MOLECULAR DETECTION AND CHARACTERIZATION OF *Pasteurella multocida* INFECTING CAMELS IN MARSABIT AND TURKANA COUNTIES, KENYA

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DECLARATION

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

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DEDICATION

It is with nalstogia to dedicate this thesis to my late father Peter Kasivalu Kavivya who passed away on 13th December, 2011, he used to encourage me to strive and excel in academics but he passed away before reaping more fruits from his investment and my mother Regina Ngina Kasivalu. They are blessed with six boys; John Mutua, Daniel Mutunga, Patrick Mbithi, Stephen Muema and the Late Shedrack Munyao To my dear wife Elizabeth Berline who has rent a shoulder to lean on in the time of need. She provided emotional and financial support throughout and taking care of our children when I was busy with studies. Special dedication to our children Brian Mule, Blessings Ngina and Bridgit Baraka you are an inspiration to me, you keep me going for every time is see you I gain a new energy to work extra hard.

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ACRONYMS AND ABBREVIATIONS

CT	cycle threshold
DNA	deoxyribonucleic acid
HS	hemorrhagic septicemia
LPS	lipopolysaccharide
OMPs	outer membrane proteins
PCR	polymerase chain reaction
PM	*Pasteurella multocida*
REA	restriction endonuclease analysis

ABSTRACT

Pasteurella multocida is considered to be one of the normal flora in the respiratory tract of camels and other animals. It becomes pathogenic and causes Pasteurellosis when the resistance of the camel body is diminished by harmful environmental influences such as sudden changes in environmental conditions, poor dietary nutrition, walking for long distances and infestation by parasites such as trypanosomiasis. *P. multocida* is a small, gram-negative, nonmotile, non–spore-forming coccobacillus with bipolar staining features. The bacteria typically appear as single bacilli on Gram stain; however, pairs and short chains can also be seen. Close herding, overwork, limited food supply, and wet climatic conditions are stresses that seem to speed the spread of the infection. A study done in northern Kenya noted that in Africa *P. multocida* infections causing death in camels (*Camelus dromedarius*) have been existing since 1890 though the real cause of this disease remains elusive and needs further study. *P. multocida* capsular type B is the possible cause of respiratory disease in camels, its role as the causative agent for Pasteurellosis in camels' remains unclear and needs further investigation. Polymerase chain reaction if done using proper primer design can enhance identification of *P. multocida* at different specificity *viz;* genus, strain, species, or all members of a domain. The study was done to detect, characterize and analyze *P. multocida* capsular types in Marsabit and Turkana Counties by application of PCR. The study was a retrospective study that used samples collected for diagnosis. *P. multocida* was detected, capsular typed and genetic diversity determined using PCR. The study was done on EDTA blood samples and nasal swabs brought to central veterinary laboratories (CVL)-Kabete collected from camels in Marsabit and Turkana Counties in the month of November 2018 and April 2019 respectively. The blood and nasal swabs preserved at -80°C were used for DNA extraction for subsequent molecular analysis. *P. multocida KMT1* gene and capsular groups' *capA (hyaD-hyaC), capB (bcbD), capD (dcbF), capE (ecbJ) and capF (fcbD*) were targeted marker genes. Capsular typing data was used to determine the genetic diversity of *P. multocida*. The genomic DNA was extracted from 132 samples (102 whole blood and 30 nasal swabs) for Molecular detection of *P. multocida* and capsular types by PCR assays. A total of 16% (21/132) samples for the two counties showed amplification product with KMT1SP6 and KMT1T7 specific primers. Marsabit County showed the highest *P. multocida* detection with 24.6% (15/61) and Turkana with the lowest detection of 8.5% (6/71). Based on the sample type the highest *P. multocida* detection was recorded in EDTA blood with 18.6% (19/102) and 6.7% (2/30) from nasal swabs. Capsular groups were determined using multiplex PCR. Among 21 DNA identified as *P. multocida*, capsular group E was detected by Multiplex PCR in twenty (21) *P. multocida* positive samples. Sequence analysis of the *P. multocida* positive samples matched capsular group E for gene ecbJ with accession number AF302466. The study concludes that PCR analysis based on KMT1T7 and KMT1SP6 primers work well for species confirmation of field strains irrespective of capsular types. The study recommends further characterization of *P. multocida* and capsular groups to provide definitive answers to the real cause of camel Pasteurellosis. Monitoring the capsular types of *P. multocida* strains circulating in a specific geographical region may be important in the formulation of vaccines. The *P. multocida* strain circulating in Marsabit and Turkana Counties is of capsular group E, there are not many capsular groups present in the two counties, and PCR assay targeting *KMT1* gene is recommended for large-scale surveillance of *P. multocida.*

