 2017), is known to be a major infectious disease that affects various mammalian species such as humans (El-Sayed & Awad, 2018). Brucellae can pass through all mucous membranes as well as intact or injured skin, hence can occur through several pathways. In domestic animals, infection happens through consumption of water or food contaminated by fetuses that have been aborted or fetal membranes, uterine discharges or as a result of licking the genitalia of infected animals. The infected males transmit the pathogens to the females when mating or even through artificial insemination (El-Sayed & Awad, 2018). It is also known that transmission can occur through mechanical means such as by arthropods like ticks (Wang *et al.*, 2018), and possibly *Stomoxys* stable flies (Baldacchino *et al.*, 2013).

Humans mainly acquire this disease through indirect or direct contact with infected animals or contaminated food items originating from animals. Brucellosis is a very diverse disease that mainly appears as malaise, myalgia and fever, that could eventually escalate into a chronic infection that affects many tissues and organs (Probert *et al.*, 2004). Due to the nature of this illness, it is often misdiagnosed as malaria or other febrile illnesses. Based on a study carried out by FAO, it was determined that in Kenya, there were 255,000 confirmed cases of human brucellosis between 2012 and 2016, which is probably less than the actual number of infections due to misdiagnosis (FAO; USAID, 2017). The causative agents for human

brucellosis have been determined to include *B. abortus*, *B. suis, B. melintesis*, *B. canis*, and some strains that bear a resemblance to *Brucella* found in marine mammals (Probert *et al.*, 2004).

The gold standard for detection of *Brucella* species in the laboratory has been based mainly on culture isolation as well as phenotypic characterization (Probert *et al.*, 2004). For clinical diagnosis in humans, several agglutination tests, for instance those that rely on serum agglutination that uses cell suspensions of whole smooth *Brucella* as antigens, have been favored in the East African region. A major example is the febrile antigen Brucella agglutination test (FBAT) (de Glanville *et al.*, 2017). However, research by de Glanville *et al.* (2017), indicates that these tests are not always reliable, with false positives being reported.

In other sources such as vector bloodmeals, detection of *Brucella* DNA has been carried out using PCR assays that target various genes specific to this genus. One such targeted gene is the outer membrane protein gene 22 (omp22), together with the IS711 gene. The omp22 gene allows for the selection of all *Brucella* species as it is found in the entire genus, whereas the IS711 gene allows for the distinction of different *Brucella* species because of its varying locations in the genome (Wang *et al.*, 2018). Other genes that have been targeted for detection of *Brucella* include 16S rRNA, omp2, the 16S-23S intergenic spacer region, and bcsp31 (*Brucella* cell surface salt extractable gene). Bcsp31, which encodes a 31-kDa antigen that is conserved among *Brucella* spp., is the most common gene utilized for the diagnosis

of human brucellosis using PCR assays (Probert *et al.*, 2004). The use of this more sensitive target, bcsp31, allowed for the successful detection of this pathogen.

2.6.2 *Bacillus anthracis*

Bacillus anthracis, which is the microbial agent that causes anthrax, is a zoonotic bacterial pathogen known to cause disease which could lead to death in animals and people (Ågren *et al.*, 2013). This pathogen is in the *Bacillus cereus* family, which has mesophilic members which are genetically closely related including *B. anthracis*, *B. cereus*, and *B. thuringiensis*, as well as some psychro-tolerant members: *Bacillus mycoides* and *B. weihenstephanensis* (Wielinga *et al.*, 2011). The members of this family are found globally and produce spores that are extremely resistant to various forms of stress, for instance, drought, enabling them to survive in varying environmental surroundings (Wielinga *et al.*, 2011). *Bacillus anthracis* is one of the most feared micro-organisms because of its life threatening effect on both animals and humans, as well as its potential use in bioterrorism (Ågren *et al.*, 2013).

The main virulence factors for *B. anthracis* are positioned on two mega plasmids, pXO1 and pXO2, which when both present in strains, cause severe disease, and even mortality in both animals and humans (Wielinga *et al.*, 2011). The pXO1 plasmid confers toxin production, whereas pXO2 confers capsule synthesis properties to the bacteria (Ågren *et al.*, 2013). One vital challenge in developing an assay that would specifically detect *B. anthracis* is the high similarity it shares with other strains in the same genus (Ågren *et al.*, 2013). The major distinction between the distinct

species in the genus is the presence of distinctive plasmids that give virulence. However, it has also been brought to light that some *B. cereus* strains also contain anthrax-specific pXO-like plasmids (Ågren *et al.*, 2013) .

Although positive identification of anthrax has been done using techniques such as immunohistochemical staining (Levine *et al.*, 2002), PCR-based protocols, especially real-time PCR, are being preferred. This is due to its closed system, which decreases the possibility for contamination and laboratory-exposure infections (Levine *et al.*, 2002).

Over 300 PCR-target sequences have been reported in previous work targeting *B. anthracis*, with various chromosomal targets being investigated, as well as plasmid targets. However, most chromosomal markers used were later determined to be shared by *B. anthracis* and a subset of very closely related *B. thuringensis* and *B. cereus*. That made these assays prone to false positives as these other species would be perfect matches (Ågren *et al.*, 2013).

The techniques that have been successfully employed require both chromosomal markers and plasmid-encoded targets to differentiate between apathogenic and pathogenic strains. The markers that have been determined to give the best discrimination include the chromosomal marker that targets the prophage known as lambdaBa03 (PL3), which is found in all *B. anthracis* strains (Wielinga *et al.*, 2011), a signal sequence of another chromosomal gene, *rpoB* (Levine *et al.*, 2002), and a

marker that targets the region coding for the *capB* gene located on the plasmid pXO2 (Levine *et al.*, 2002; Wielinga *et al.*, 2011).

2.6.3 *Leptospira* spp.

Some bacteria from the genus *Leptospira* are pathogenic, causing the zoonotic disease leptospirosis, through both direct and indirect transmission from animals to humans (Ahmed *et al.*, 2009). Although leptospirosis occurs globally, with an estimated 500,000 severe cases, it is most frequent in subtropical and tropical areas which experience high rainfall. While cases of mild leptospirosis are unknown, it is thought to be higher than the number of severe cases, indicating that the actual prevalence of the disease is underestimated (Ahmed *et al.*, 2009). This underestimation can be attributed to the symptoms of the disease, which are many times, mistook for other illnesses that are epidemic or endemic in the same environmental regions, such as enteric fevers, dengue, influenza, meningitis, rickettsiosis, and malaria (Musso & La Scola, 2013). Unlike many zoonoses, leptospirosis can be treated easily using antibiotics if diagnosis is made before the 5th day after disease onset (Ahmed *et al.*, 2009).

Various animals, including mammals are sources of these bacteria, with rodents being the main reservoirs (Musso & La Scola, 2013). *Leptospira* are normally localized in the kidneys and renal system of the infected mammals, especially in chronic infections. It can be transferred to humans through indirect or direct exchange with the urine or tissues of animals that are infected, where the bacteria

goes through cuts or abrasions, or through the conjunctiva (Musso & La Scola, 2013). This contact can occur during the slaughtering processes and handling of infected tissues.

The major approaches for detection and classification of *Leptospira* rely on serology, with the genus being divided into over 300 serovars, which are further grouped into close to 30 serogroups, which are both saprophytic and pathogenic (Guernier *et al.*, 2018). The microscopic agglutination test (MAT) is one of the current major diagnostic tests, often used as the gold standard (Ahmed *et al.*, 2009). Another serological test commonly used is the cross agglutination absorption test (CAAT) (Guernier *et al.*, 2018). However, serological tests usually only confirm the disease when it has reached a later, acute phase because the anti-*Leptospira* antibodies can usually only be detected five to seven days after the onset of the illness (Ahmed *et al.*, 2009).

The use of faster techniques for diagnosis, such as the detection of DNA from pathogenic *Leptospira* present in tissues like blood, using PCR techniques is being embraced (Ahmed *et al.*, 2009). Several genes have been targeted for PCR amplification of these pathogens. The gene that has been determined to be most discriminating is the *secYIV* gene, which is a housekeeping gene present within many prokaryotic species (Ahmed *et al.*, 2009).

2.6.4 *Coxiella burnetti*

This is a small, obligate gram-negative bacterium, known to be the etiological agent of the zoonotic disease Q fever (Selim *et al.*, 2018). It has a small, circular chromosome, roughly 5 Mbps in size and may also contain one of four plasmids which are 32-51 kb in size and carry around 2% of its genomes information (Angelakis & Raoult, 2010) . It is known to infect vertebrates, including humans and is of great public health importance (Ndeereh *et al.*, 2017). This pathogen can be transmitted through inhalation of the urine or placental fluids of infected animals (Angelakis & Raoult, 2010) or consumption of unpasteurized milk or contaminated meat (Cantas & Suer, 2014). They can also be transmitted through arthropod vectors such as ticks.

Although most animals infected with Q fever remain asymptomatic, this pathogen can be found in the animal tissues such as lungs, blood, and liver during the phase of infection which is acute. When the infection becomes chronic, the bacterium is continually shed in urine, feaces, and sometimes in milk (Ndeereh *et al.*, 2017). Infection in humans manifests as atypical pneumonia, influenza-like symptoms, and/ or hepatitis with impulsive recovery in the critical stage (Selim *et al.*, 2018). In its chronic manifestation, it may present as endocarditis, especially in immunocompromised individuals (Angelakis & Raoult, 2010). It can also lead to spontaneous abortion in women (Ndeereh *et al.*, 2017).

Various analytical techniques, including isolation through cell culture, immunodetection, and serology can be utilized for the diagnosis of Q fever (Angelakis & Raoult, 2010). However, molecular methods such as real-time PCRs, conventional and nested are being used for early diagnosis of chronic Q fever (Angelakis & Raoult, 2010). The gene targeted for amplification is the repetitive element IS1111, due to its high specificity and sensitivity. Although it has been determined that the same repeat in *Coxiella*-like endosymbionts can be maternally inherited by ticks (Duron, 2015), it is still commonly used to screen for *Coxiella burnetti*.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Study Site

The study was carried out in the central region of Nairobi county, within Kamukunji, Starehe, Mathare and Makadara constituencies. Nairobi is the capital city of Kenya, with its population reported at almost 4.4 million, during the national population and housing census in 2019 (KNBS, 2019). This population is expected to increase as the country's population reaches the projected 97.2 million by 2050 (Alarcon *et al.*, 2017b).

The Shauri Moyo meat market, also known as Burma market, was the main target. This was due to reports of the sale of bushmeat misrepresented as legal meat (Kiage 2019; Gitonga 2019; Kimuyu 2019), as well as its frequent closure by the Nairobi City Government because of lack of compliance to quality standards. The sampling extended to the suburbs located around this major meat market.

Archived meat samples obtained from Naivasha subcounty, Nakuru county were identified by Ouso *et al.*, 2020. This included meat covertly obtained from butcheries located in the towns Kinamba, Kambi Somali, Gilgil, Kambi Daraja, Kabati town, Kasarani, Mirera Elementaita Kongoni, Kikopey and Langalanga. Sub-samples had then been stored at -80°C for downstream analysis. This site was selected due to its vicinity to various game parks and reports of the illegal sale of bushmeat. Species identified were already published in a previous study (Ouso *et*

al., 2020). Screening for specific bacterial pathogens would be carried out on these samples.

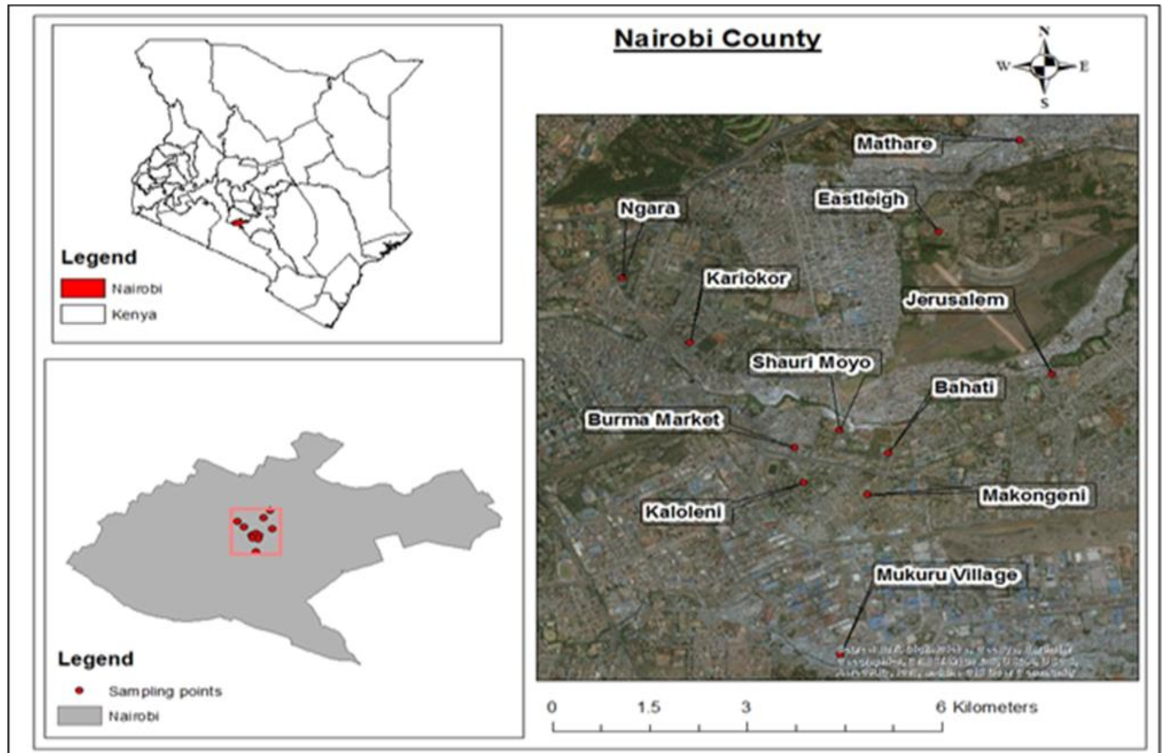

Figure 1: Image showing the meat sampling sites in Nairobi.

3.2 Sampling (Meat samples)

A total of 115 meat samples were purchased in November 2018, from randomly selected stalls in Burma Market and butcheries located around it. This involved targeting stalls within Burma market as well as establishments located in the suburbs of Eastleigh, Kariokor, Kaloleni, Mukuru Village, Mathare, Jerusalem, Jericho, City Stadium, Ngara, and Makongeni. The butcheries in this area mostly sold beef exclusively. However, to get chevron and pork, butcheries that also sold these

species were targeted. The desired species was declared during purchase. The meat species of interest were the animals most commonly bought by households, including: 61 cattle samples; 30 goat samples; 3 camel samples; 9 pig samples; 4 chicken samples; 4 tilapia samples and 4 Nile perch samples. Each 250 g sample was re-wrapped separately in aluminum foil to prevent cross-contamination. They were then transported in cooler boxes with ice-packs to the lab for later sub-sampling. The samples were then sub-sampled into two replicates of 1-2 g, with care taken to use sterile blades and fresh gloves per sample. All archived samples were stored in 1.8-mL cryovials at -80 °C, to prevent growth of microbiota, until DNA extraction.

3.3 Molecular Identification of vertebrate sources of meat

3.3.1 DNA extraction protocols from meat

Initially, 50 mg of each meat sub-sample was thawed and genomic DNA extracted using the ISOLATE II Genomic DNA Extraction kit (Bioline, UK) according to the manufacturer's protocol.

3.3.2 Normalization of extracted DNA

After elution, the nucleic acid concentration of samples extracted using different protocols was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). Thereafter, the DNA concentration of each sample was normalized to ~10 ng/µl by further eluting calculated

concentrations of DNA with the appropriate elution buffers based on the extraction protocol used. Quality of extracted DNA was also assessed on 0.8% agarose gel.

3.3.3 PCR-HRM for identification of vertebrate sources of meat

All the 115 meat samples obtained from the meat markets were analyzed by polymerase chain reaction coupled with high-resolution melting analysis (PCR-HRM) to determine the vertebrate species. Genetic markers targeting sections of mitochondrial cytochrome b (Cyt b), Cytochrome oxidase subunit 1 (CO1) and vertebrate 16S rRNA gene (Ouso *et al*., 2020; Ogola *et al*., 2017; Omondi *et al.*, 2015) were used in DNA amplification of the different sources of meat (Table 1). This involved carrying out 10-µl PCR reactions comprising of 2 µl of 5X HOT FIREPol® EvaGreen® HRM Mix no ROX (Solis BioDyne, Tartu, Estonia), 0.5 µM of both forward and reverse primers (Omondi *et al.*, 2015; Ogola *et al.*, 2017), 2 µl of DNA template and topped up with nuclease free water. The PCR reactions were carried out in a RotorGene Q thermocycler (Qiagen, Germany) beginning with PCR amplification immediately followed by high-resolution melting. Each run included non-template controls, known positive control samples (Appendix 1) as well as the unknown samples for identification. The PCR conditions used were provided in previous work (Ouso et al., 2020). This included an initial denaturation step at 95°C for 15 minutes followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 56°C for 20 seconds followed by an extension step at 72°C for 30 seconds. This cycle was followed by a final extension step of 72°C for 5 minutes before proceeding to the melt stage. Here, the amplicons were steadily melted from

75°C to 95°C at gradual 0.1 °C increments with fluorescence recorded every 2 seconds. This generated graphs of fluorescence against temperature.

Table 1: Oligonucleotide primers used for vertebrate identification

Target gene	**Primer name**	**Primer sequence (5'-3')**	**Target size (bp)**	**Citation**
CO1	VFId_t1	TCTCAACCAACCACAARGAYATYGG	750	(Ivanova *et al.*,2012)
	VRId_t1	TAGACTTCTGGGTGGCCRAARAAYCA		
	New Uni-Minibar-F1	TCCACTAATCACAARGATATTGGTAC	205	(Lee *et al.*, 2015; Meusnier *et al.*, 2008)
	Ronping_R	TATCAGGGGCTCCGATTAT		
Vertebrate 16S	Vert16S For	GAGAAGACCCTRTGGARCTT	200	(Omondi et al, 2015)
	Vert16S Rev	CGCTGTTATCCCTAGGGTA		
Cytochrome b	Cyt b For	CCCCTCAGAATGATATTTGTCCTCA	383	(Boakye at al., 1999)
	Cyt b Rev	CATCCAACATCTCAGCATGATGAAA		

3.3.4 Confirmation of goat positive control samples

The positive controls used as references for meat source identification were archived samples that had been positively identified during collection of the tissue samples (morphologically), as well as through sequencing (Appendices 1 and 4). However, in the selection of positive controls for the goat samples there was a slight challenge brought about by mislabeling of the archived samples. The samples labeled 'Goat

Meat' (GM) and 'Goat Meat B' (GMB) were both believed to belong to the species *Capra hircus.* Amplification products targeting the Cyt b marker were cleaned using the ExoSAP-IT protocol (USB Corporation, Cleveland, OH) and sent to Macrogen, Europe for Sanger sequencing.

For confirmatory identification of goat samples, specimen were collected from Kiamaiko area of Kariobangi estate, which is a main abattoir for sheep and goats being sold to residents of Nairobi (Juma *et al.*, 2010). Here, small sections of goat ears were taken from seven goats, for use in confirming the actual HRM profile of goat species. The DNA from these tissues was extracted using the ISOLATE II Genomic DNA kit (Bioline, UK) and PCR-HRM analysis carried out using these seven as the positives and GM and GMB as the unknowns.

Additionally, PCR using the primers that target the long barcoding region of the CO1 gene was carried out for molecular identification. The PCR products were then cleaned as described previously and sent for sequencing.

3.4 Experimental analysis of meat samples

3.4.1 Analysis of effect of different DNA extraction protocols

To study the impact of four different extraction protocols in species identification using PCR-HRM, a subset of nine meat samples: two cattle; four goats; one sheep as well as two camels was subjected to four DNA extraction protocols. Briefly, each sample was cut into four pieces, each about 50 mg. The first replicate was extracted using the ISOLATE II Genomic DNA kit (Bioline, UK) and the second using the

DNeasy Blood and Tissue Kit protocol (Qiagen, Valencia, CA). The third replicate was extracted using a lab optimized protocol described by Kipanga *et al.*, 2014. Briefly, this method included using a cell lysis buffer, together with 25 µl of proteinase K for the breakdown of 50 mg of meat tissue. After incubation at 65 °C for two hours, protein precipitation was carried out using a salt; ammonium acetate, and incubation in ice for 15 minutes. This was followed by centrifugation at maximum speed for 15 minutes. The supernatant was pipetted into fresh sterile 1.5 mL Eppendorf tubes. After this, 300 µl of isopropanol was added to the tubes, and the tubes inverted 100 times to ensure adequate mixing before centrifugation for 1 hour. The resulting supernatant was discarded and 300 µl of ice-cold 70% ethanol added to the tubes; followed by mixing through inversion of the tubes and centrifugation for 30 minutes. The ethanol was then discarded and the tubes left inverted to dry overnight. Finally, 50 µl of nuclease-free water was added to the tubes and the DNA eluted at 65 °C for 1 hour.

The fourth replicate was extracted using a modified version of the aforementioned protocol, where proteinase K was not added during the cell-lysis step. The centrifuge used for all extractions was a 5417R Eppendorf centrifuge (Eppendorf, Hamburg, Germany).

The extracted DNA was then analyzed using PCR-HRM of CO1, Cyt b, and mitochondrial 16S rRNA gene as described above. Standardized DNA samples, extracted using the protocols described above were compared to check for any

change in melt temperature or profile. The marker CO1 was selected for use in this comparison. For ease of analysis, six single-peak profiles; obtained from two camel and three goat samples were assessed.

3.4.2 Application of various treatments to meat samples

Meat samples were selected from archived samples obtained from Naivasha region (Ouso *et al.*, 2020) - three from each of cow (*Bos taurus*), goat (*Capra hircus*), sheep (*Ovis aries*), pig (*Sus scrofa domesticus*), as well as known chicken (*Gallus gallus domesticus*) and camel (*Camelus dromedarius*) samples, and exposed to four different treatments. This was to determine the effect of different treatments to the melt profile. Each meat sample was cut into four pieces, each about 250 mg. The first piece was placed in an oven at 65°C for 2 hours, the second piece was cooked in a microwave oven for 12 minutes whereas the third batch was placed on the lab bench and left to decompose for 72 hours. The fourth piece was used as the control, where no treatment was applied. Total DNA was extracted from the samples using the ISOLATE II Genomic DNA kit (Bioline, UK) and analysis carried out using PCR-HRM. The markers used for the analysis were the CO1, mitochondrial 16S rRNA gene and Cyt b genes.

3.4.3 Analysis of species admixtures in the meat

To determine whether PCR-HRM could be used successfully to identify meat adulteration in commonly sold processed meat, six meat mixtures were made. The mixtures prepared included: cattle + sheep; sheep + goat; cattle + goat; cattle +

camel; chicken + pork; and chicken +Nile perch. About 50 mg from each species was placed into their respective tubes and extraction carried out using the ISOLATE II Genomic DNA kit (Bioline, UK). Each mixture and subsequent extraction were carried out in triplicate. Conventional PCR as well as PCR-HRM analysis of the mixed and pure domestic samples targeting the three mitochondrial genes 16S rRNA, CO1, and Cyt b were carried out to determine the most efficient marker in investigating meat species adulteration.

3.5 PCR amplification and sequencing of vertebrate samples.

For confirmation of vertebrate origin of samples, the DNA was amplified using primers that target a longer segment (750 bp), the barcoding region of the CO1 gene. This involved amplification with conventional PCR using 15-µl reaction volumes consisting of 3 µl of 5X HOT FIREPol® Blend Master Mix ®, 0.5 µM of both forward and reverse primers and topped up with 9 µl of PCR-ready water. The cycling conditions are similar to those described in Ouso *et al.,* 2020 and included the first denaturation step of 95°C for 15 minutes, then 40 cycles of 95°C for 20 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute per cycle. This was immediately followed by the final extension of 72°C for 7 minutes (Ouso et al., 2020). Five microliters of the amplicons were then analyzed using gel electrophoresis for confirmation of amplification before cleaning of the remaining volume using the ExoSAP-IT protocol (USB Corporation, Cleveland, OH). The cleaned product was then sent to Macrogen, Europe for Sanger sequencing.

3.6 Detection of zoonotic bacterial pathogens in meat sold for human consumption

3.6.1 Meat samples

The samples obtained from Nairobi that were screened for bacteria included the 115 samples obtained from Burma market and its surroundings and the seven goat ears obtained from Kiamaiko as positive controls. The ear tips were sliced from morphologically identifiable goats and stored in a 50mL falcon tube, which was placed in an ice cooler. This was important as they would be used as controls to positively identify or exclude unknown samples for detection of species–substitution. The ears were then cleaned with water and ~5mm^3 of the inner muscle cut off using sterile forceps and a scalpel. The inner muscle was selected to exclude skin microbiota. DNA was extracted from these samples using the ISOLATE II Genomic DNA extraction protocol. For comparison, DNA from 99 archived samples obtained from Naivasha area; including Kambi Somali, Gilgil, Kinamba, Kasarani, Kabati town, Mirera Elementaita and Langalanga (Ouso *et al*., 2020) were extracted using the commercially available DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). All the samples analyzed were archived at -80°C immediately after sampling to prevent the growth of microbiota, therefore no fresh samples were used. In total,221 samples were screened for zoonotic pathogens.

3.6.2 Design of synthetic constructs

Synthetic constructs that would act as positive controls for PCR screening of the zoonotic bacterial pathogens *Brucella* spp., *Bacillus anthracis* and pathogenic

Leptospira spp. were designed by aligning at least ten sequences of the target genes per pathogen and creating a consensus sequence. The publicly accessible sequences were obtained from GenBank and the consensus sequence sent to GenScript® (New Jersey, USA) where they were synthesized and packaged in pUC57 plasmids for DNA delivery.

The 193-bp construct used for the detection of *Brucella* would anneal to primers targeting the *bcsp31* gene, (GenBank accession number M20404) (Probert *et al.*, 2004) whereas the one used as a control for detection of pathogenic *Leptospira* would be a 245-bp sequence that anneals to primers targeting *SecYIV* gene (GenBank accession number AF115283) (Ahmed *et al.*, 2009). For the species-specific detection of all *Bacillus anthracis* strains, we selected the 181-bp construct that anneals to primers targeting the *PL3* gene (GenBank accession number AE017334) (Wielinga *et al.*, 2011).

3.6.3 Linearization of plasmid DNA controls

To allow use of the plasmid DNA as positive controls, the protocol provided by Genscript® was used to linearize them. In summary, they were first centrifuged at 6000 x g for 1 minute at 4°C. The vial was then opened and 20 µl of sterile water was added to dissolve the DNA. The vial was then closed and vortexed for 1 minute before being incubated at 50°C for 15 minutes to dissolve the DNA. After this, standard PCR was carried out on each using the specific primers and PCR conditions

for the gene targets. The PCR was carried out in 50-µl reactions. The amplicons were then each serially diluted 1:10 five times in water.

3.6.4 Detection of *Brucella* spp.

A *Brucella* genus-specific single-plex PCR-HRM assay targeting a 151-bp section of the gene *bcsp31*, was used. This was done in 10-µl reaction volumes consisting of 2 ul of 5X HOT FIREPol® EvaGreen® HRM Mix no ROX (Solis BioDyne, Estonia), 6 µl of nuclease free water, 0.5 µl of both forward and reverse primers (Table 2) and 1 µl of the DNA template. Each run included non-template controls as well as the linearized, diluted synthetic positive control for *bcsp31*. The cycling conditions had been described in earlier work (Probert *et al.*, 2004) were as follows: Initial denaturation at 95°C for 15 minutes, then 45 cycles of 95°C for 15 seconds and a combined annealing and extension step at 57°C for 1 minute. This was followed by a final extension at 72°C, and finally, the amplicons were gradually melted from 75°C to 95°C at 0.1 °C increments and the fluorescence recorded every 2 seconds. This step results in graphs showing HRM and fluorescence melt rates against change in temperature.

Table 2: Primers used for the identification of bacterial zoonotic pathogens

Pathogen	**Target gene**	**Primer name**	**Primer sequence (5'-3')**	**Target size (bp)**	**citation**
Pan-*Brucella*	Brucella bcsp31	BCSP31-brucella-F	GCTCGGTTGCCAATATCAATGC	151	(Probert *et al.*, 2004)
		BCSP31-brucella-R	GGGTAAAGCGTCGCCAGAAG		
Pathogenic *Leptospira* sp.	SecYIV	SecYIV-Lepto-F	GCGATTCAGTTTAATCCTGC	~220	(Al-Kahtan *et al.*, 2017; Ahmed *et al.*, 2009)
		SecYIV-Lepto-R	GAGTTAGAGCTCAAATCTAAG		
Bacillus anthracis	PL3	PL3-Anthrax-F	AAAGCTACAAACTCTGAAATT TGTAAATTG	139	(Wielinga *et al.*, 2011; Ågren *et al.*, 2013)
		PL3-Anthrax-R	CAACGATGATTGGAGATAGAG TATTCTTT		
Coxiella burnetti	IS1111	IS1111 F	GCTCCTCCACACGCTTCCAT		(Tokarz *et al.*, 2009)
		IS1111R	GGTTCAACTGTGTGGAATTGAT GAGT		
General bacterial 16S	16 S rDNA	Ehr/Ana-27F	AGAGTTTGATCCTGGCTCAG	~1350 bp	(Fredriksson *et al.*, 2013; Srinivasan *et al.*, 2015)
		Ehr/Ana-1492R	GGTTACCTTGTTACGACTT		

3.6.5 Detection of pathogenic *Leptospira* spp.

PCR-HRM targeting the *SecYIV* gene which is present in all pathogenic *Leptospira* species was used to determine if any of the meat samples had this pathogen. The PCR was carried out in 10-µl reaction volumes with 1 µl DNA template as described previously, with primers shown in Table 2, using the described cycling conditions (Ahmed *et al.*, 2009) as follows: Initial denaturation at 95°C for 15 minutes, 40 cycles of 95°C for 5 seconds, annealing at 54°C for 5 seconds and extension at 72°C for 15 seconds, a final extension step at 72°C for 5 minutes followed by two hold steps, 95°C for 2 minutes and then 25°C for 1 minute. This was directly followed by the melt step as was described above. Each run included non-template controls as well as the linearized and serial-diluted synthetic construct of the *SecYIV* gene as the positive control.

3.6.6 Detection of *Bacillus anthracis*

To screen for presence of *Bacillus anthracis,* both conventional PCR and PCR followed by high-resolution melting was used. These techniques target a 139-bp segment of the chromosomal marker *PL3* present on all *B. anthracis* strains. PCR reaction volumes of 10-µl were used with 1 µl DNA template as described above. The cycling conditions used had been described in earlier work (Wielinga *et al.*, 2011; Ågren *et al.*, 2013) and included an initial denaturation step of 95°C for 15 minutes, followed by 30 cycles of 95°C for 30 seconds, annealing at 57°C for 30 seconds and an extension step of 72°C for 30 seconds. This was immediately

followed by a final extension at 72°C for 7 minutes before the melt stage. Each run also included the non-template control as well as the pre-amplified and diluted *pl3* synthetic construct as the positive control.

3.6.7 Detection of *Coxiella*

To determine if *Coxiella burnetti*, the causative agent for Q fever was present in the meat samples, PCR-HRM targeting the specific insertion gene *IS1111* was carried out. Each run was a 10-µl reaction volume containing 2 µl of the 5X HOT FIREPol® EvaGreen® HRM Mix no ROX, 0.5 µM of both forward and reverse primers, 2 µl of the DNA template and topped up with PCR water (Tokarz *et al.*, 2009). This was carried out in a touch-down PCR described in (Ndeereh *et al.*, 2017).

3.6.8 Universal 16S rRNA gene for detection of other bacteria

To investigate the presence of other pathogenic bacteria that may be present in the meat samples, a set of general primers targeting the 16S rRNA gene of bacteria was used. The sole way used for detection using these primers was conventional PCR followed by gel-electrophoresis due to the large expected size of the amplicon, ~1350 bp. This involved 15-µl PCR reactions consisting of 3 µl of 5X HOT FIREPol® Blend Master Mix® (Solis BioDyne, Estonia), 0.25 µl of both forward and reverse primer at 10 pmol/µl s, 2 µl of DNA template and topped up with nuclease free water as described in previous studies (Fredriksson *et al.*, 2013; Srinivasan *et al.*, 2015). The cycling conditions for these runs included the initial denaturation at 95°C for 15 minutes followed by 35 cycles of 95°C for 20 seconds,

annealing at 58.3°C for 30 seconds and annealing at 72°C for 1 minute. This was followed by the final extension of 72°C for 7 minutes. The amplified products were electrophoresed in a runVIEW gel tank (Cleaver Scientific, United Kingdom) at 125V for 30 minutes in 1.5% agarose gel made of 1XTAE buffer (40mM Tris Base; 20mM Acetic Acid; 1mM EDTA). This involved dissolving 1.5 g of agar in 100ml of TAE buffer and addition of ethidium bromide before casting the gel. For 5 µl of each sample, 2 µl of loading buffer was added before loading them into individual wells. Visualization was done using GenoPlex (VWR International, Leicestershire, United Kingdom). To confirm that only the intended target was amplified, a 100-bp ladder (Solis BioDyne, Estonia) was used to confirm amplicon size.

3.6.9 Sequencing of positive samples

Samples that were successfully amplified using the pan- or specific primers were cleaned using the ExoSAP-IT protocol (USB Corporation, Cleveland, OH) and the cleaned product was sent to Macrogen, Europe for Sanger sequencing.

3.7 Sequence Analysis

All the sequences were trimmed and analyzed using Geneious v 11.1.5 software, available from Biomatters (Kearse *et al.*, 2012). The sequences were then queried against the NCBI GenBank database using BLAST parameters, as well as the BOLD (http://www.boldsystems.org/) database for vertebrate identification. The GenBank database was also used for the identification of bacterial sequences. Multiple

sequence alignments for bacterial sequences were carried out using Clustal Omega (EMBL-EBI).

3.8 Statistical Analysis

Data analysis was carried out to determine if the results obtained relating the treatments applied to the melting temperatures observed had any statistical significance. The relationship between the extraction protocols and melting temperatures was also examined. Both of these relationships were determined using two-way ANOVA. All the statistical analysis was carried out using RStudio v 1.1.453 in conjunction with the R console v 3.5.0. To carry out some summary statistics, the libraries 'ggpubr' and 'dplyr" were used. The function aov () was used to carry out the analysis of variance.

CHAPTER FOUR

4.0 RESULTS

4.1 Molecular identification of meat samples

4.1.1 DNA Quantification

DNA was successfully extracted from all the 221 samples. However, the range of DNA concentration was different based on the extraction protocol used. Those extracted using the kits had a lower concentration than those using the lab-optimized protocol afore-mentioned. Based on the data of 9 samples extracted using each of the four protocols in section 3.4.1, the following was determined: The DNeasy Blood and Tissue Kit protocol (Qiagen, Valencia, CA) and the ISOLATE II Genomic DNA Extraction kit (Bioline, UK) had a lower yield, with an average of 39.46 ng/ µl and 37.06 ng/ µl respectively. The lab optimized protocol had a high yield of 297.74 ng/ µl while using the same protocol without adding proteinase K in the cell-lysis yielded a slightly lower average DNA concentration of 251.45 ng/ µl (Figure 2).

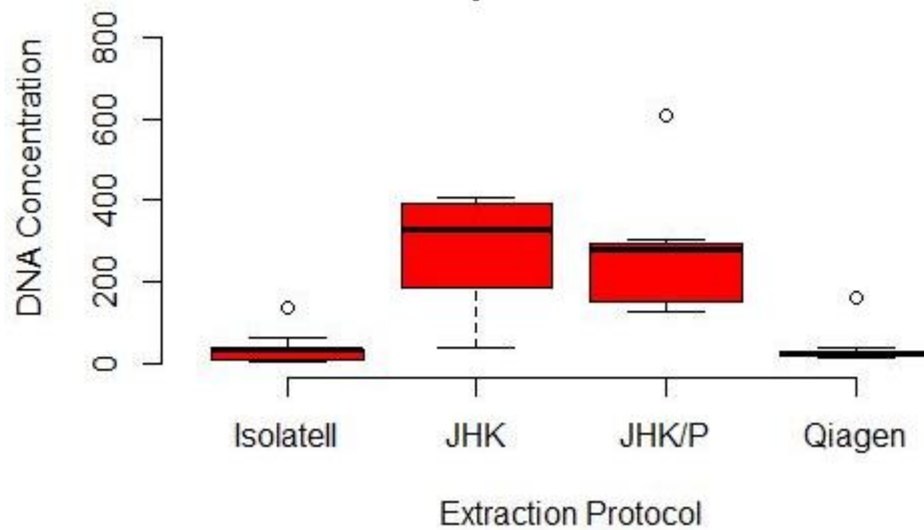

Figure 2: Box plot showing DNA concentration obtained using different extraction protocols

Key: IsolateII- ISOLATE II Genomic DNA kit (Bioline, UK), JHK- lab-optimized protocol, JHK/P- lab-optimized protocol without proteinase K, Qiagen-DNeasy Blood and Tissue Kit protocol (Qiagen, Valencia, CA).

4.1.2 Identification of positive control for goat meat

Initial analysis of the melt profiles produced by amplifying DNA from the samples labeled Goat Meat (GM) and Goat Meat B (GMB) showed varying melting temperatures and peaks. A look at the profiles produced targeting the marker CO1, GM had two peaks, whereas GMB had a single peak. The difference was seen in the other markers, Cyt b and mitochondrial 16S rRNA. This brought the need for sequencing of both amplicons to determine the true goat sample for use in our analysis. Analysis of the HRM profiles produced by the seven known goat samples allowed for the confirmation of 'Goat Meat B' as *Capra hircus* (goat) as well as excluded the sample labelled 'Goat Meat' (Figure 3). Additionally, the Sanger

sequencing carried out on both samples positively identified GM as originating from *Ovis aries* (sheep), and confirmed GMB as goat (Appendix 4) hence providing positive controls that could be used for further analysis.