CHAPTER ONE

INTRODUCTION

1.1 Background information

Respiratory tract infection is ranked among the health problem to camels worldwide that result in enormous death of camels and loss in production (Khan, 2012). *Pasteurella multocida* (*P. multocida*) is considered to be one of the normal flora in the respiratory tract of camels and other animals. It becomes pathogenic and causes Pasteurellosis when the resistance of the camel body is diminished by harmful environmental influences such as sudden changes in environmental conditions, poor dietary nutrition, walking for long distances and infestation by parasitic such as trypanosomiasis (Elsayed *et al*., 2021). *P. multocida* causes Pasteurellosis in camels within 10-24 hours, accompanied by fever, swelling of subcutaneous tissues and mandibular and/ or cervical lymph nodes, throat region, pulmonary edema, fibrinous pneumonia, and diarrhea. Pasteurellosis has low morbidity, mortality rate may be 80% or higher and carrier or sick camels are considered as source of infection to other animals especially young calves (Abdeltawab *et al*., 2016; Elsayed *et al*., 2021). Affected animals seldom recover and usually die within 24-48 hours. Signs of dyspnea such as dilated nostrils or open mouth breathing and cyanotic mucous membranes are commonly observed (Brenda *et al*., 2013; Tahamtan *et al*., 2016). Therefore, Pasteurellosis is a hazardous disease in camels that drastically affects productivity (Brenda *et al*., 2013; Abdeltawab *et al*., 2016). Pasteurellosis is prevalent in Kenya and globally, the available approaches for prevention, diagnosis, and control have many limitations that need to be improved (OIE, 2012).

Morphologically, *P. multocida* has a characteristic small, nonmotile, gram negative, nonspore forming coccobacillus that shows bipolar staining morphology. On gram stain, *P. multocida* typically looks like single bacilli, also seen are pairs and short chains (Abdeltawab *et al*., 2016; Abbas *et al.,* 2018). Abbas and Omar (2005) reported that agents causing diseases most commonly isolated from pneumonic camels are *P. multocida*.

P. multocida, which contain more genes due to varied antigenic specificity express polysaccharide capsule on their surface, based on capsule antigen. The capsule contain distinct genes that encode proteins that take part in the formation of distinct capsular proteins (Teleb *et al*., 2019). The capsular composition of *P. multocida* strains are characterized into five capsular groups A, B, D, E, F (Bhimani *et al*., 2014; Teleb *et al*., 2019; Smith *et al*., 2021; Aski and Tabatabaei, 2016). The *hya*D gene is distinct for capsular group A strain purposely for the synthesis of hyaluronic acid, *fcb*D gene specific for capsular group F encode chondroitin, *dcb*F gene take part in synthesis of heparin in capsular group D, *bcb*D and *ecb*J gene encode glycosyltransferase specific for group B and E capsule. The understanding of genes for capsule biosynthesis enabled the application of Multiplex PCR for laboratory typing of *P. multocida* (Teleb *et al*., 2019). *P. multocida* has various host range, but the source of agents causing disease responsible for most sporadic infections remains unclear (Aski and Tabatabaei, 2016).

Close herding, overwork, limited food supply, and wet climatic conditions are stresses that seem to speed the spread of the infection (Elsayed *et al*., 2021). Viable bacteria are rarely found in pastures or soil after 2 to 3 weeks though the bacteria can be viable for several hours and days in wet soil or water and biting arthropods are not

significant vectors (OIE, 2012). A study done in northern Kenya by Gluecks *et al.*, (2017) noted that in Africa Pasteurellosis infections causing death in camels (*Camelus dromedarius*) have been existing since 1890 though the real cause of this disease remains unclear and needs further study.

P. multocida capsular type B is the possible cause of infections in respiratory system of camels in Iran while capsular type E in Africa though their function as Pasteurellosis causing agents in camels is not definitive requiring further investigation (Tahamtan *et al*., 2016). According to Tahamtan *et al*., (2016) detection of *P. multocida* and molecular characterization can be done by application of biochemical, molecular assays and other methods. Various molecular methods have been designed to enhance *P. multocida* characterization such as ribotyping, restriction enzyme analysis (REA), multilocus sequence typing, pulsed field gel electrophoresis, and polymerase chain reaction (PCR) based fingerprinting (Abbas *et al.*, 2018).

PCR if done using proper primer sequences design can enhance identification of *P. multocida* genus, strain, species and all members of a domain. Tahamtan *et al.*, (2016) and Townsend *et al.*, (2001) used the PCR amplicon size of 460 bp band to identify *P. multocida.* Tahamtan *et al.*, (2016) and Vidhya *et al.*, (2007) used 760 bp amplicon size corresponding to capsular type B and confirmed that a majority of *P. Multocida* isolates causing Pasteurellosis belong to serotype B. Kebede and Gelaye (2010) also reported capsular group B as being responsible for Pasteurellosis in camels. The available comparative genomic and phylogenetic analyses of this family indicate that many members of Pasteurellaceae are poorly classified (Brenda *et al.,* 2013). Brenda *et al.,* (2013) noted that while phylogenetic analysis is useful in distinguishing *P. multocida*

strains from other members of the family, ancestral relatedness among strains are more difficult to differentiate. The study was done to detect, characterize *P. multocida* capsular types circulating in Marsabit and Turkana Counties by application of PCR.

1.2 Statement of the Problem

A number of fungi, bacteria, viruses, and parasites may possibly cause respiratory infection in camels. *P. multocida* causes Pasteurellosis in camels leading to low production, economic losses due to high cost of vaccination and medication followed by the death of the camels. Pastoralists in Lapur division of Turkana district in Kenya identified Pasteurellosis as one of the important camel diseases in Kenya (Mochabo *et al*., 2005). A study conducted in North Kenya by Gluecks *et al*., (2017) focused on *P. multocida* capsular type B and E that where not detected, instead their study confirmed the presence of *P. multocida* capsular type A and D specific DNA sequences though at very low frequency. Gluecks *et al*., (2017) concluded there was a circulation of *P. multocida* strain in camels that lack established capsular types. Their study failed to specify the counties where the study was conducted rather mentioning northern Kenya. The current study was conducted in Marsabit and Turkana counties. It is important to have appropriate methods to detect and characterize *P. multocida* capsular types in order to develop better control and prevention methods (Abdeltawab *et al*., 2016), Therefore, these study was done to detect, characterize and analyze *P. multocida* capsular types in Marsabit and Turkana Counties by application of PCR.

1.3 Justification of the study

P. multocida camels' infection is common in Kenya though there is little knowledge on the genetic diversity of the pathogen. Pasteurellosis is ranked among common causes of economic losses in camels in Kenya especially in Marsabit and Turkana counties where there were cases of camels death reported at the Directorate of Veterinary Services. In the month of November 2018 and April 2019 farmers experienced fatalities, drop in productivity and enormous cost on vaccination and medication. Diagnosis of Pasteurellosis in camels in Kenya is limited with very few studies focusing on the disease. The diagnostic techniques applied focus on clinical signs and history that cannot give a correct status of *P. multocida* infection in the field. It has been observed that identification of *P. multocida* in the clinical samples is greatly enhanced by the application of PCR method (Abbas *et al.,* 2018). Using well-standardized PCR can enable not only detection of the circulating agents but also their characterization through sequencing. Furthermore, *P. multocida* is a zoonotic disease, the community living in Marsabit and Turkana counties value camels for meat, milk, blood, fat that can be a viable source of *P. multocida* infection. This study focus on the detection and characterization of *P. multocida* in Marsabit and Turkana counties, Kenya. Therefore, the study has provided important information on the detection and genetic diversity of *P. multocida* in the two counties. This information may assist in understanding the molecular epidemiology of *P. multocida* infection in camels and the capsular type's circulation in the area.

1.4 Research hypotheses

i. There is no *P. multocida* infection in camels in Marsabit and Turkana Counties.

ii. *P. multocida* is not diversified across Marsabit and Turkana counties.

1.5 General objective

To detect, characterize and determine genetic diversity of *P. multocida* infecting camels in Marsabit and Turkana Counties.

1.6 Specific objectives

i. To determine prevalence of *P. multocida* in camels in Marsabit and Turkana Counties using PCR.

ii. To determine the genetic diversity of capsular types of *P. multocida* in camels in Marsabit and Turkana Counties.

1.7 Significance of the study

This study will reveal the presence of *P. multocida* and the capsular types circulating in Marsabit and Turkana counties in Kenya as the cause of Pasteurellosis in camels. Identification of *P. multocida* in the clinical samples is greatly enhanced by the application of PCR thus avoiding the tedious and time-consuming laboratory protocols such as biochemical and morphological characterization. The findings would be of major importance for diagnosis, study of disease epidemiology, development of vaccine and detection of any shift or mutations of strain circulating in a region. Farmers will understand how to manage camels to avoid *P. multocida* infection by; not underfeeding camels, ensuring appropriate medication and vaccination programs, and minimizing

herding of camels in crowded areas especially in wet conditions in order to slow the spread of *P. multocida* infection.