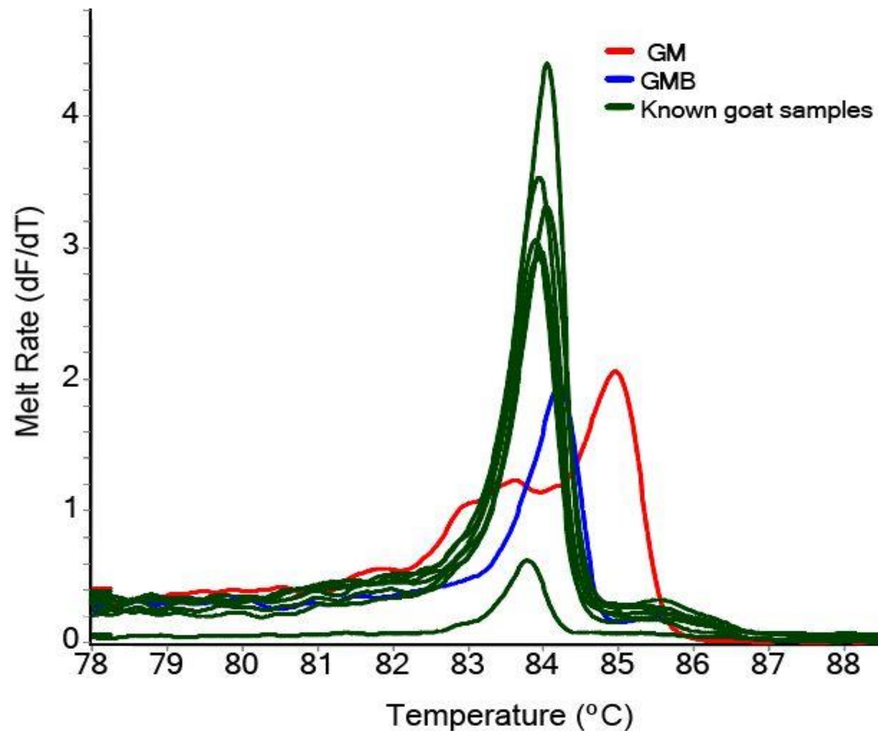

Figure 3: Cytochrome c oxidase subunit 1 melt profiles to determine goat samples. Key: GM – 'Goat Meat'; GMB- 'Goat Meat B'

4.1.3 HRM identification of meat sampled from butcheries in Nairobi

Through PCR-HRM analysis of the three genetic markers, CO1 (Figure 4), Cyt b (Figure 5) and mitochondrial 16S rRNA (Figure 6), as well as confirmation by sequencing of the long CO1 marker, 15/115 of the samples (13.04%) were determined to have been misrepresented (Figure 7).

The identification of the unknown samples was done through comparison of the high-resolution melting curves they generated against the curves produced by the reference samples; known positive controls. Each gene produced varying curves that allowed the distinction of species as indicated in the figures below. The curves produced by CO1 allowed the easiest distinction between species (Figure 4). This was followed by Cyt b, where although the curves were closer together, there was still clear delineation between species. However, using the mitochondrial 16S rRNA gene alone would have made distinguishing some of the species e.g. pig and camel, difficult.

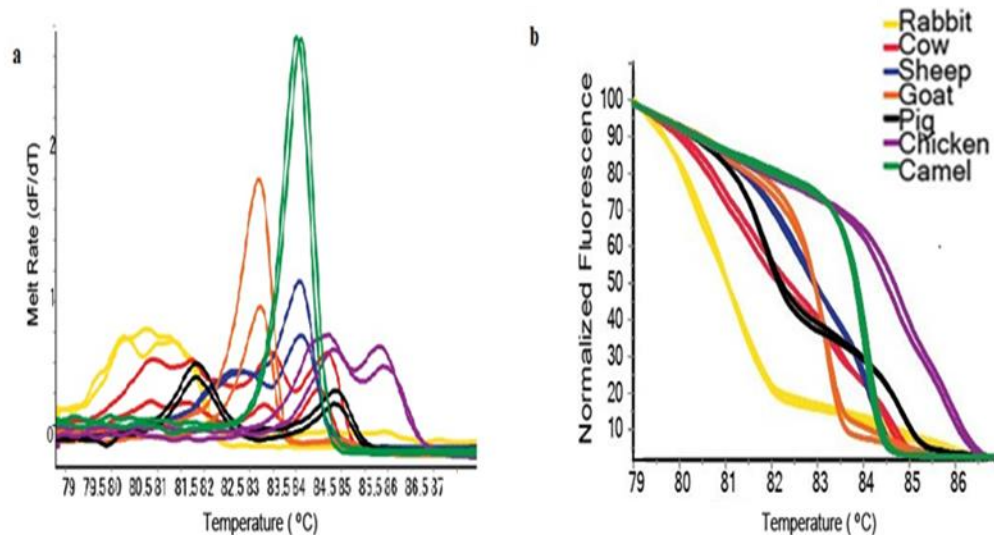

Figure 4: High-resolution melting analysis through comparing a) melt profiles and b) normalized curves of the known species targeting the gene cytochrome oxidase subunit 1.

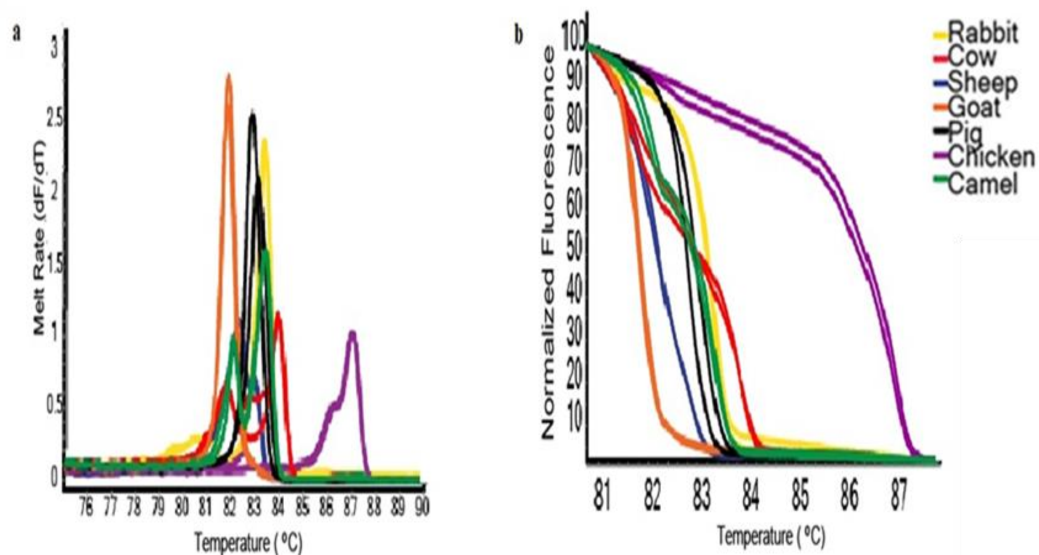

Figure 5: High-resolution melting analysis through comparing a) melt profiles and b) normalized curves of the known species targeting the gene cytochrome b.

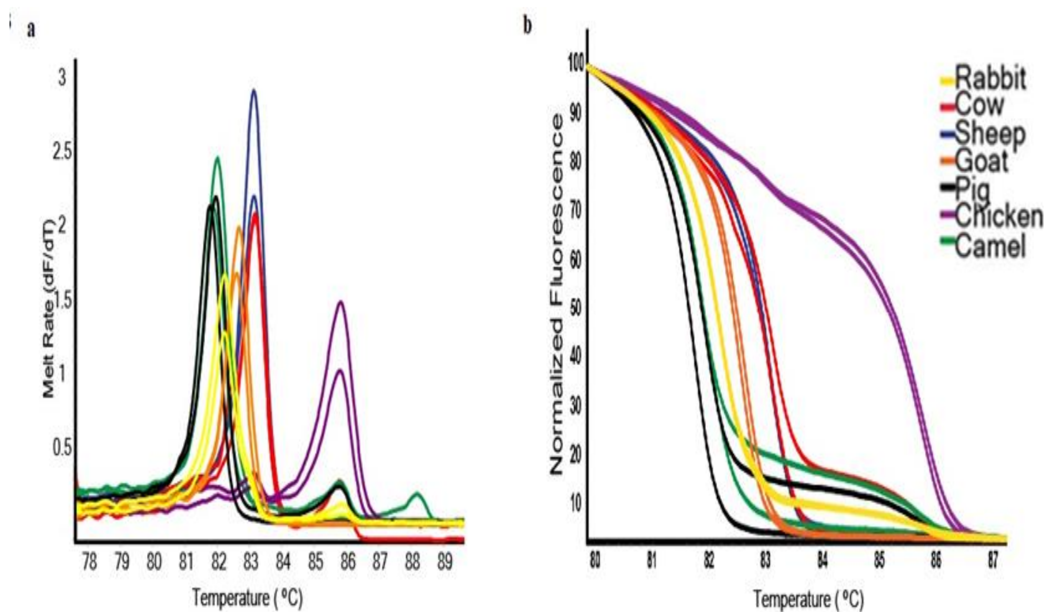

Figure 6: High-resolution melting analysis through comparing a) melt profiles and b) normalized curves of the known species targeting the mitochondrial gene 16S rRNA.

4.1.4 Species substitution in Nairobi

It was determined that although most of the samples sold to consumers were correctly labeled or represented, there was a total of 15 cases out of the 115 samples misrepresented. As the desired species was specified during purchase, it was possible to detect misrepresentation. Of the 61 expected to be cattle, two (3.27%) were goat, and one (1.64%) was camel, whereas one of the nine purchased pig samples was cattle. It was also found that four (13.3%) of the expected goat samples were sheep, while three of them were cattle (Figure 7). The case of species substitution was higher in the fish samples, where all the tilapia samples purchased were found to be Nile perch.

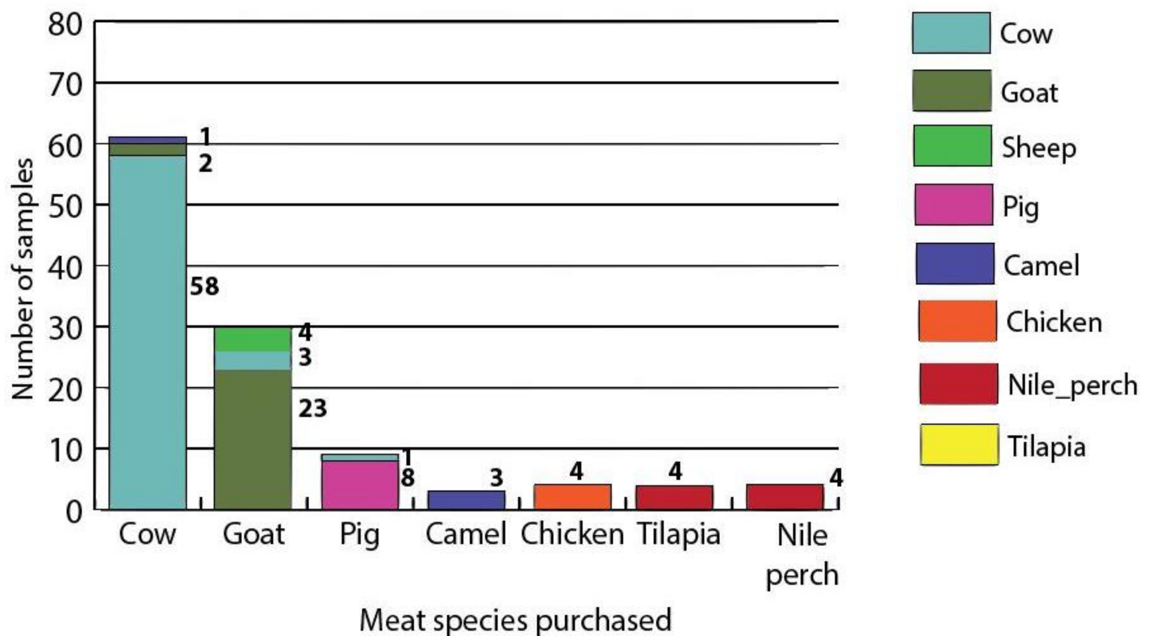

Figure 7: Stacked graph showing species substitution in Nairobi meat value chain (November 2018)

4.2 Experimental analysis of meat samples

4.2.1 Effect of different treatments on PCR-HRM profiles

The application of different treatments has minimal effect on the melting temperatures of the PCR products. Analysis was carried out on CO1, where single peaks could be viewed and compared (Figures 7 and 8). Although there was a slight shift in the Tm, there was no correlation between heat treatment and higher melting temperature. The shift in Tm of the CO1 amplicons compared to the control (untreated meat) ranged between -0.54°C and +0.75°C for microwaved samples, -0.25°C and +0.56 °C for oven dried samples and -0.23°C and +0.65°C for the rotten meat samples. There was a slight shift in Tm based on the different treatments applied. However, the standard deviation is low showing that various conditions meat is found in will not affect PCR-HRM results (Table 3).

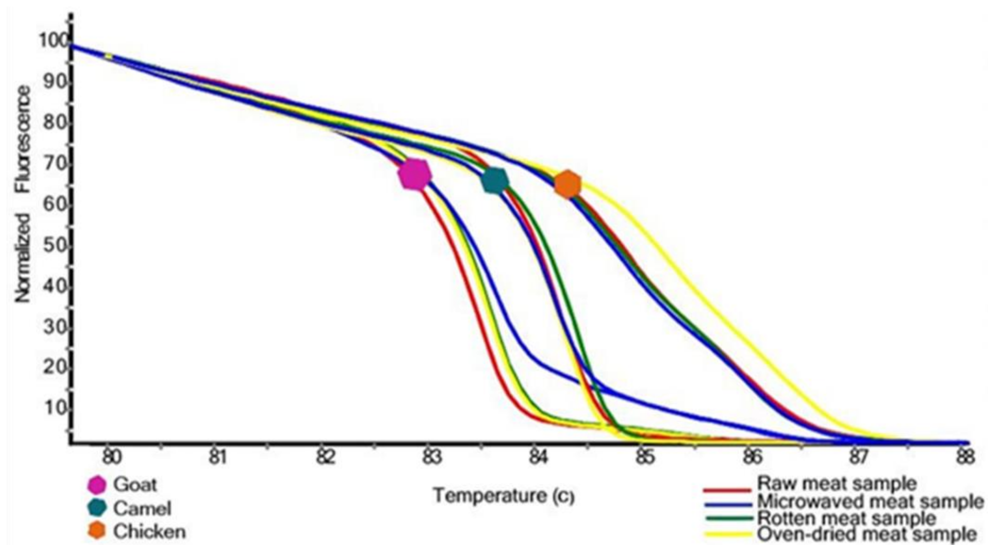

Figure 8: Normalized HRM profiles showing how the melting temperature of the CO1 region of the DNA samples was affected by the different treatments applied.

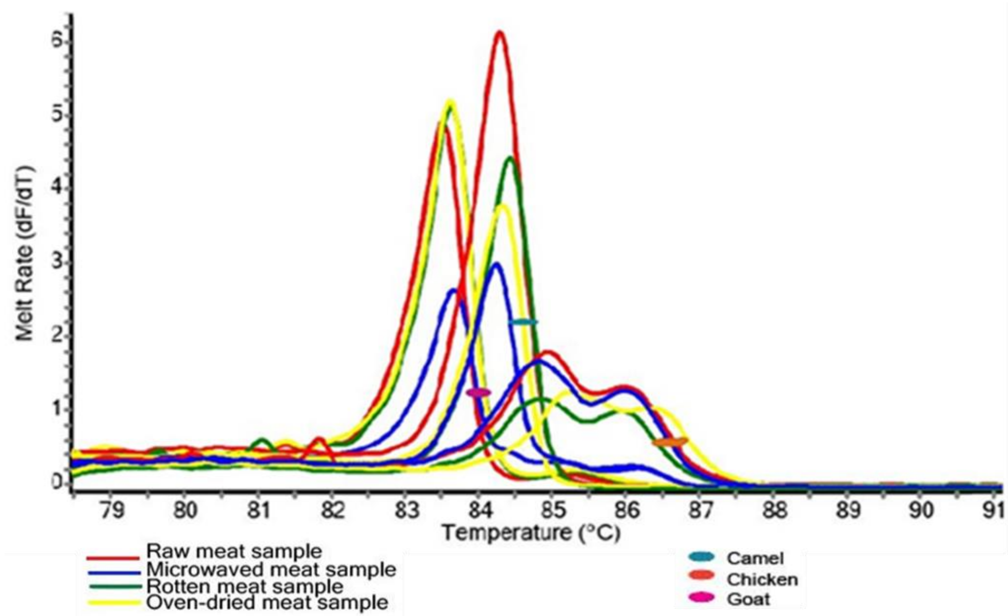

Figure 9: Melt rate profiles of CO1 regions from DNA extracted from meat exposed to different treatments.

Table 3: Melting temperatures of single peaked profiles targeting CO1 marker

		Melting temperature per treatment					
Sample ID	**Species**	**Raw**	**Rotten**	**Oven-dried**	**Cooked**	**Standard deviation**	**Variance**
Goat64	Goat	83.63	83.42	83.38	84.17	0.3636	0.1322
Goat27	Goat	83.52	83.60	83.52	83.70	0.0854	0.0073
Goat08	Goat	83.50	83.61	83.61	83.65	0.0645	0.0042
Camel1	Camel	84.30	84.42	84.30	84.25	0.0723	0.0052
Camel2	Camel	84.35	84.80	84.25	84.25	0.2626	0.0690

Two-way ANOVA was carried out based on the two factors; treatment applied and species. Due to the unbalanced nature of the data a Type III ANOVA test was carried out using R, giving the results shown in Table 4.

Table 4: ANOVA test looking at effect of treatments applied on the melting temperature.

Source	**Sum of Squares**	**Degrees of Freedom**	**F-Value**	**Pr (>F)**
(Intercept)	14196.1	1	5.7006e+05	< 2e-16 ***
Species	0.2	1	8.1003e+00	0.01473 *
Treatments	0.2	3	2.2206e+00	0.13838
Species: Treatments	0.3	3	3.4829e+00	0.05028
Residuals	0.3	12		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

The only significant difference in melting temperature is caused by species of origin; which determines the GC content of the DNA segment, hence the melting temperature. The intercept value caters for imbalance between the number of samples i.e. two camels and three goats. Both the PCR-HRM analysis and statistical analysis show that application of different treatments will not hinder the correct identification of meat samples obtained from suspected channels.

4.2.2 Effect of different extraction protocols on PCR-HRM

To compare the different extraction protocols, the Qiagen extraction protocol was used as the standard (control). Other extraction protocols were compared to this protocol. The melt temperatures varied with a range of -0.1°C to +0.29°C for Isolate II, between less than + 0.1°C to + 0.2°C for the lab optimized protocol and less than + 0.1°C to + 0.2°C for the modified lab-optimized protocol which excludes proteinase K, respectively. The melt profiles were unchanged at the species level (Figure 10 and Figure 11). In all the samples compared, Qiagen- extracted samples had the lowest melting temperature (Table 5). These curves show that the use of extraction protocol used has little effect on the melting behavior of the DNA. The protocols cause even less variation between the T_ms than the treatments used. Species of origin is still the major determinant of the final melting temperature (Table 6).