CHAPTER TWO

LITERATURE REVIEW

2.1 Camel distribution

Most Camels are found in dry and semi dry regions of the world characterized by short rainfall followed by long dry season (Bourzat and Wilson, 1987). Camel population worldwide is approximately 35 million (FAO, 2019), majority being found in Sudan, Niger, Somalia, Chad, Kenya, Ethiopia, Mali, Mauritania and Pakistan as well as Middle East and some countries in southern Europe, Japan and North America. African camel population can be classified into East Africa (Ethiopia, Djibouti, Somalia, Kenya, Sudan), West Africa (Mali, West Sahara, Burkina Faso, Mauritania, Nigeria, Niger, Senegal and Chad) and north Africa (Egypt, Algeria, Libya, Morocco and Tunisia) (Bourzat and Wilson, 1987). In Asian countries camels are found in Yemen, united Arab Emirates, India, Mongolia, China, Saudi Arabia and Oman. The Marsabit camel population is approximately 132,215 while the Turkana camel population is approximately 456,826 (Census, 2019).

2.2 Aetiology of Pasteurellosis in camel

The causal agent of camel's Pasteurellosis is capsular type of *P. multocida* mostly residing as a commensal in the nasopharynx of the animal (OIE, 2012). *P. multocida* is assumed to be the causative agent of most respiratory infections both in wild and domestic animals (OIE, 2012). *P. multocida* is frequently isolated from cats, birds, rabbits, dogs, cattle, camel and pigs. It causes nasal mucosa atrophy in pigs and bovine respiratory infection in cattle (OIE, 2012). It is assumed to be the cause of death in saiga

antelopes. Humans get infected by *P. multocida* following dog or cat bites (Narsana and Farhat, 2015; Smith *et al*., 2021). The cause of Pasteurellosis in camels needs further study, previous reports on Pasteurellosis pathogen isolation proved futile hence providing limited characteristic information of the isolates (Gluecks *et al*., 2017). Clinically *P. multocida* present no symptom or just mild upper respiratory inflammation, progressing to pneumonic and wide spread disease (Peng *et al.*, 2018).

Figure 2.1: Presentation of Pasteurellosis in camel. The camel appear dull, rough coat, extended neck and the swelling of submandibular.
Source; Yahya Tahamtan Razi Vaccine and Serum Research Institute- Shiraz, Iran Bacteriology Department (2016).

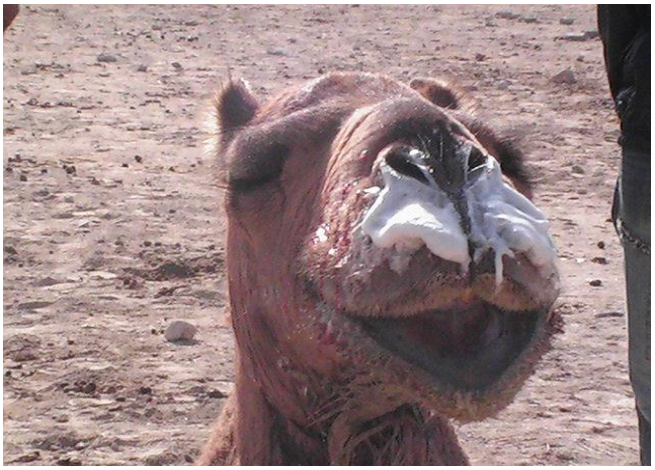


Figure 2.2: Fibrinous nasal discharge in a camel suffering from Pasteurellosis

Source; Yahya Tahamta Razi Vaccine and Serum Research Institute- Shiraz, Iran · Bacteriology Department (2016).

2.3 Taxonomy of *P. multocida*

The genus Pasteurella that infect camels belongs to Pasteurellaceae family that has various members of Gammaproteobacteria that are Gram negative. They are common human or animal respiratory flora that are not only opportunistic pathogens but also possible pathogens. The available comparative genomic and phylogenetic analysis of this family indicate that many members of Pasteurellaceae are poorly classified (Brenda *et al.*, 2013). Classification of strains of *P. multocida* is done based on capsular and somatic antigens. There are five capsular groups; A, B, D, E and F (Carter, 1955). Classification of *P. multocida* is summarized in the table 2.1 below.