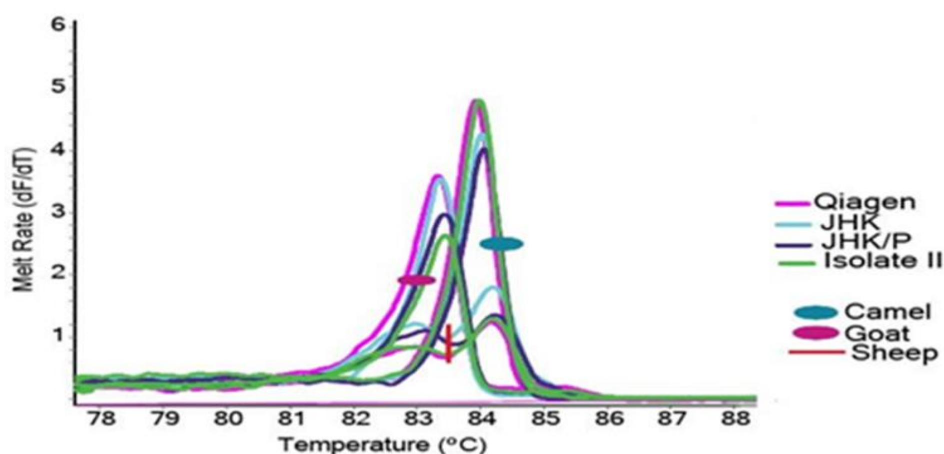

Figure 10: Melt rate profiles of the CO1 gene showing the shift in melting temperature of three species: camel, goat and sheep.

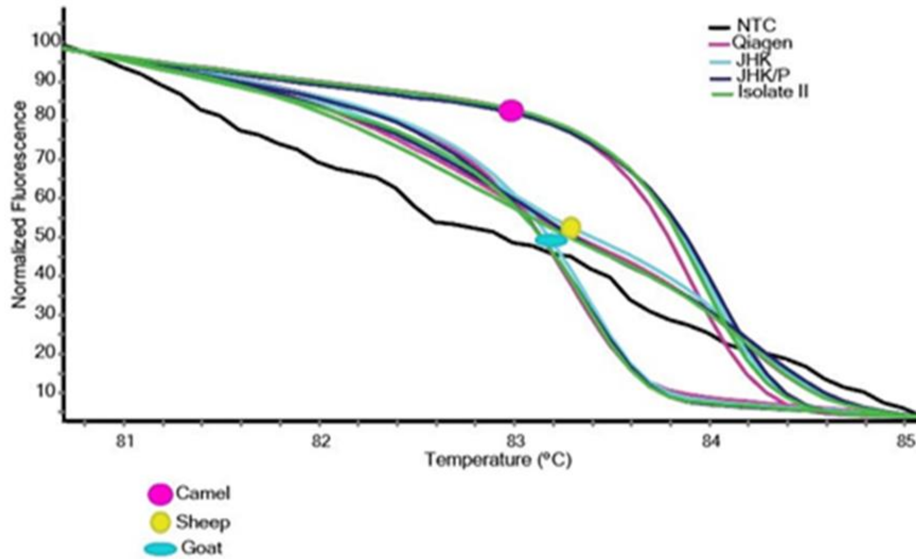

Figure 11: Normalized HRM curves of the CO1 region of three species (camel, goat and sheep) showing decrease in fluorescence with increase in temperature.

Table 5: Changes in melting temperatures based on DNA extraction protocol used

		Melting temperature per Protocol					
Sample ID	Species	Qiagen	IsolateII	JHK	JHK/P	Standard deviation	Variance
41BM_Goat	Goat	83.41	83.70	83.41	83.45	0.1396	0.0195
43BM_Goat	Goat	83.30	83.38	83.38	83.38	0.0400	0.0016
44BM_Camel	Camel	83.90	84.00	84.03	84.04	0.0640	0.0041
92BM_Goat	Goat	83.33	83.43	83.39	83.42	0.0450	0.0020
Camel1	Camel	83.90	84.00	84.03	84.04	0.0640	0.0041

Table 6: ANOVA results of association between extraction protocol, species of origin and the melting temperature

Source	Sum of Squares	Degrees of Freedom	F-Value	Pr(>F)
(Intercept)	14112.0	1	2.4662e+06	< 2.2e-16 ***
Species	0.3	1	5.1730e+01	1.098e-05 ***
Protocol	0.0	3	1.4301e+00	0.2825
Species: Protocol	0.0	3	8.8910e-01	0.4746
Residuals	0.3	12		

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

4.2.3 Distinction of meat sources using PCR-HRM

The distinction of meat mixtures was not possible using conventional PCR-gel analysis targeting of any of the markers we used for vertebrate identification (Figure 12). This is because the amplicons produced targeting the mitochondrial markers are the same size across species (200bp), which was different from the expected band size of the universal 16S rRNA marker used for screening of bacteria (1350 bp). However, gel analysis of amplicons using species-specific primers would have allowed this distinction. The melting profiles obtained from the short CO1 marker showed slight variations between pure samples and the mixed samples but could not be used to show the individual sources that made up the mixture.

The Cyt b marker was successful in showing separate curves for the white meat mixtures including chicken and pork and tilapia and pork (Figure 13a), with DNA from mixed meat sources having two distinct peaks. The individual curves corresponded with the distinct meat sources. However, differentiating sources of red meat using this marker was not possible. For instance, all mixtures that contained any goat meat had a melt profile indistinguishable from pure goat samples (Figure 13b).

Amplification targeting the marker mitochondrial 16S rRNA gave the best resolution in isolating the individual sources making up the mixtures. With two distinct peaks, each matching with the source species, visible in the samples with DNA extracted from mixed meat. The only mixed samples that could not be determined using this marker contained cattle and sheep sources due to their similar melt temperature and profile (Figure 14).

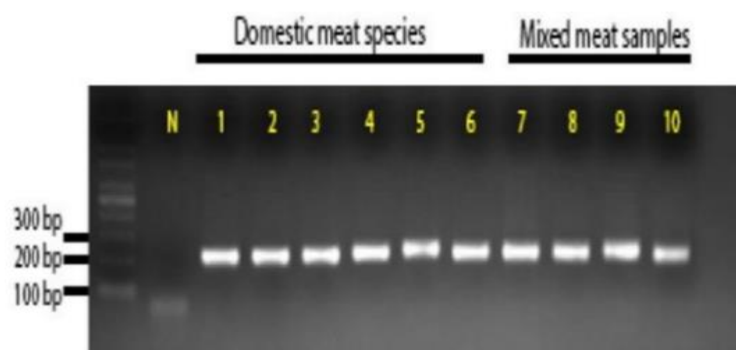

Figure 12: Agarose gel image of mitochondrial 16S rRNA gene amplification of domestic and mixed meat samples using PCR.

Expected band size - 200bp

Key: Lane- N- non-template control, 1-cattle, 2-goat, 3-camel, 4-pig, 5-chicken, 6-sheep, 7-cow + goat, 8-cow + camel, 9- pork + chicken, 10-goat + sheep.

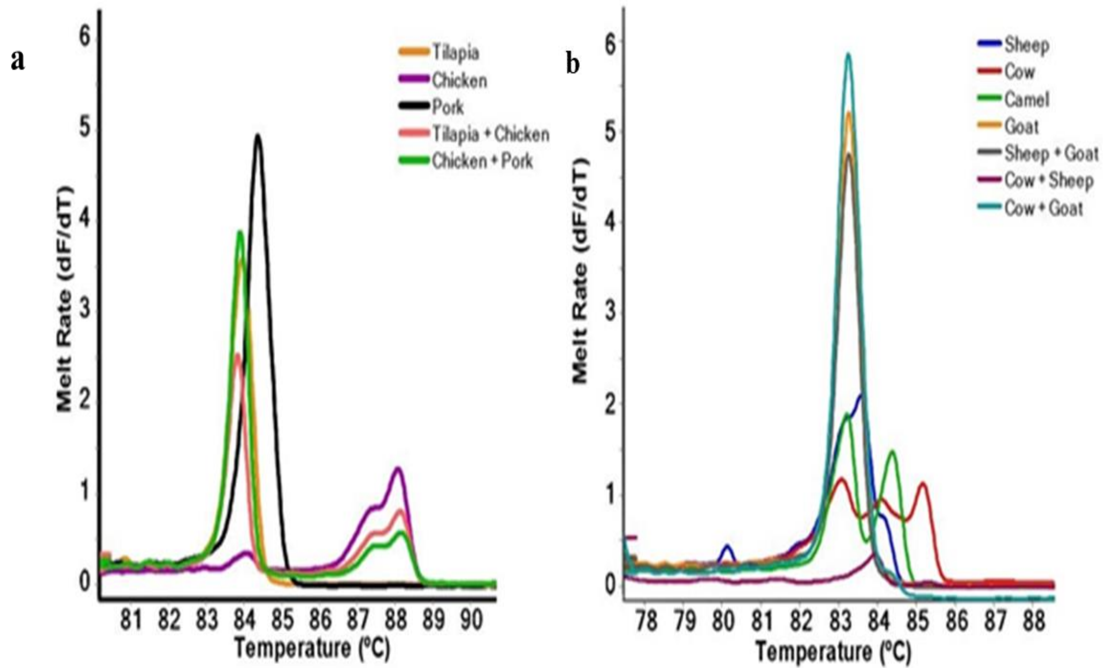

Figure 13: Melt rate profiles showing how Cyt b distinguishes pure and mixed samples. (a) Double peaks that correspond to mixed samples in white meats. (b) Red meat mixtures cannot be clearly distinguished using the same marker.

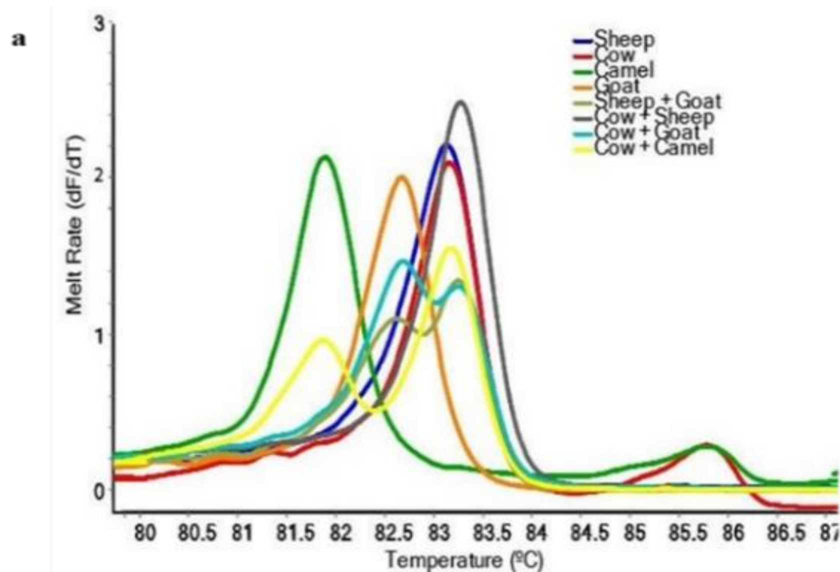

Figure 14: High-resolution melting analysis of the mitochondrial 16S rRNA gene of pure and mixed meat samples. Melting profiles produce double peaks in mixed samples that correspond with source species.

4.3 Molecular screening of zoonotic bacterial pathogens

4.3.1 *Brucella* spp.

PCR-HRM analysis targeting the bcsp31 gene present in all *Brucella* species was carried out successfully (Figure 15), yielding preliminary results with 27 positive samples out of the 221 samples (Appendix 3). Of these 27 samples, 18 were samples obtained in Nairobi, while the rest were from Naivasha. The majority of these samples were from cattle, at 42.86%, followed by 8 goat samples and 5 sheep. This was also confirmed using conventional PCR where the expected band size, 151 bp, was observed. The presence of this gene shows the presence of pathogenic *Brucella* species in the meat sample. Representative positive samples, after amplification and clean-up were sent for sequencing. Thereafter, comparison of sequences against the GenBank database confirmed that *Brucella* species highly similar to the species *Brucella abortus*, *B. suis* and *B. melitensis* were present in the sample (Appendix 5). PCR-HRM targeting the *bcsp31* gene could not be used to distinguish multiple *Brucella* species in the same sample. This is because it is common in the *Brucella* genus.

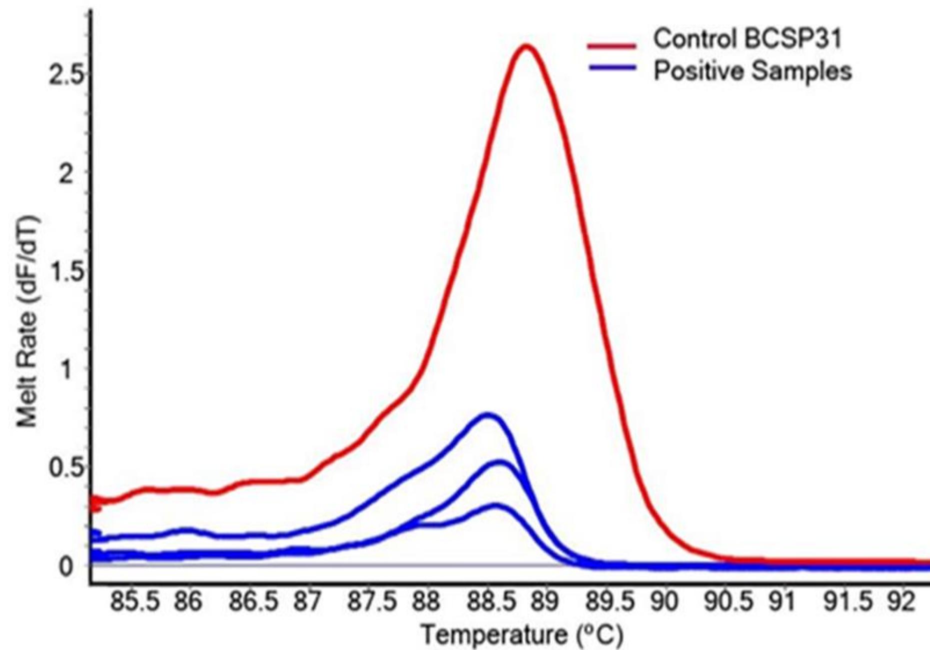

Figure 15: Melt rate profiles obtained during screening for *Brucella*. All samples with peaks similar to the positive controls were considered as positive in the preliminary screening.

4.3.2 *Leptospira* spp.

Out of the total 221 samples, only one meat sample, a cattle sample from Kinamba, Naivasha area was found positive for pathogenic *Leptospira* species. Although 3 samples had peaks similar to the secYIV marker, sequencing confirmed that only one of those samples was pathogenic *Leptospira* while the other two were merely artifacts (Figure 16). Re-running the PCR-HRM assay, showed that only that single sample had peaks corresponding with the positive control. Using NCBIs Basic Local Alignment Search Tool (BLAST) confirmed that the positive sample had 99.1% identity with *Leptospira interrogans* (Appendix 6).

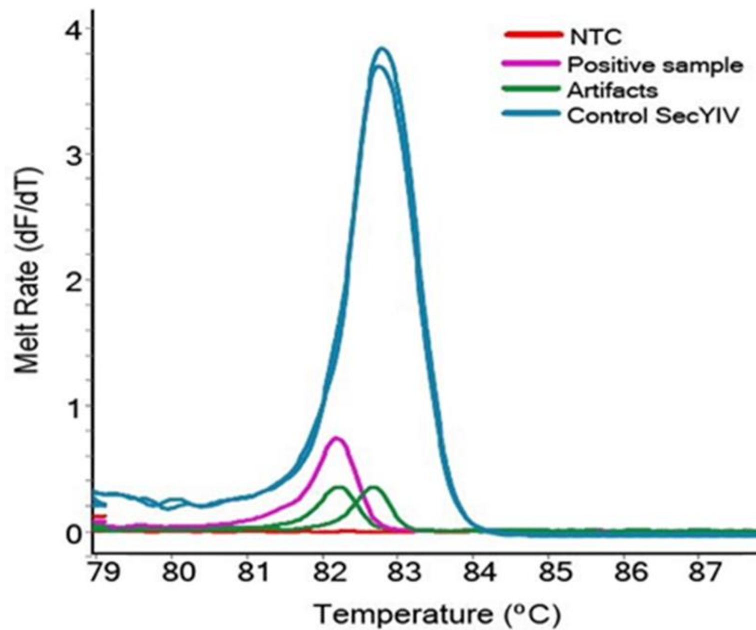

Figure 16: Melt rate profiles of the SecYIV gene that is present in all pathogenic Leptospira species.

Key: NTC- Non-template control

4.3.3 *Bacillus anthracis*

Using the optimized conditions to screen for possible risk of exposure to anthrax through the food chain, yielded no positive samples. The marker PL3 was used to optimize both standard PCR and PCR-HRM, but there were no similar peaks or bands within the samples (Figure 17).

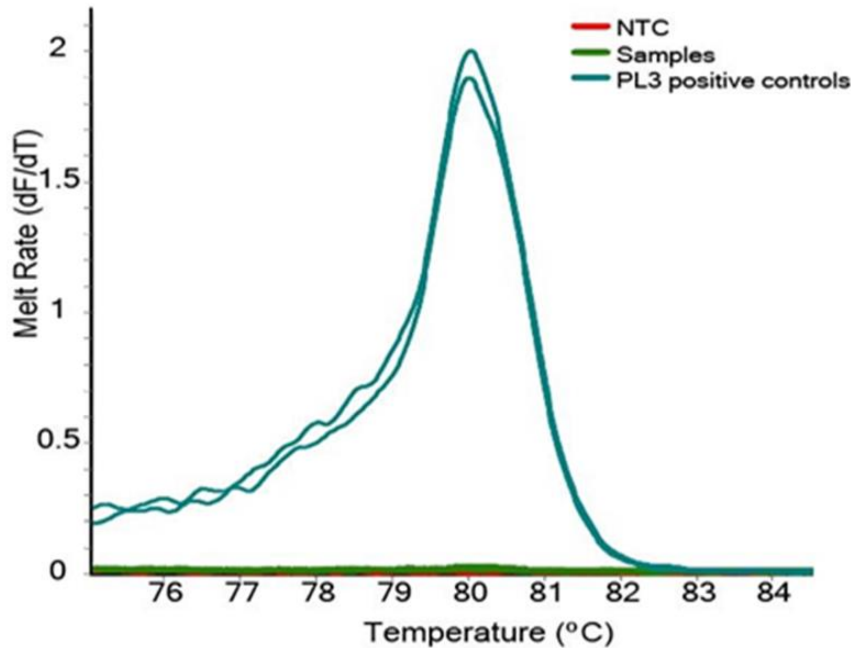

Figure 17: PCR-HRM melt profiles for Bacillus anthracis screening. No samples were positive for the pathogen.

Key: NTC- Non-template control

4.3.4 *Coxiella burnetti*

Screening for this pathogen using lab -optimized conditions, yielded no positive samples. There were no positive samples used in this run although the PCR-HRM primer conditions had been optimized previously.

4.3.5 Universal 16S rRNA gene pathogens

The use of the general primers targeting the 16S rRNA gene sections of bacteria allowed us to identify both apathogenic and pathogenic bacteria. The primers amplified the region between 27 bp and 1492 bp of the bacterial 16S rRNA gene. Due to the large expected band size, PCR-HRM was not ideal for their identification. Hence, conventional PCR was used (Figure 18a and 18b). Of the 221 samples

screened using these primers, 59 were positive, with visible bands of the expected band size (Figure 18a).