Table 2.1: Classification of *P. multocida* (Cabi, 2019)

Taxonomy	
Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Pasteurellales
Family:	Pasteurellaceae
Genus:	*Pasteurella*
Species:	*Pasteurella multocida*

2.4 Epidemiology and geographical distribution

Camel's Pasteurellosis presents with an acute septicemia followed by high morbidity and mortality (OIE, 2012). Pasteurellosis is widely distributed with the most occurrence in south East Asia. Camels' Pasteurellosis is fatal in Africa, Middle East and some countries in southern Europe as well as in Japan and North America. African form of Pasteurellosis occurs sporadically, limited in extent and mostly associated with stress conditions (Sah, 2011; FAO, 2019). In humans, about 50% of dog bites present *P. multocida* infection (Cross, 2018).

Infected camels are the source of infection to other camels by direct contact, on fomites, through ingesting or breathing in of the *P. multocida* that probably comes from the nasopharynx of infected animals (OIE, 2012; Brenda *et al.,* 2013). Serious epidemics occurs during rainy season, in camels in poor physical condition. Susceptibility to infection is thought to be increased by closely herding animals while wet conditions

enhance the fast spreading of the disease (OIE, 2012; Brenda *et al.*, 2013; Tahamtan *et al*., 2016). Viable bacteria are rarely found in pastures or soil after 2 to 3 weeks though the bacteria can be viable for several hours and days in wet soil or water, biting arthropods are not significant vectors (OIE, 2012).

Pasteurellosis has low morbidity though mortality may be higher than 80% (Abdeltawab *et al*., 2016). Within 24 hours of infection by *P. multocida* there is soft tissue inflammation accompanied by increased leukocytes numbers leading to generally a diffuse, localized cellulitis (OIE, 2012). The camel appear dull with rough coat, extended neck, fibrinous nasal discharge and swelling of submandibular (Figure 2.1 and 2.2). Other parts of the body are also infected such as the respiratory tract causing regional lymphadenopathy (Brenda *et al.,* 2013). Bacteremia can result in other serious cases causing endocarditis or osteomyelitis. When the bacteria cross the blood – brain barrier it may cause meningitis (OIE, 2012; Brenda *et al.,* 2013).

A number of virulence factors are expressed by *P. multocida* contributing to the pathogenesis. Genes that play part in the biosynthesis of capsule, fimbriae and adhesins, lipopolysaccharide (LPS), hyaluronidase, iron –regulated toxins, iron acquisition proteins, sialic acid metabolism and outer membrane proteins (OMPs) are part of virulence factors (Peng *et al*., 2018; Elsayed *et al*., 2021). The capsules found in capsular type B and A are important in resisting phagocytosis by host immune cells and also complement-facilitated lysis respectively (Peng *et al*., 2018). *P. multocida* is able to invade, replicate and cause disease to the host owing to the presence of polysaccharide capsule vital for its virulence. The capsule serves several function, which include; protecting the bacteria cell wall from drying by being highly hydrated, keeping away

phagocytic cell by giving them anti-phagocytic activity through their serum resisting bacterial complement activity evasion (Teleb *et al*., 2019).

The capsule block the attachment of bacterial to phagocytes by preventing pathogens- associated molecular patterns (PAMPs) that are components of common molecules such as glucans, peptidoglycan, lipopolysaccharides, teichoic acids and mannans from attaching to endocytic recognition receptors on the surface of the phagocytes. The PAMPs trigger complement three pathways; the classical pathway, lectin pathway and the alternative pathway. The three pathway end in the formation of C3 and C5 convertases that generate anaphylatoxins C3a and C5a the membrane attack complex (MAC). Capsule type A and B disrupt the host's complement pathways by preventing the formation of C3 convertase thus preventing the formation of opsonins C3b and C4b that take part in enhanced attachment and complement protein C5a (Kachooei *et al*., 2017**).**

2.5 Biology of *P. multocida*

P. multocida appear as small, nonmotile, gram negative, nonspore forming coccobacillus which have the bipolar staining morphology. On gram stain the coccobacillus typically looks like single bacilli however, pairs and short chains can also be observed (Abdeltawab *et al*., 2016). The bacterium does not survive when exposed to temperature of about 55°C and to most hospital disinfectants (OIE, 2012). *P. multocida* expresses virulent factor such as; hyaluronidase, sialic acid metabolism, toxins, iron regulated and iron acquisition proteins, fimbriae adhesins, invasins, antiphagocytic, polysaccharide capsule and variable carbohydrate surface molecules (Harper *et al*,.