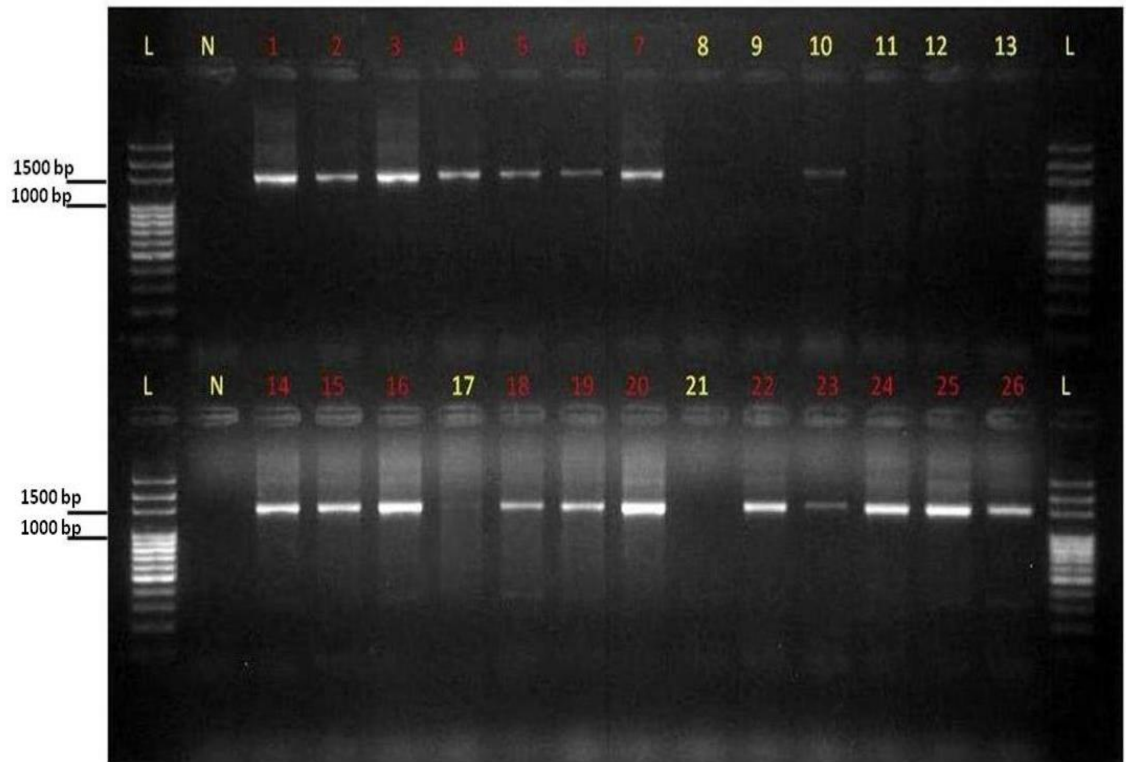

Figure 18a: Gel electrophoresis image of representative samples from Nairobi that were screened for bacteria using the universal 16S rRNA primers

Expected band size – 1350bp

Key: L-Ladder, N- Non-template control. Those numbered in red were positive for bacteria.

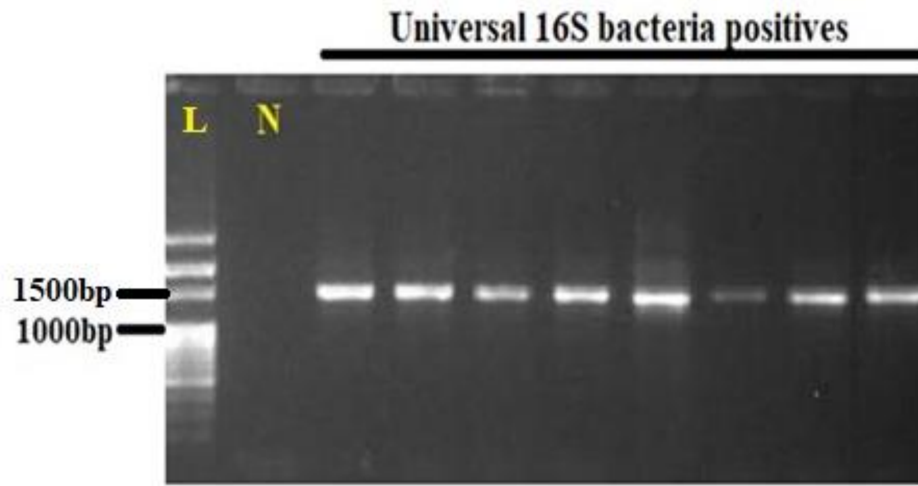

Figure 18b: Gel electrophoresis image of selected positive samples that were screened for bacteria using the universal 16S rRNA primers and cleaned for sequencing.

Expected band size – 1350bp

Key: L-Ladder, N- Non-template control, Lanes 2-8 contain representative samples found to contain bacteria.

Of the 59 positive samples identified and sequenced, only 7 were proven to be pathogenic using NCBIs BLAST sequence alignment (Appendix 2). Those with both the Query cover and percentage of higher than 90% were considered as positively identified. (Sequences in Appendix 7) Four of them were determined to be *Lactococcus garvieae*, one *Clostridium perfringens* species, one *Clostridium septicum* and *Aeromonas caviae*. Four of seven contaminated samples were from Naivasha region where as the rest were from Nairobi. The *Clostridium* species were found in cattle samples collected from Burma Market whereas the *Aeromonas* was found in a giraffe meat (Ouso *et al*., 2020) sample misrepresented and sold in Kambi Somali, Naivasha region. Three of the *Lactococcus* species were found in samples

sold in Naivasha region (2 sheep and 1 cattle) whereas one was found in a pig sample obtained in Jerusalem, Nairobi. The small proportion of pathogens (35/221) or roughly 8%, indicates that in the samples obtained in this study, meat fraud did not pose a public health risk.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Vertebrate species identification

The use of mitochondrial DNA markers for species determination has proven useful, reliable and is even used in barcoding of species (Barcaccia *et al.*, 2016). These markers are favored due to their high copy numbers in cells as well as high mutation rates, which allow for distinction of closely related species (Klomtong *et al.*, 2016). Mitochondrial DNA (mtDNA) markers are used to track down food piracy and mislabeling because they are highly conserved in the species level as well as preserved even in processed food products (Barcaccia *et al.*, 2016). Comparison of unknown samples with HRM profiles of known meat sources was successfully used in the identification of animal sources of meat. The mitochondrial 16S rRNA, Cyt b, and CO1 markers used have been used previously in blood-meal (Omondi *et al.*, 2015; Ogola *et al.*, 2017) as well as bushmeat (Ouso et al., 2020) analysis. The use of these three mtDNA markers for meat source analysis gave us a more robust way of species determination and distinction as some samples amplified well with one marker and not another (Omondi *et al.*, 2015; Ouso *et al.*, 2019). Using these markers, we were able to detect 15cases of species substitution, the majority of which occurred between goat meat and cheaper alternatives such as cattle (three cases) and sheep (four cases). This occurrence is similar with those reported before (Cawthorn *et al.*, 2013; Di Pinto *et al.*, 2015), where most adulteration is linked to financial gain, and may cause allergic reactions but no public-health risk. Growing

evidence that relates the consumption of beef and mutton with a severe allergic reaction termed "*midnight anaphylaxis*" reveals that substitution with meat from these species may pose a health risk to susceptible populations (Gray et al., 2016).

Generally, differences in the profiles produced between species were far greater than differences within species of all genes. Although CO1 gave the best differentiation between species, it had lower PCR amplification and peaks in the melt profiles of some species such as cattle and pig when compared to the other two markers. These species also had multiple peaks in CO1 amplification making analysis of the melting temperature difficult in those cases. The Cyt b and mitochondrial 16S rRNA markers had higher peaks and amplification in the majority of samples. A study carried out by Nicolas *et al.* (2012) found that intra- and inter-specific divergences were significantly higher for CO1 and Cyt b genes than for mitochondrial 16S rRNA. Their work showed that 16S is 2.5 less variable than Cyt b and CO1, giving it lower discriminatory power. An interesting look into this phenomenon is shown in known pig samples which had a wide range of melting temperatures when analyzed using the mitochondrial 16S rRNA gene and cyt b markers as shown in Appendix 8.

The application of various treatments to the meat samples allowed us to mimic the conditions that meat samples may be found in. Often, meat analyzed for species determination in food fraud cases may have undergone some post-slaughter changes such as processing, cooking, sun-drying or smoking, for instance in bushmeat cases (Ramanan & Khapugin, 2017). A comparison of the melting temperature between

the DNA extracted from raw (controls), oven-dried, cooked and degraded meat samples allowed us to note that the application of different meat treatments had minimal effect on the HRM curve profiles of the different domestic animals. The HRM profiles obtained from using CO1 were more reliable as there was less intra-specific variation unlike those produced targeting mitochondrial 16S rRNA. The similar melting profiles and slight shifts in the melting temperature show that high-resolution melting targeting mitochondrial markers will give similar results in same-species meat samples even if they have undergone different treatments. Based on the HRM analysis, the largest shift in melting temperature was seen in microwaved meat, where there was a more significant deviation from control samples. Sakalar *et al.* (2012) showed that application of heat treatments such as baking and boiling decrease the detectable number of copies of some genes. The temperature applied, as well as the length of heat treatment would affect the quantification of DNA from the samples. This could also explain the slight shift in the HRM curves displayed.

The use of four different extraction protocols; i.e. manual vs kit-based extraction, yielded even less of a variation between the same-species samples. The variation in melting temperature was probably caused by the difference in salt concentrations of the DNA yielded using different protocols. For instance, cations such as Mg^{2+} and Na^{+} interact with the highly charged DNA polyanion, with lower Na^{+} concentrations favoring the denaturation of double stranded DNA (Tan & Chen, 2006). Notably, in the samples tested, QIAGEN kit extracted samples had the lowest melting temperature whereas the lab-optimized protocols had higher temperatures. Although

the T_m shifted, their variation did not cause a change in the melting profile. This technique is hence reliable with the use of various extraction protocols.

Additionally, based on melting profiles with double-peaks, we were able to detect mixed meat samples by targeting the mitochondrial markers Cyt b as well as 16S rRNA. With increased need to check for meat-adulteration (O'Mahony, 2013; Di Pinto *et al.*, 2015), faster, more affordable techniques are also required. HRMA allows one to filter through all the samples hence saving on cost of sequencing tools from all to only those that have questionable profiles or some representative samples (Lopez-Oceja *et al.*, 2017). Although Cyt b enabled the distinction of several mixtures from domestic animal sources, all mixtures containing goat meat were indistinguishable from pure goat samples. Using mitochondrial 16S rRNA gene, allowed for more distinction of the sources of meat. However, this technique was limited in cases where the vertebrate sources have similar melt profiles and temperatures such as *Bos taurus* and *Ovis aries*.

Despite its proven advantages, HRM analysis is limited by its need to have a set of reference species that are to be run side by side with samples in each run. The samples selected as references are also quite subjective, determined by what the researcher may deem as important. However, unique HRM profiles that do not match any controls can be identified by amplicon sequencing of representative samples. The use of the mitochondrial genes, which are commonly used for DNA barcoding and species fingerprinting, made species differentiation possible.

However, although mitochondrial markers are preferred in species identification, (Ballin *et al.*, 2009; Cai *et al.*, 2017) they are not appropriate for quantification of meat samples in mixtures because there are major differences in mtDNA levels in diverse species (Cai *et al.*, 2017).

5.2 Pathogens identified using molecular analysis.

The screening of the meat samples showed that although most of the meat sold in Nairobi and Naivasha during the time period were free from zoonoses, bacterial contaminants had come in contact with zoonotic pathogens. As the meat samples were obtained from the final selling point (butcheries), it was not possible to determine at which point in the meat value chain that contamination with these pathogens occurred. This is because the pathogens could have originated from the meat handlers or the meat handling environment from the slaughterhouses up to the butcheries. These findings could vary depending on the season of sampling (D'Andrea *et al.*, 2012) as well as tissue sample collected. For instance, pathogens of the genus *Leptospira* tend to colonize the renal tubules of the kidneys (Jobbins & Alexander, 2015) and would hence be hard to find in the muscular tissue most often sold as meat. Despite this, one meat sample from Naivasha was found to contain this pathogen, hence determining that those who handled the animal and its bodily fluids, for instance during slaughter, were exposed to this pathogen.

The risk of transmission of brucellosis was even higher due to the higher number of samples found with the causative pathogens. This transmission could occur in cases

of consumption of undercooked contaminated meat. Since it is often regarded as an occupational hazard, it could also be transmitted to the farmers, slaughterhouse workers and butchers who had contact with any of the bodily fluids of these infected animals (Garshasbi *et al.*,2014). Given that a majority of the *Brucella* positive samples were cattle, this also hints that any female cattle within the herd could also transmit these pathogens through the milk they produce. There could also be cases of spontaneous abortions within the herd causing economic losses.

The absence of *Bacillus anthracis* in the samples correlated with the prevalence reports indicated in the country. Although dead animals have been reported to be sold to meat butcheries in slums, hence posing a risk of the spread of this pathogen (Alarcon *et al.*, 2017a), none were found in the samples collected. Anthrax outbreaks in Kenya are often recurrent in hotspot areas such as Nakuru West subcounty where livestock, humans, as well as wildlife are affected (Muturi *et al.*, 2018). However, none of our samples contained this pathogen.

Targeting the universal 16S rRNA gene to detect bacterial pathogens only resulted in the conclusive identification of 7/59 of the positive samples. This could be attributed to the nature of the primers, which amplified most bacteria present. Hence, any sample that could have more than one profile would be difficult to sequence and confidently identify. Another reason for this was that the majority of bacteria found were not pathogenic, but instead consisted of those normally found in nature. *Lactococcus garvieae,* one of the pathogens identified using the universal bacterial

primers, is a non-motile Gram-positive, catalase negative, facultatively anaerobic coccus (Rubião, 2018). It has optimum growth at 45°C, pH of 9.6, and at 6.5% NaCl (Rubião, 2018). It was determined to be the etiological agent of lactococcosis discovered in rainbow trout in Japan in the 1950s (Ferrario *et al.*, 2013) and has since spread to numerous countries and has been associated with outbreaks of this disease in many fish and marine species. Improvement in molecular techniques over the years has led to the isolation of this pathogen in other animals such as cattle and buffaloes with mammary infection (Ferrario *et al.*, 2013). Several strains of this species have been isolated from several food products such as milk, dairy products, meat, seafood and fish. They have also been isolated from human clinical samples (Ferrario *et al.*, 2013; Rubião, 2018), making *L. garvieae* gain recognition as an emerging human pathogen (Reguera-Brito *et al.*, 2016).

Although infective endocarditis is the most common manifestation of *L. garvieae*, it has been linked to other clinical manifestations such as liver abscess, urinary infections, infective spondylodictic and peritonitis in the immuno-compromised and elderly people (Reguera-Brito *et al.*, 2016). The presence of this pathogen in meat, could pose a hazard to such consumers, especially if the meat is undercooked or contaminated by this bacterium during handling. This is because epidemiological data from clinical studies shows a relationship between infection with *L. garvieae* and consumption of contaminated food (mostly seafood and fish) (Gibello *et al.*, 2016).

Pathogens of the genus *Clostridia*, which were isolated from meat samples obtained in Nairobi region, are opportunistic in nature (Nanjappa *et al.*, 2015). They are responsible for various deadly diseases such as gas gangrene, botulism and tetanus. *Clostridium perfringens* is a rod-shaped spore-forming, anaerobic, Gram-positive pathogenic bacterium normal to the intestinal flora of humans and animals. It can grow at temperatures that range between 20°C and 50°C. *C. perfringens* is classified into 5 types (A-E) (Uzal *et al.*, 2014; Dave, 2017) and produces over 20 toxins (Kiu & Hall, 2018; Yibar *et al.*, 2018). Consumption of food contaminated by this enterotoxin-producing *C. perfringens* (CPE) vegetative cells leads to food poisoning associated with abdominal pain and watery diarrhea (Yibar *et al.*, 2018). By 2013, this pathogen was the second most frequent cause of food-borne illness in the United states, causing around one million illnesses annually (Grass *et al.*,2013). Meat and poultry outbreaks reported to the U.S. Centers for Disease Control and Prevention's (CDC) Foodborne Disease Outbreak Surveillance System between the years 1998 and 2010 accounted for 92% of outbreaks linked to this pathogen (Grass *et al.*, 2013). Although *C. septicum* is generally found in nature and transmitted especially through contact of wounds with contaminated soil (originally reported during wartime), there have been clinical cases of infections in unwounded, otherwise healthy individuals. It was suggested that the source of the organism was the individuals own intestinal tract (Alpern & Dowell, 1969). The presence of these pathogens, especially *C. perfringens*, a known food-borne pathogen, shows that consumers could be at risk of infection by them.

Bacteria of the genus *Aeromonas* are Gram-negative, oxidase-positive, glucose-fermenting, facultatively anerobic rods (Isonhood & Drake, 2002) and usually found ubiquitous in aquatic environments. They have been isolated from environmental, clinical and food samples (Castilho *et al.*, 2009). These species have been identified as pathogens to both humans and poikilothermic animals, with clinical isolates obtained from patients with soft tissues infections, otitis, cystitis, septicaemia, diarrhea and extraintestinal diseases (Castilho *et al.*, 2009).These species have been discussed as potential or emerging food-borne pathogens, though this matter has been considered controversial because the results from human volunteer studies were inconclusive (Isonhood & Drake, 2002). However, the epidemiological evidence being produced from further research shows that these bacteria are able to cause gastroenteritis (Isonhood & Drake, 2002). Although the best growth temperature of aeromonads is believed to be 28°C, there has been a wide variability and range in the optimum temperature. Many strains can still grow at less than 5°C, and hence can grow in refrigerated foods. *Aeromonas cavieae*, which was found in one of the misrepresented samples, where giraffe meat was sold as livestock meat (Ouso et al., 2020), are highly cytotoxic and slightly enterotoxigenic in nature (Martins *et al.*, 2002). The presence of this food-borne pathogen in the meat sold to consumers also indicates a risk of infection.

5.3 The meat value chain

The present study has shown that there are cases of meat fraud in the meat value chain in Nairobi, where consumers are sold different animal species other than the meat requested and paid for. The case of species substitution was also shown in archived samples used in this study (Ouso et al., 2020). The presence of species substitution in the meat value chain could also bring about the violation of various religious beliefs; for instance, in cases where cattle and pig species were interchanged. The most substituted meat species was goat, which has a higher commercial value than the rest. The case of camel meat sold in place of beef is a cause for concern as camels are known to be significant point of transmission of zoonotic diseases to humans (Zhu *et al.*, 2019).

Aside from financial fraud, investigating the meat value chain also showed that although bacterial contaminants were found, this was expected as livestock are known reservoirs of zoonotic pathogen. This was made clear by the presence of the zoonotic pathogens *Leptospira interrogans*, *Brucella* spp., as well as the food-borne pathogens uncovered in these two regions: Naivasha subcounty and Nairobi county. The incidence of bacteria was not seen as more significant in either of the locations with roughly 14% and 18% of the samples obtained from Naivasha and Nairobi respectively having any of the target pathogens.

Although no bushmeat was found in this study, investigation by the Kenya Wildlife Services (KWS) uncovered the sale of wild animals in the target site, Burma Market.

On June 28th 2019, 800 kgs of bushmeat being sold as beef to consumers were revealed to be Zebra meat, leading to the arrest of 15 traders (Kiage, 2019). A few weeks later, on August 6th 2019, 200kgs of buffalo meat was impounded by KWS officials on its way to Nairobi from Naivasha where it would have been sold to consumers in the same meat market (Gitonga, 2019), bypassing the inspection protocols required to protect them (Kenya Bureau of Standards, 2017; GOK, 2012). The study's inability to collect any bushmeat could be due to lack of intelligence from KWS officials, as was done in the archived samples obtained from Naivasha (Ouso et al., 2020) concerning recently poached animals that would lead to sale of suspected bushmeat. The sampling strategy used, where small quantities of meat was purchased from several stalls could have aroused suspicion among the traders who would consequently only sell the meat obtained from legal species. Despite this, the study showed that meat fraud takes place in the sampling areas.

5.4. Conclusion and Recommendations

5.4.1 Conclusions

1. The use of the three genes, CO1, mitochondrial 16S rRNA and Cyt b to identify the vertebrate species was efficient as it allowed for the proper distinction of species.
2. High-resolution melting analysis is a robust analytical technique that can be used in meat species determination as well as to screen for various pathogens.

5.4.2 Recommendations

- PCR-HRM could be incorporated in the meat quality control system for fast, relatively affordable and accurate species-identification in the meat value chain.
- The use of PCR-HRM can be used for the quick detection of pathogen contaminants in the meat sector.
- Routine surveys checking for meat substitution should be carried out in the major meat markets across the country to determine if meat traders are abiding by the laws put in place.

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APPENDICES

Appendix 1: Positive controls used for identification of vertebrate sources of meat.

Species	Origin of sample	GPS Address	Description	No. of replicates
Chicken	City Stadium	-1°17'22.80"S 36°50'19.19" E	Vendor	2
Goat	Kiamaiko market	1°15'13.9"S 36°52'25.6"E	Abattoir	7
Rabbit	*icipe*	1°13'13.8"S 36°53'48.7"E	small animal unit	3
Cow	*icipe*	1°13'13.8"S 36°53'48.7"E	archived sample (Ouso et al., 2020)	2
Pig	*icipe*	1°13'13.8"S 36°53'48.7"E	archived sample (Ouso et al., 2020)	2
Camel	Eastleigh	-1°15'60.00"S 36°50'59.99" E	Stall	2

Appendix 2: BLAST results of bacteria identified using Universal 16S rRNA gene

Sample ID	Species	Location	Bacteria	Accession Number	% identity	Query Cover	E-value
4BM	Cattle	Burma Market	*Clostridium septicum* gene for 16S ribosomal RNA, partial sequence, strain: JCM 7278.	AB558163.1	99.93%	100%	0.0
2BM	Cattle	Burma Market	*Clostridium perfringens* strain HBUAS55083 16S ribosomal RNA gene, partial sequence	MN537509.1	99.66%	100%	0.0
45BM	Pig	Jerusalem	*Lactococcus garvieae* strain FJAT-18104 16S ribosomal RNA gene, partial sequence	MF385039.1	99.93%	100%	0.0
88N	Giraffe	Kambi Somali	*Aeromonas caviae* strain T25-43 16S ribosomal RNA gene, partial sequence	MN733089.1	90.43%	95%	0.0
91N	Sheep	Unknown (Nakuru)	*Lactococcus garvieae* JRC-LG3 gene for 16S ribosomal RNA, partial sequence	MK990006.1	100%	100%	0.0
11N	Sheep	Kasarani (Nakuru)	*Lactococcus garvieae* strain FJAT-18104 16S ribosomal RNA gene, partial sequence	KX671995.1	100%	100%	0.0
96N	Cattle	Unknown (Nakuru)	*Lactococcus garvieae* strain FJAT-18104 16S ribosomal RNA gene, partial sequence	MN220581.1	99.32%	100%	0.0

Appendix 3: Samples that tested positive for *Brucella* spp.

Sample ID	Species	Location
N58	Sheep	Mirera (MIR)
N59	Cattle	Karagita (KAR)
N61	Goat	Unknown locations (UL)
N73	Cattle	Langalanga (LG)
N26	Cattle	Kwa Muya (KM)
N71	Sheep	Langalanga (LG)
N63	Sheep	Kabati town (Kab)
N62	Cattle	Town/Kanjoo estate
N78	Sheep	Mutaita (MTT) - Elementaita
BM4	Cattle	Burma Market
BM7	Cattle	Burma Market
BM11	Cattle	Burma Market
BM25	Goat	Burma Market
BM27	Cattle	Burma Market
BM31	Cattle	Burma Market
BM34	Cattle	Burma Market
BM46	Cattle	Jerusalem
BM68	Sheep	Ngara
BM69	Goat	Ngara
BM97	Cattle	Mathare
G1	Goat	Kiamaiko Market
G4	Goat	Kiamaiko Market
G5	Goat	Kiamaiko Market
G6	Goat	Kiamaiko Market
G7	Goat	Kiamaiko Market
Til3	Nile perch	City Market
Til4	Nile perch	City Market

Appendix 4: FASTA DNA sequences of selected vertebrate species

>3_VId_Chicken1, *Gallus gallus* mitochondrion, cytochrome c oxidase subunit 1

CACTCTTTACCTAATTTTCGGCACATGGGCGGGCATAGCCGGCACAGCACTTAGCCTTCTAATTCG
CGCAGAACTAGGACAGCCCGGAACTCTCTTAGGAGACGATCAAATTTACAATGTAATCGTCACA
GCCCATGCTTTCGTCATAATCTTCTTTATAGTTATACCCATCATGATCGGTGGCTTCGGAAACTGA
CTAGTCCCACTTATAATCGGTGCCCCAGACATAGCATTCCCCCGCATAAATAACATAAGCTTCTGA
CTCCTCCCTCCCTCCTTCCTTCTCCTACTAGCCTCATCTACCGTAGAAGCTGGGGCCGGCACAGGA
TGGACAGTTTACCCCCCTTTAGCCGGCAACCTAGCCCACGCTGGCGCATCAGTAGACCTAGCCAT
CTTTTCATTACACTTAGCAGGTGTTTCCTCCATTCTAGGAGCCATCAACTTTATCACTACCATCATC
AACATAAAACCCCCCGCACTGTCACAATACCAAACACCCCTATTCGTATGATCCGTCCTCATTACT
GCCATCCTACTACTCCTCTCCTTACCCGTCCTAGCAGCTGGGATTACCATACTACTTACCGACCGC
AACCTTAACACCACATTCTTCGACCCAGCTGGAGGAGGAGACCCAATCCTATACCAACACCTATT
CTGATTCTTCGGCCACCCAGAAGTCTAGT

>15_VID_GM, *Ovis aries* mitochondrion, cytochrome c oxidase subunit 1

CACCCTTTACCTTCTATTTGGTGCCTGAGCTGGTATAGTAGGAACCGCCTTAAGCCTACTAATTCG
CGCCGAACTAGGCCAACCCGGAACTCTACTCGGAGATGACCAAATCTACAACGTAATTGTAACC
GCACATGCATTTGTAATAATTTTCTTTATAGTAATGCCTATTATAATCGGTGGATTCGGCAACTGA
CTAGTTCCTCTGATAATTGGAGCCCCTGATATAGCATTTCCTCGGATAAATAACATAAGCTTTTGA
CTTCTTCCCCCATCTTTCCTGTTACTCCTAGCATCCTCTATGGTTGAGGCCGGAGCAGGAACAGGT
TGAACCGTATACCCTCCTCTAGCAGGCAACCTAGCCCATGCAGGAGCCTCAGTAGATCTAACTAT
TTTCTCCCTACATCTGGCAGGTGTCTCTTCAATTCTAGGAGCCATTAATTTTATTACAACTATTATT
AATATAAAACCCCCTGCGATGTCACAGTATCAAACCCCCTTGTTTGTATGATCTGTACTAATTACT
GCCGTACTTCTCCTTCTCTCACTTCCTGTATTAGCAGCTGGTATCACAATACTACTAACGGACCGA
AACCTGAATACAACCTTTTTTGACCCAGCAGGAGGAGGAGACCCTATCCTATATCAACACCTATG
CTGATTCTTCGGCCACCCAGAAGTCTAGTCATA

>26_VID_24BM_R, *Bos taurus* mitochondrion, cytochrome c oxidase subunit 1

ATAGGGTCTCCTCCTCCTGCCGGGTCGAAGAAGGTTGTATTTAGGTTCCGGTCTGTTAATAGCAT
TGTGATGCCGGCTGCTAATACAGGGAGCGAGAGTAGTAGTAGTACGGCGGTAATTATTACGGAT
CATACGAACAGAGGGGTTTGGTATTGTGACATTGCGGGGGGCTTTATGTTGATAATTGTTGTAAT
GAAGTTGATGGCTCCTAAAATTGAGGAAACTCCTGCTAAGTGTAAAGAGAAAATGGTTAGATCT
ACTGAAGCTCCTGCATGGGCTAGGTTGCCTGCTAAGGGAGGGTACACGGTTCAGCCTGTTCCTG
CCCCAGCTTCAACTATAGAGGATGCGAGGAGTAGTAGGAATGAGGGAGGGAGGAGTCAGAAG
CTTATATTATTTATTCGGGGAAATGCTATATCGGGAGCACCAATTATTAGGGGAACAAGTCAGTT
ACCGAATCCTCCAATTATGATTGGTATTACTATGAAGAAGATTATTACAAATGCGTGTGCGGTTA
CAACTACGTTGTAGATTTGGTCGTCTCCGAGCAGAGTTCCGGGTTGGCCTAATTCAGCGCGAATT
AGAAGGCTTAGAGCTGTTCCTACTATACCGGCCCAAGCACCAAATAGTAGATAAAGGGTACCGA
TATCCTTGTGGTTGGTTGAGAA

>27_VId_68BM, *Ovis aries* mitochondrion, cytochrome c oxidase subunit 1

GTGCCTGAGCTGGTATAGTAGGAACCGCCTTAAGCCTACTAATTCGCGCCGAACTAGGCCAACCC
GGAACTCTACTCGGAGATGACCAAATCTACAACGTAATTGTAACCGCACATGCATTTGTAATAAT
TTTCTTTATAGTAATGCCTATTATAATCGGTGGATTCGGCAACTGACTAGTTCCTCTGATAATTGG
AGCCCCTGATATAGCATTTCCTCGGATAAATAACATAAGCTTTTGACTTCTTCCCCCATCTTTCCTG
TTACTCCTAGCATCCTCTATGGTTGAGGCCGGAGCAGGAACAGGTTGAACCGTATACCCTCCTCT
AGCAGGCAACCTAGCCCATGCAGGAGCCTCAGTAGATCTAACTATTTTCTCCCTACATCTGGCAG
GTGTCTCTTCAATTCTAGGAGCCATTAATTTTATTACAACTATTATTAATATAAAACCCCCTGCGAT
GTCACAGTATCAAACCCCCTTGTTTGTATGATCTGTACTAATTACTGCCGTACTTCTCCTTCTCTCA
CTTCCTGTATTAGCAGCTGGTATCACAATACTACTAACGGACCGAAACCTGAATACAACCTTTTTT
GACCCAGCAGGAGGAGGAGACCCTATCCTATATCAACACCTATGCTGATTCTTCGGCCACCCAGA
AGTC

>47_VId_GMB, *Capra hircus* mitochondrion, cytochrome c oxidase subunit 1

TCCTCCTCCTGCTGGGTCAAAGAAGGTTGTGTTTAGGTTTCGGTCTGTTAGTAGTATTGTGATGCC
AGCTGCTAATACAGGAAGTGAAAGGAGGAGTAGTACGGCAGTAATTAAGACAGATCACACAAA
CAGGGGAGTTTGATATTGTGATATTGCGGGTGGTTTCATGTTAATGATAGTTGTGATAAAATTAA
TGGCTCCTAGAATTGAAGAGATGCCTGCTAGGTGTAGGGAAAAAATAGTTAGGTCTACTGAGGC
TCCTGCATGGGCTAGATTACCTGCTAGAGGAGGATATACGGTTCAACCTGTTCCTGCTCCGGCTT
CAACTATAGAGGATGCTAGAAGTAATAGGAAAGAGGGGGGAAGGAGTCAAAAGCTTATATTAT
TTATCCGAGGAAATGCTATATCGGGGGCTCCAATTATTAGAGGGACTAGTCAGTTGCCAAACCCT
CCAATCATAATAGGTATTACTATAAAGAAAATTATTACGAATGCGTGTGCAGTTACAATTACATT
GTAGATCTGGTCATCTCCAAGTAGGGTTCCGGGTTGACCTAGTTCGGCGCGAATTAGTAAGCTCA
AGGCGGTCCCTACTATGCCAGCTCAGGCACCGAACAGAAGGTAGAGGGTGCCAATATCCTTGTG
GTTGGTTGAGAACTGG

>49_VId_11BM, *Bos taurus* mitochondrion, cytochrome c oxidase subunit 1

TACTATTTGGTGCTTGGGCCGGTATAGTAGGAACAGCTCTAAGCCTTCTAATTCGCGCTGAATTA
GGCCAACCCGGAACTCTGCTCGGAGACGACCAAATCTACAACGTAGTTGTAACCGCACACGCAT
TTGTAATAATCTTCTTCATAGTAATACCAATCATAATTGGAGGATTCGGTAACTGACTTGTTCCCC
TAATAATTGGTGCTCCCGATATAGCATTTCCCCGAATAAATAATATAAGCTTCTGACTCCTCCCTC
CCTCATTCCTACTACTCCTCGCATCCTCTATAGTTGAAGCTGGGGCAGGAACAGGCTGAACCGTG
TACCCTCCCTTAGCAGGCAACCTAGCCCATGCAGGAGCTTCAGTAGATCTAACCATTTTCTCTTTA
CACTTAGCAGGAGTTTCCTCAATTTTAGGAGCCATCAACTTCATTACAACAATTATCAACATAAAG
CCCCCCGCAATGTCACAATACCAAACCCCTCTGTTCGTATGATCCGTAATAATTACCGCCGTACTA
CTACTACTCTCGCTCCCTGTATTAGCAGCCGGCATCACAATGCTATTAACAGACCGGAACCTAAAT
ACAACCTTCTTCGACCCGGCAGGAGGAGGAGACCCTATT

>50_VID_12BM, *Sus scrofa* mitochondrion, cytochrome c oxidase subunit 1

ACCCTGTACCTACTATTTGGTGCCTGAGCAGGAATAGTGGGCACTGCCTTGAGCCTACTAATTCG
CGCTGAACTAGGTCAGCCCGGAACCCTACTTGGCGATGATCAAATCTATAATGTAATTGTTACAG
CTCATGCCTTTGTAATAATCTTCTTTATAGTAATACCCATTATGATTGGGGGTTTTGGTAACTGACT
CGTACCACTAATAATCGGAGCTCCCGATATGGCCTTTCCACGTATAAACAACATAAGTTTCTGACT
ACTTCCACCATCCTTCCTATTACTACTGGCATCCTCAATAGTAGAAGCCGGGGCGGGTACTGGAT
GAACCGTATACCCACCTTTAGCTGGAAACTTAGCCCATGCAGGAGCTTCAGTTGATCTAACAATT
TTCTCCCTACACCTTGCAGGTGTATCATCAATCCTAGGGGCTATTAATTTCATTACCACAATTATTA
ACATAAAACCTCCCGCAATGTCTCAATACCAAACACCCCTGTTTGTCTGATCAGTACTAATCACAG
CCGTACTACTTCTACTATCCCTGCCAGTTCTAGCAGCTGGCATTACTATACTACTGACAGACCGCA
ACCTGAACACAACCTTTTTTGATCCAGCAGGTGGTGGAGACCCTATCCTTTATCAACACTTGTTCT
GATTCTTCGGCCACCCAGAAGTCTAGTCAT

>10_CytB_44BM, *Camelus dromedarius* mitochondrion, cytochrome b

AAGCTGTGGCTATGGTTCGTGAACAAGAAAGCAATTCCCACTGTTTCATGTTTCTGAAAAGGTAT
ATGATCCGTAATATAGCCCTCGTCCCACGTGAATATATAGGCAAATGAAGAATATGGAAGCTCCG
TTAGCATGTAATTATCGAATGATTCAGCCTTAGTTAACATCTCGACAGATGTGAGTTACAGAAGA
AAAATCTGTTGTTGTGTCGGATGTATAATGGATTGCCTAGGAATAATCCTGGCCAGGA

>Vert16S_T3, *Lates niloticus* 16S ribosomal RNA gene, partial sequence; mitochondrial

AGCTTTAGACGCCAGGATAGCTCATGTTAAGACCCCCTAAATAAAGGCCTGAACTTAGTGACCCC
CTATTCTAATGTCTTCGGTTGGGGCGACCATGAGGAACAAAAACCCCTCACGTGGAATGGGAGT
ACCAAAGCCCACTATTATTTTTTCCTACACTCCTACAACTAAGAGCCACAGCTCAATTTAACAGAA
ATTCTGACCAACAATGATCCGGCAATGCTGATCAACGGACCAAGTTACCCTAGGGATAACAG

Appendix 5: FASTA DNA sequences of selected pathogenic *Brucella* positive samples

>1_BCSP_25BM, *Brucella melitensis* strain VB12455 chromosome 1, complete sequence

GTTGCCAATATCAATGCGATCAAGTCGGGCGCTCTGGAGTCCGGCTTTACGCAGTCAGACGTTG
CCTATTGGGCCTATAACGGCACCGGCCTTTATGATGGCAAGGGCAAGGTGGAAGATTTGCGCCT
TCTGGCGACGCTTTACCC

>3_BCSP_48BM, *Brucella suis* bv. 1 strain 60 chromosome 1, complete sequence

TAGCTCGGTTGCCAATATCAATGCGATCAAGTCGGGCGCTCTGGAGTCCGGCTTTACGCAGTCA
GACGTTGCCTATTGGGCCTATAACGGCACCGGCCTTTATGATGGCAAGGGCAAGGTGGAAGATT
TGCGCCTTCTGGCGACGCTTTACCC

>11_BCSP_81BM, *Brucella melitensis* strain VB12455 chromosome 1, complete sequence

TCGGTTGCCAATATCAATGCGATCAAGTCGGGCGCTCTGGAGTCCGGCTTTACGCAGTCAGACG
TTGCCTATTGGGCCTATAACGGCACCGGCCTTTATGATGGCAAGGGCAAGGTGGAAGATTTGCG
CCTTCTGGCGACG

>3_BCSP_N73, *Brucella arbortus* isolate 5 BCSP31 gene, partial cpds.

CCAATGCCTCATAAAGGCCGGTGCCGTTATAGGCCCAATAGGCAACGTCTGACTGCGTAAAGCC
GGACTCCAGAGCGCCCGACTTGATCGCATTGATATTGGCAACCGAGCTG

>1_BCSP_Til3, *Brucella melitensis* strain M1981 chromosome 1, complete sequence

CGGTGCCGTTATAGGCCCAATAGGCAACGTCTGACTGCGTAAAGCCGGACTCCAGAGCGCCCGA
CTTGATCGCATTGATATTGGCAACCGAGCA

>2_BCSP_Til4, *Brucella suis* bv. 1 strain 60 chromosome 1, complete sequence

GCCGGTGCCGTTATAGGCCCAATAGGCAACGGTCTGACTGCGTAAAGCCGGACTCCAGAGCGCC
CGACTTGATCACATTGATATTGGCAACCGAGCAA

Appendix 6: FASTA DNA sequence of pathogenic *Leptospira* positive sample

>4_secYIV_N24, *Leptospira interrogans* serovar Autumnalis preprotein translocase (secY) gene

CGATTCAGTTTAATCCTGCAGAATTGGCTGAGAATTTGAAAAAATACGGTGGGTTCATTCCAGGA
ATTCGTCCGGGTTCTCACACAAAAGAATACATTGAAAAAGTGTTAAATAGAATCACTCTTCCCGG
AGCTATGTTTCTTGCAGGTTTGGCATTAGCACCTTATATTATTATAAAATTCTTAGATTCAAGCTCT
AACT

Appendix 7: FASTA DNA sequences of bacteria identified using Universal 16S rRNA primers

>1Uni2BM, *Clostridium perfringens* 16S ribosomal RNA gene, partial sequence

GCTGCCTCCTTGCGGTTAGCTCACGGACTTCGGGTATTGCCAACTCTCATGGTGTGACGGGCGGT
GTGTACAAGACCCGGGAACGTATTCACCGCGACATTCTGATTCGCGATTACTAGTAACTCCAGCT
TCATGTAGGCGAGTTTCAGCCTACAATCCGAACTGAGACTGGTTTTTAAGTTTGGCTCCACCTCG
CGGTATTGCATCTCTCTGTACCAGCCATTGTAGCACGTGTGTAGCCCTACACATAAGGGGCATGA
TGATTTGACGTCATCCCCACCTTCCTCCTGGTTAACCCAGGCAGTCTCGCTAGAGTCCTCAACTTA
ATGGTAGTAACTAACGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACG
AGCTGACGACAACCATGCACCACCTGTCACCTTGTCCCCGAAGGGATTTCCTCGATTAAGAGTAA
TGCAAGGGATGTCAAGTGTAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCGCT
ACTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGAATA
CTTAATGCGTTAGCGGCGGCACGGAGGTGTTGAAACCCCCACACCTAGTATTCATCGTTTACGGC
GTGGACTACCAGGGTATCTAATCCTGTGTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAG
TCCAGAGAGTCGCCTTCGCCACTGGTGTTCTTCCTAATCTCTACGCATTTCACCGCTACACTAGGA
ATTCCACTCTCCTCTCCTGCACTCTAGATAACCAGTTTGGAATGCAGCACCCAAGTTGAGCCCGG
GTATTTCACATCCCACTTAATCATCCGCCTACGCTCCCTTTACGCCCAGTAAATCCGGATAACGCT
CGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTCCTCCTTGGGTACCGT
CATTATCTTCCCCAAAGACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTGC
ATCAGGGTTTCCCCCATTGTGCAATATTCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTC
AGTCCCAATGTGGCCGATCACCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTAGGCGTTA

>3Uni_4BM, *Clostridium septicum* gene for 16S ribosomal RNA gene, partial sequence

GGTTACGCTCACGAACTTTGGGTATTGCCAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGC
CCGGGAACGTATTCACCGCGACATGCTGATTCGCGATTACTAGCAACTCCAGCTTCATGTAGGCG
AGTTTCAGCCTACAATCCGAACTGAGACAAGTTTTATAGTTTAGCTCCACCTCGCGGTATTGCATC
TCGTTGTACTTGCCATTGTAGCACGTGTGTAGCCCTAGACATAAGGGGCATGATGATTTGACGTC

ATCCCCACCTTCCTCCCGGTTAACCCGGGCAGTCTCGCTAGAGTGCTCAACTAAATGGTAGCAAC
TAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACA
ACCATGCACCACCTGTCATCCTGTCCCCGAAGGGACTTCCTCGATTAAGAGTAATGCAGGAGATG
TCAAGTCTAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCGCTGCTTGTGCGGG
CCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGGATACTTAATGTGTT
AACGGCGGCACGGAAGGAGTTGATACCTCCCACACCTAGTATCCATCGTTTACGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGCGTCAGTTACAGTCCAGAGA
GTCGCCTTCGCCACTGGTGTTCTTCCTAATCTCTACGCATTTCACCGCTACACTAGGAATTCCACTC
TCCTCTCCTGCACTCTAGACTTCCAGTTTGAAATGCAGCCCCCAGGTTGAGCCCGGGTATTTCACA
TCTCACTTAAAAGTCCGCCTACGCTCCCTTTACGCCCAGTAAATCCGGACAACGCTCGCCACCTAC
GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTCCTCCTCAGGTACCGTCATTATCGTC
CCTGAAGACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTGCATCAGGGTT
TCCCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAA
TGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAAC
TAGCTAATGCGCCGCGGGCCCATCTTGTAGCGGATTACTCCTTTAATTGCTGCTCCATGCGAAGC
TGCAATGTTATGCGGTATTAATCTCCCTTTCGGGAGGCTATTCCCCTCTACAAGGCAGGTTGCCCA
CGTGTTACTCACCCGTCCGCCGCTAGGTTTGTTTCCGAAGAAACTCCC

>5Uni25BM, *Romboutsia lituseburensis* gene for 16S ribosomal RNA, partial sequence

CGGGGTGTACAAAACCGGGAACGCATTCACCGCAGCATTCTGATCTGCGATTACTAGTAACTCCA
GCTTCATGTAGGCGAGTTTCAGCCTACAATCCGAACTGAGAATGGCTTTAAGGGATTAGCTCCGC
CTCACGACTTGGCTGCCCTCTGTACCACCCATTGTAGCACGTGTGTAGCCCTAAGCATAAGGGGC
ATGATGATTTGACGTCATCCCCACCTTCCTCCAGGTTATCCCTGGCAGTCCCTCTAGAGTGCCCAA
CTTAATGCTGGCAACTAAAGGGAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGA
CACGAGCTGACGACAACCATGCACCACCTGTCACTTCTGTCCCCGAAGGGAAATCTCCGATTAAG
GAGAGGTCAAAAGGATGTCAAGCTTAGGTAAGGGTCTTCGCGTTGCTTCGAATTAAACCACAAG
CTCCGCTACTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCACTCTTGCGAGCGTACTTCCCAGG
CGGAGTACTTAATGCGTTAGCTGCGGCACCGAGGGGGGTAACCCCCGACAGCTAATACTCATCG
TTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGTGTCA
GTTACAGTCCAGAGAGCCGCCTTCGCCACTGGGGTTTCCTCCTAATATCTACGCATTTCACCGCTA
CACTAAGGAAATTCTACTCCTCCTCTCCTGCACTCAAGTCTCTTAGTTTTCAAAAGCTTACTACCGG
TTGAGCCGGTAGCGCTTTCACTTCTGAACTT

>13Uni82BM, *Lactococcus garvieae* 16S ribosomal RNA gene, partial sequence

GTGGTGTGACGGGCGGTGTGTACAAGGACCCGGGAACGTATTCACCGCGGCGTGCTGATCCGC
GATTACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAATGGTTT
TAAGAGATTAGCTCACCCTCGCGGGTTGGCTGCTCGTTGTACCTTCCATTGTAGCACGTGTGTAG
CCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAG
TCTCATTAGAGTGCCCAACTTAATGATGGCAACTAATAATAAGGGTTGCGCTCGTTGCGGGACTT
AACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTATCCCGTGTCCCGAAGG
AACTCCTATCTCTAAGGATAGCACGAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGA

ATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGT
CGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCGATACAGAGAACTCATAGCTCCCTACAT
CTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCG
AGCCTCAGTGTCAGTTACAGGCCAGAGAGCCGCTTTCGCCTCCGGTGTTCCTCCATATATCTACG
CATTTCACCGCTACACATGGAATTCCACTCTCCTCTCCTGCACTCAAGTCTCCCAGTTTCCAATGCA
CACAATGGTTGAGCCACTGCCTTTTACATCAGACTTAAGAAACCACCTGCGCTCGCTTTACGCCCA
ATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCC
TTTCTGGTTAGATACCGTCACTGAATGAATTTTCCACTCCACTAACGTTCTTCTCTAACAA

>14Uni46BM, *Clostridium sordelii* 16S ribosomal RNA gene, partial sequence

GGTTAGCTAACCGGCTTCGGGCGCCCCCAACTCCCATGGTGTGACGGGCGGTGTGTACAAGACC
CGGGAACGCATTCACCGCAGCATTCTGATCTGCGATTACTAGTAACTCCAGCTTCATGTAGGCGA
GTTTCAGCCTACAATCCGAACTGAGAATGGCTTTAAGGGATTAGCTCCACCTCACGGCTTGGCAA
CCCTCTGTACCACCCATTGTAGCACGTGTGTAGCCCTAAGCATAAGGGGCATGATGATTTGACGT
CATCCCCACCTTCCTCCGAGTTATCCTCGGCAGTCCCTCTAGAGTGCCCAACTTAATGCTGGCAAC
TAAAGGCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACA
ACCATGCACCACCTGTCACCACTGTCCCCGAAGGGAAATCTCCGATTAGGGAGAGGTCAGTGGG
ATGTCAAGCTTAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCGCTACTTGTGCG
GGTCCCCGTCAATTCCTTTGAGTTTCACTCTTGCGAGCGTACTTCCCAGGCGGAGTACTTAATGCG
TTAGCTGCGGCACCGAGGGGGGTAACCCCCGACACCTAGTACTCATCGTTTACGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCAGCGTCAGTTACAGTCCAGAGA
GCCGCCTTCGCTACTGGTGTTCCTCCTAATATCTACGCATTTCACCGCTACACTAGGAATTCCACT
CTCCTCTCCTGCACTCAAGTCCTACAGTTCCAAAAGCTTACTACGGTTGAGCCGTAGCCTTTCACT
TCTGGCTTGAAAGACCGCCTACGCACCCTTTACGCCCAGTAATTCCGGATAACGCTAGCCCCCTA
CGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTCCTCCTCAAGTACCGTCATTATCTTC
CTTGAGGACAGAGCTTTACGACCCGAAGGCCTTCATCGCTCACGCGGCGTTGCTGCATCAGGCTT
TCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTTTGGACCGTGTCTCAGTTCCAA
TGTGGCCGATCACCCTCTCAGGTCGGCTACTGATCGTTGCCTTGGTAAGCCGTTACCTTACCAACT
AGCTAATCAGACGCGGGTCCATCCTGTACCGCCGGAGCTTTGATACAAAAGCCATGCGACTCTCA
TATGTTATCCCGTATTAGCATACCTTTCGGTATGTTATCCGTGTGTACAGGGCAGGTTACCCACGC
GTTACTCACCCGTCCGCCGCTCACCCCGAAGGGTTCGCTCGACTGCA

>21Uni45BM, *Lactococcus garvieae* 16S ribosomal RNA gene, partial sequence

GCGGTTAGGCAACCTACTTTGGGTACTCCCAACTTCCGTGGTGTGACGGGCGGTGTGTACAAGG
CCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGCAGGC
GAGTTGCAGCCTCCAATCCGAACTGAGAATGGTTTTAAGAGATTAGCGCACCCTCGCGGGTTGG
CGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGA
CGTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCACTAGAGTGCCCAACTTAATGATGGC
AACTAGTAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG
ACAACCATGCACCACCTGTATCCCGTGTCCCGAAGGAACTCCTTATCTCTAAGGATAGCACGAGT
ATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGC

GGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATG
CGTTAGCTGCGCTACAGAGAACTTATAGCTCCCTACAGCTAGCACTCATCGTTTACGGCGTGGAC
TACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGAGCCTCAGTGTCAGTTACAGGCCAGA
GAGCCGCTTTCGCCTCCGGTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCA
CTCTCCTCTCCTGCACTCAAGTCTCCCAGTTTCCAATGCACACAATGGTTGAGCCACTGCCTTTTAC
ATCAGACTTAAGAAACCACCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGGGACCT
ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTTAGATACCGTCACTTAAG
TAATTTTCCACTCTACTTAACGTTCTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCAC
TCACGCGGCGTTGCTCGGTCAGGGTTGCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTA
GGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGTATCATC
GCCTTGGTAGTCCTTTACACTACCAACTAGCTAATACAACGCGGGATCATCAAGTAGTGAAGCAA
TTGCTTCTTTCAAATAAGAATCATGCGATTCTCATTGTTATGCGGTATTAGCGTTCGTTTCCAAAC
GTTGTCCCCCGCTACTCGGCAGATTTCCCACGCGTTACTCACCCGTTCGCCGCTCTTCATGAAAAT
AGCAAGCTATCTTCAATCATCGCTCGACTGCA

>2_Uni_23N, *Vagococcus fessus* 16S ribosomal RNA, partial sequence

CCGGAAACTTTTTCACCGCGCCGTCTGATCCCCGATTTACAACGAATTCCGATTTCATGTAGGCAA
TTTGCAGCCAAAAACCCGAACTGAAAAAACTTTAAAAAAATATCTTGGCCTCCCGAGGTTGCGAC
TCCTTGTACCTTCCATTGGGACACGTGTGTAGCCCAGGGCATAAGGGGCATGATGATTTGACGTC
CTCCCCACCTTCCTCCGGGTTATCACCGGCAGTCTCCCTAGAGTGACCAACTTAATGATGGCAACT
AACAATAAGGGTTGCGCTCGTTGGGGGAATTAACCCAACATCTCACGAAACGAGCTGACGACCA
ACCATGCACCACCTGTTTCCTTTGTCCCGAAGGGAAACTTCTCTTTCAGGATGGTTCAAGGATGTC
AAGACCTGGTAAGGGTCTTCCCGTTGCTTCCAATTAAACCACATGCTCCACCGCTTGTGCGGGCC
CCCGTCGAATTCCTTTGAATTTCAACCTTGCTGTCCTAGTCCCCAGGGGGAATGCTTAATGCCTTC
ACTGATG

>5_Un_57N, *Staphylococcus saprophyticus* 16S ribosomal RNA gene, partial sequence

CCAAATGGGGAAGAATTTTAGGGGATTGGCTGGACCTCCGGGTTTACCTGCCTTTTGTATTGCCC
TTGGTGGCACGGGGGAGGCCCAAACCTAAGGGGGATGGAGGATTTGACGCCTTCCCCCCCTTCC
TCCGGTTGGTCCCCGGCAGCTCCACCTAAATGGCCCAACTTATGGAGGGCAACTAAGATTAAGG
GTGGCCCTCGTTGCGGAACTTAACCCACCTTCTCACAACCCAACCTGACAACAACCTGGCCCCAC
CGTGTACATTTTGTCCCCCAAAGGGAAAGGTCTCTATCTCTAGAGTTTTCAAAGGATGTCAAAATT
TGGTAAGGTTCTTCGCGTTGCTTCAAATTAAACCCACATGCTCCACCGTTGGGGCGGGTCCCCGT
CAATTCCTTTAGATTTTCAACCTTGCAGTCGTACTCCCCAGGCGGAGGGCTTAAGGCGTTACCTG
CAGCACTAGGGGGCGAAACCCCCCTAACACTTAGCTCTCATCGTTTACGGCGTGAACTCCCAGGG
TATCTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCTT
CCGCCACTGGTGTTCCTCCATATCTGTGCGCATTTCACCGACTACACATGGGAATTCCTCTCTTCTC
TTGCGCACTCAAGTCTTCCCAGTTTCCAATGAACCCTCTCCACGGGAGAGCCGTGGGCTTTCACA
TCAGACATAAGAAACCGCATACGCGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACATA
CGTATTACCGCGGGTGGTGGCACGTAGTTAGCCGGGGCTTTGGGATTAGGCACCGTCAAGACGT

GCACAGTTAATTACACATTTTTTCTTCCCTAATAACAGAGTTTTGAGATGCCGAACCCTTTCATCAC
TCGCGGGGCGGTGCTCTGTCAGGCTTTTGCCCATCCCGGAAGATTCCCTACTGCCCCCTCCCGTA
G

>7_Un-66N, *Macrococcus caseolyticus* 16S ribosomal RNA gene, partial sequence

GATCCGAGGGCCTCACTAAACCATCCAATCGGTAGTAGCGACGGGCGGTGTGTACAAAGGGCA
GGGACTTAATCAACGCAAGCTTATGACCCGCACTTACTGGGAATTCCTCGTTCATGGGGAATAAT
TGCAATCCCCGATCCCCATCACGAATGGGGTTCAACGGGTTACCCGCGCCTGCCGGCGTAGGGT
AGGCACACGCTGAGCCAGGATCAAACTCTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGG
CAGTCTCTCTAGAGTGCCCAACCTAATGATGGCAACTAAAGATAAGGGTTGCGCTCGTTGCGGG
ACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTTGTCCCCCG
AAGGGGAAAGCTCTATCTCTAGAGTTGTCAAAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTG
CTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCT
TGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTGAGGGGCGGAAACCC
CCCAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATCCCCA
CGCTTTCGCACCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCATA
TCTCTGCGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGTCTCCCAGTTT
CCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAGACCGCCTACGCGCG
CTTTACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAG
TTAGCCGTGGCTTTCTGGTAAGGTACCGTCAAGGTACGTTCAGTTACTAACGTACTTGTTCTTCCC
TTACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCCGTCAGACTTTCG
TCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCA

>9_Un_85N, *Macrococcus caseolyticus* 16S ribosomal RNA, partial sequence

TCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAACATGCTGATCTAC
GATTACTAACGATTCCAGCTTCATGTAGTCCAATTGCAGACTACAATCCGAACTGAGAATGGTTTT
ATGGGATTTGCTTGACCTCCCGGTTTTGCTGCCCTTTGTACCATCCATTGGAGCACGTGTGTAGCC
CAAATCATAAGGGGGATGATGATTTGACGTCATCCCCCCCTTCCTCCGGTTTGTCACCGGCAGTC
TCTCTAAAGTGCCCAACCTAATGATGGCAACTAAAGATAAGGGTTGCGCTCGTTGCGGGACTTAA
CCCAACATCTCACGACACGAGCTGACGACCACCATGCACCACCTGTCACTTTGTCCCCCGAAGGG
GAAAGCTCTATCTCTAGAGTTGTCAAAAGGATGTCAAGAATTGGTAAGGGTCTTCGCGTTGCTTC
CAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAATTTCAGTCTTGCG
ACCGTACTCCCCAGGGGGAATGCTTAATGCGTTAGCTGCAGCACTGAGGGGCGGAAACCCCCCA
ACACTTAACACTCATCGTTTACGGCGTGGACTACCAAGGTATCTAATCCTGTTTGATCCCCACGCT
TTCGCACCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCC

>10_Uni_87N, *Proteus vulgaris* 16S ribosomal RNA gene, partial sequence

TCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC
GTAGCATTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATC
CGGACTACGACAGACTTTATGAGTTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATCTGCCAT
TGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCC

GGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGCCATTACGCGCTGGCAACAAAGGATAAGGGTT
GCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCT
GTCTCATGGTTCCCGAAGGCACTCCTCTATCTCTAAAGGATTCGATGGATGTCAAGAGTAGGTAA
GGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCAT
TTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACGCGTTAGCTCCAGAAGCCA
CGGCTCAAGACCACAACCTCTAAATCGACATCGTTTACAGCGTGGACTACCAGGGTATCTAATCC
TGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCG
GTATTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCTACCCCCCTCTACAAGACTCT
AGCCGAACAGTTTCAGATGCAATTCCCAAGTTAAGCTCGGGGCTTTCACATCTGACTTAATTGAC
CGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTG
CTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAAATGATAAAGGTATTAACTTCAT
CACCTTCCTCCCCGCTGAAAGTACTTTACAACCCTAAGGCCTTCTTCATACACGCGGCATGGCTGC
ATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTC
TCAGTCCAGTGTGGCTGATCATCCTCTCAGACCAGCTAG

>12_Uni_91N, *Lactococcus garvieae* 16S ribosomal RNA gene, partial sequence

TGGGTACTCCCAACTTCCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCG
CGGCGTGCTGATCCGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCC
GAACTGAGAATGGTTTTAAGAGATTAGCGCACCCTCGCGGGTTGGCGACTCGTTGTACCATCCAT
TGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCC
GGTTTATCACCGGCAGTCTCACTAGAGTGCCCAACTTAATGATGGCAACTAGTAATAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTA
TCCCGTGTCCCGAAGGAACTCCTTATCTCTAAGGATAGCACGAGTATGTCAAGACCTGGTAAGGT
TCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTG
AGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCGCTACAGAGAA
CTTATAGCTCCCTACAGCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTT
TGCTCCCCACGCTTTCGAGCCTCAGTGTCAGTTACAGGCCAGAGAGCCGCTTTCGCCTCCGGTGT
TCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTCCTGCACTCAAGTC
TCCCAGTTTCCAATGCACACAATGGTTGAGCCACTGCCTTTTACATCAGACTTAAGAAACCACCTG
CGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGGGACCTACGTATTACCGCGGCTGCTGGC
ACGTAGTTAGCCGTCCCTTTCTGGTTAGATACCGTCACTTAAGTAATTTTCCACTCTACTTAACGTT
CTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGG
GTTGCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTC
CCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGTATCATCGCCTTGGTAGTCCTTTACACTACC
AACTAGCTAATACAACGCGGGATCATCAAGTAGTGAAGCAATTGCTTCTTTCAAATAAGAATCAT
GCGATTCTCATTGTTATGCGGTAT

>15_Un_96N, *Lactococcus garvieae* 16S ribosomal RNA gene, partial sequence

AGCTTGTTGGTGATGGGGACGGGGTTTGCATTATTCCCCGTGAGAGAAGAATTCGGGAAATCTG
CGGGTCATCGGCTTGCGTTGATTGGATCCCTGCCCTTTGTCCACACCGCCCTTCGATACTCCCGAT
TCTTTGGTTTAATGAGGCCCTCGGATCTACCCCGCGATCGCCCGCCCACTGCCCTGGCGGATCGC

TGAGAAGACTGCCGAAGTTGAGATTCTTAAGGAACCTGAGTCGGTGATCGGTCACCATGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCC
TGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAG
AACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAACCAGAAAGGGACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCG
AGCGCAGGTGGTTTCTTAAGTCTGATGTAAAAGGCAGTGGCTCAACCATTGTGTGCATTGGAAA
CTGGGAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGAGAAATGCGTAGAT
ATATGGAGGAACACCGGATGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTACACGATGAGTGCTAGCTG
TAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCATTAAGCACTCCGCCTGGGGACTACGAC
CGCAAGGTTGAAACTCAGAGGAATTGACGGGCGCCCGCACAAGCGGAGGAGCATGTGGTGTAA
TTCGAAGCGACGCGAAGAATCTTACCAGGTCTTGACATACTCGTGCTACCCTTAGAGATAAGGA
GTTCCTTCGGGACACGAGATACAGGTGCTGCATGGTTGTCATCAACTCATGTCGTGAGATGTGG
GATAACTCCCGCAACGAGCGCACCCTTATTACTAGTGCCATCATAACGTGGGCACTCTAGGAGGA
CCGCCGGTGATAACCGGAAGAGGTGGGGATGACGTAAATCTCAGCCCCTTAGGACCGGGCAAC
CCCTGCTCCATGGAGGGACACCA

>16_Uni_97N, *Weissella ceti* 16S ribosomal RNA gene, partial sequence

CCGTGGGCCGACCCCGGCGGGGCCGATCCGATGGCCTCACTAAACCATCCAATCGGTAGTAGCG
ACGGGCGGTGTGTACAAAGGGCAGGGACTTAATCAACGCAAGCTTATGACCCGCACTTACTGGG
AATTCCTCGTTCATGGGGAATAATTGCAATCCCCGATCCCCATCACGAATGGGGTTCAACGGGTT
ACCCGCGCCTGCCGGCGTAGGGTAGGCACACGCTGAGCCAGGATCAAACTCTATGCGGCATTTT
CTTCCCCTCCTTCCTCCGGGTTGTCACCGGCAGTCTCGCTAAAATGACCAACTGAATGCTGGCAAC
TAGTAAAGTAAGGGTTGCTGACGTTGCGGGACTTAACCCAACATCTCCCTACTGGAATTTCCGAA
CAGGCCATGCACCCACTAGCACTTTGTCCCCAGGAAGGGAAAACGCCCTTTCTGGCGAAGTCAA
AAGATGTCATGACCTGGTTAAGGTTCTTCGCGTTGCTTCTAATTAAACCACATGCTCCACCGCTTG
TGGTGGTCCCCGTCTTTTCCTTTGAAGTTTCAACCTTGCGGCCGTACTCCCCACGCGGAGTGCTGA
ATGCGTTATCTGCGACACTTATGAGCGGAAAATGCCCCCCACACATCTATCACTCATCGTTTAACG
GTGTGGAACTACCAGGGTATCTATATCCTGTTGGGCTACCCACACTTTCGAGCGCTCAACGTCAG
TCACAGTCTCAGAGAGCCGTCATTCGCCACTGGTGTTCCTCCATATATCTTACGCATTTCACCGCT
ACACATGGAATTCCACTCTCCTCTACTGCACTCAAGTTATCCAGTTCCAAAAGCACTTCCACAGTT
GAAGCTGTGGGGCTGTCACTTCGGACTTAAATAAACCGTCTGCGCTC

>4_Un_47N, *Weissella ceti* strain 1119-1A-09 16S ribosomal RNA, partial sequence

GCCGACCCCGGCGGGGCCGATGCGAGGGCCTCACTAAACCATCCAATCGGTAGTAGCGACGGG
CGGTGTGTACAAAGGGCAGGGACTTAATCAACGCAAGCTTATGACCCGCACTTACTGGGAATTC
CTCGTTCATGGGGAATAATTGCAATCCCCGATCCCCATCACGAATGGGGTTCAACGGGTTACCCG
CGCCTGCCGGCGTAGGGTAGGCACACGCTGAGCCAGGATCAAACTCTAACGTCATCCCCACCTTC
CTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTGAATGCTGGCAACTAGTGATAAGG
GTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCAC

CTGTCACATTGTCCCCGAAGGGAAAGCGCCATTTCTGGAGTGATCAAAGGATGTCAAGACCTGG
TAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTGCACCGCTTGTGCGGCTCCCCGTCAATT
CCTTTGAGTATCAACCTTGCGGCCATACTCCCCAGGCGGAGTGCTGAATGCGTTAGCTGCTACAC
TTAGGGGCGGAAACCCCCAAACATCTAGCACTCATCGTTTACGGTGTGGACTACCAGGGTATCTA
ATCCTGTTTGCTACCCACACTTTCGAGCCTCAACGTCAGTTACAGTCCAGAGAGCCGCCTTCGCCA
CTGGTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTACTGCAC
TCAAGTTATCCAGTTTCCAAAGCACTTCCACAGTTAAACCTGTGGGCTTTCACTTTCAGACTTAAA
TAACCGTCTGCGCTCGCTTTACGCCCAATAAATCCAGGATAACGCTTGGCAACATACGTATTACC
GCGGGCTGCTGGCACGTATTTAGCCGTTCCTTTTCTGGTAAGAATACCCGTCAGACACTGAGCAG
GTAACTATCAA

>11_Un_89N, *Proteus vulgaris* strain FDAARGOS_556 chromosome, complete genome

TCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGCCATTACGCGCTGGC
AACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACG
ACAGCCATGCAGCACCTGTCTCATCGTTCCCGAAGGCACTCCTCTATCTCTAAAGGATTCGCTGG
ATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGC
GGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGATTTAACG
CGTTAGCTCCAGAAGCCACGGCTCAAGACCACAACCTCTAAATCGACATCGTTTACAGCGTGGAC
TACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGG
GGCCGCCTTCGCCACCGGTATTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCTAC
CCCCCTCTACAAGACTCTAGCCAACCAGTTTCAGATGCATTTCCCAAGTTAAGCTCGGGGCTTTCA
CATCTGACTTAATTGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCT
CCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTTTTCTGCGGGTAACGTCAATTGAT
AAAGGTATTAACCTTATCACCCTTCCTCCCCGCTGAAAGTACTTTACAACCATAAGGCCTTTCTTCA
TACACGCGGCATGGGTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCCGCCTCCCGT
AGGA

>17_Uni_98N, *Macrococcus* sp. 'vitulae' strain DPC7159 16S ribosomal RNA gene, partial sequence

GGCCGTGGGCCGACCCCGGCGGGGCCGATCCGAGGGCCTCACTAAACCATCCAATCGGTAGTA
GCGACGGGCGGTGTGTACAAAGGGCAGGGACTTAATCAACGCAAGCTTATGACCCGCACTTACT
GGGAATTCCTCGTTCATGGGGAATAATTGCAATCCCCGATCCCCATCACGAATGGGGTTCAACGG
GTTACCCGCGCCTGCCGGCGTAGGGTAGGCACACGCTGAGCCAGGATCAAACTCTAAACGTCTT
CCCCCCCTGCCTCCGGTTTGTCACCGGCAGTCTCTCTGCAGTGCCCACTTTAATGATGGCATCTAA
AGAAAAGGGTTGTGCTCGATGCAGGACTTAACCCAATATCTCACCACACTAGCTGACTACAACCA
TGCACCACCTGTCACTTTGTCCCCCGAAGGGGAAAGCTCTATCTCTAGAGTTGACAAAGGATGTC
AAGATTTGGTAAGGTTCTTCGCGTTGCTTCAAATTAACCCACATGCTCCACCGCTTGTGCGTGTCC
CCGTCATTTTTTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCCGAATGCTTAATGTTTTAGCT
GCAACACTGAGGGGCGGAAACCCCCAACAGTTAGCACTCATCAGTGACGGCGTGGACTACCTG
GGTATCTAAGCCTGATTGATCCCCACGCTTTCGCATCTCAGCGTCAGTTACAGACCAGAGAG

>18_Un_99N, *Lactococcus lactis* 16S ribosomal RNA gene, partial sequence

GGGCCGACCCCGGCGGGGCCGATCCGAGGGCCTCACTAAACCATCCAATCGGTAGTAGCGACG
GGCGGTGTGTACAAAGGGCAGGGACTTAATCAACGCAAGCTTATGACCCGCACTTACTGGGAAT
TCCTCGTTCATGGGGAATAATTGCAATCCCCGATCCCCATCACGAATGGGGTTCAACGGGTTACC
CGCGCCTGCCGGCGTAGGGTAGGCACACGCTGAGCCAGGATCAAACTCTAACGTCATCCCCACC
TTCCTCCGGTTTATCACCGGCAGTCTCGTTAGAGTGCCCAACTTAATGATGGCTACTAACAATAG
GGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACTAGCTGACTACAACCATGCACC
ACCTGTATCCCGTGTCCCGAAGGAACTTCCTATCTCTAGGAATAGCACGAGTATGTCAAGACCTG
GTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAA
TTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTATTGCGTTAGCTGCGATA
CAGAGAACTTATAGCTCCCTACATCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTA
ATCCTGTTTGCTCCCCACTCTTTCGAGCCTCAGTGTCAGTTACAGGCCAGAGAGCCGCTTTCGCCA
CCGGTGTTCCTCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTCCTGCAC
TCAAGTCTACCAGTTTCCAATGCATACAATGGTTGAGCCACTGCCTTTTACACCAGACTTAATAAA
CCACCTGCGCTCGCTTTACG

>2_ML-11N_Assembly; *Lactococcus garvieae* 16S ribosomal RNA gene, partial sequence

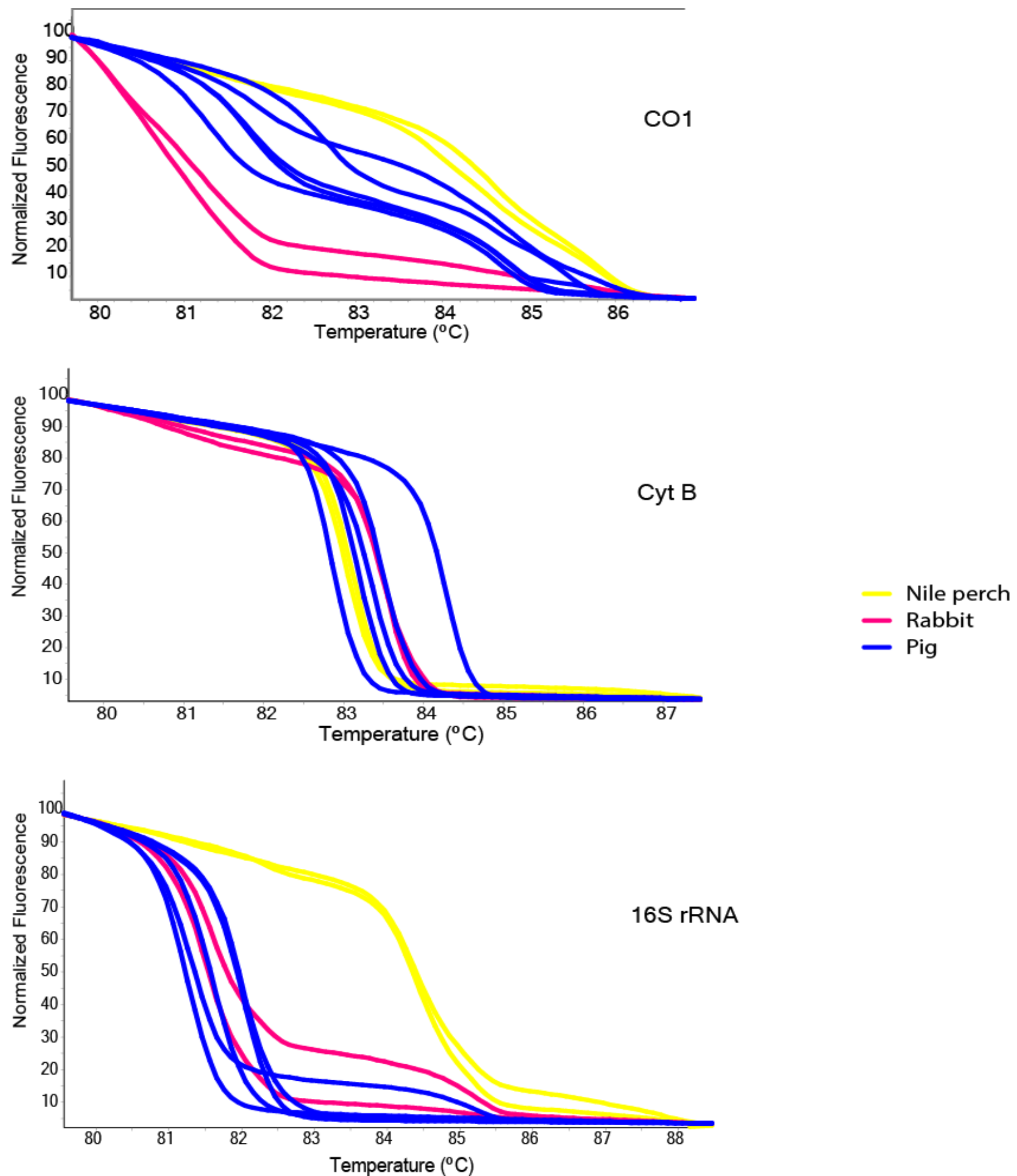
CATGCAGTCGAGCGATGATTGAAGATAGCTTGCTATTTTCATGAAGAGCGGCGAACGGGTGAGT
AACGCGTGGGAAATCTGCCGAGTAGCGGGGGACAACGTTTGGAAACGAACGCTAATACCGCAT
AACAATGAGAATCGCATGATTCTTATTTGAAAGAAGCAATTGCTTCACTACTTGATGATCCCGCG
TTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCAAGGCGATGATACATAGCCGACCTGAGAGG
GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
CTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGT
AAAACTCTGTTGTTAGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTATCTAA
CCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAAGCGTTGTCC
GGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCTTAAGTCTGATGTAAAAGGCAGTGGCTC
AACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGT
GTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGGCTCTCTGGCCTGT
AACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAGCTGTAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCATTAAG
CACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTC
GTGCTATCCTTAGAGATAAGGAGTTCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCA
TCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGT
CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGC
CAACCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGC
CTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGC

CTTGTACACACCGCCCGTCACACCACGGAAGTTGGGAGTACCCAAAGTAGGTTGCCTAACCGCA
A

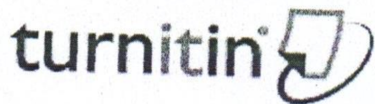
>6_ML-N88, *Aeromonas caviae* 16S ribosomal RNA gene, partial sequence

TGCAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAG
TAATGCCTGGGAATTTGCCCTGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATA
CGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCTATTGGATGTGCCCATGTGGGATT
AGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCA
GCCACACTGGAACTGACACACGGTCCACACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA
ATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACT
TTCAGCGAGGAGGAAGGGTCATTAGCTAATATCTGCTGGCTGTGACGTTTCTCACAAAAAAAAC
ACCGGCTAACTCCGTGCCAACACCCGCGGTAATACGGAGGGTGCAAGCGTTAATCCGAATTACT
GGGCGTAAAGCGCACGCATGCGGTTGGATAAGTTATATGTGAAAGCCCCGGGCTCAACCTGGG
AATTGCATTTAAAACTGTCCATCTAGAGTCTTGTAGAGAGAGGTACAATTCCACGTGTATCGGTG
AAATGTGTATAGATCTGGAAGAATACCGGTGGCGAACGCGCCCCTGTGTACACAGACTGACACT
CAGGTGCGAAAGCGTGTGGAGCACACACGATTATATACACTGGGAGTCCACGCCCTAACTATAT
CAATATGGAGGCTGTGTCCTTGAAACGTGTCTTCCAGAGCTAAGCTTTAAATCTACCGGCTGGGG
AATACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGCACCCACAAGCGGTGGAACATGTG
GTTTTATTCGATGCACGCGAAAAACCTACCTGGCTTGACTGTCTGGAATCTTTAGAAATACGGTA
GTGCCTTCGGAATCAGAAAACAGGGCTGCATGGGTGTCCTCACCTCCGGTCCGGAGAATTTGGG
TTAATTCCGAAGGAGGCCACCCTTTTCTTTTTTGCCACACGTAAGGGGGGAAATCAAGGGAAACT
GCCGTAAAAACCGAAGGAAGGGGGGGATAAACTCAATTTTCCTGGCCTTAAGGCAGGCCCCCCC
TGCTCAATGGGCCTTAAAAAGGGTCCACCTACGGAAGGGAGCC

Appendix 8: Supplementary Figure showing normalized curves of pig samples using the mitochondrial markers CO1, Cyt b and 16S rRNA.



The melting temperature of the DNA segments (markers) being amplified is the temperature at which 50% of the double stranded DNA is changed to single stranded DNA, with pig samples having a wide range in melting temperature.



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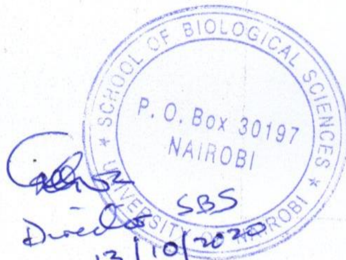

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