2006). Characterization of *P. multocida* is based on capsule and lipopolysaccharides (LPS) surface components. The bacterium is serologically classified into capsular serogroup A, B, D, E and F (Carter, 1955; Heddleston *et al*., 1972). The serological methods are tedious and complicated (Peng *et al*., 2016). Therefore, the development of molecular typing methods has made classification of *P. multocida* to be easy (Townsend *et al*., 2001)

2.6 Clinical signs and symptoms of Pasteurellosis

Most Pasteurellosis cases in camel are acute or peracute and first signs of infections are fever, dullness, and reluctance to move. Majority of the risk factors that predispose animals to pneumonia are associated with poor management conditions such as environmental stress, crowdedness, poor sanitary conditions, poor nutrition and nutritional management, extreme climatic swings and general herd health (OIE, 2012; Brenda *et al.,* 2013; Tahamtan *et al*., 2016). Normally, there is salivation and development of serous nasal discharge, Oedematous swellings appear in the pharyngeal region which extend to the ventral cervical area and brisket with congestion of mucous membranes (OIE, 2012; Elsayed *et al*., 2021). The camel experiences difficulties in breathing and usually collapses and dies 6 to 24 hours after the first infection signs are seen (OIE, 2012). Death may be sudden or may prolong for up to 5 days. Most deaths in endemic areas occur in older calves and young adults. Enormous epizootics may be found in endemic and/or non-endemic areas. Where there is confirmation of Pasteurellosis as a secondary complication, mortality may approach 100% if interventions are not applied at the beginning of the infection (OIE, 2012; Brenda *et al.,* 2013).

2.7 *P. multocida* infection medication

Treatment of Pasteurellosis should be initiated immediately the first signs of symptoms are noticed. *P. multocida* is susceptible to a broad spectrum antibiotics such as erythromycin, lincosamides or some β- lactams(dicloxacillin or cephalexin), Amoxicillin/clavulanic acid, minocycline, fluoroquinolones and trimethoprim-sulfamethoxazole (Brenda *et al.*, 2013, Cross, 2019). Vaccination is done by using bacterins, toxins- based component vaccines, live attenuated bacteria that works against toxin caused diseases (Brenda *et al.*, 2013).

2.8 Molecular detection and identification of *P. multocida*

Camel Pasteurellosis is a neglected disease in Kenya, with very few studies focusing on the disease. Only one outstanding study done in northern Kenya by Gluecks *et al* 2017 is reported. *P. multocida* causes Pasteurellosis, an acute septicemic disease with high morbidity and mortality in sheep, goat, cattle, and camels, that results in economic losses (OIE, 2012.

PCR is a powerful, very specific molecular method that can be rapidly applied for detection of several bacteria species including *P. multocida* (Ara *et al*., 2016). *P. multocida* is detected using Polymerase chain reaction or a single copy (or more) of *KMT1* gene DNA sequence exponentially amplified using specific primers to generate thousands to millions of more copies (Abbas *et al.*, 2018). *KMT1* gene is a cell wall transmembrane protein, which is highly conserved and distinct for *P. multocida*. *P. multocida* Kabete isolate (A73/17) was used as a positive control and nuclease free water as a negative control. Several studies have used *KMT1* gene primers to screen for the

presence of *P. multocida* species and run gel electrophoresis of the PCR products giving a unique band appearing at 460bp to confirm the presence of *P. multocida* (Deressa *et al*., 2010; Townsend *et al*., 2001; Ara *et al*., 2016; Tahamtan *et al*., 2016 and Abbas *et al.*, 2018). Multiplex PCR is further carried out on all *P. multocida* positive sample using specific primers for each capsular type to detect serotype A, B, D, E, F. Positive gel electrophoresis of each PCR products give unique bands for each serotypes; *capA* at 1044bp, *capB* at 760bp, *capD* at 657bp, *capE* at 511bp, and *capF* at 851bp (Townsend *et al*., 2001; Abdeltawab *et al*., 2016 and Abbas *et al.*, 2018). The information has been used to deduce the genetic diversity of *P. multocida* capsular types based on numbers obtained per *P. multocida* capsular type.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Eight locations in two separate counties were investigated for *P. multocida* infections, five locations in Marsabit County and three locations in Turkana County (Figure 3.1). Marsabit County (37°58' E, 2°19' N) is situated in northern Kenya bordering Wajir County to the east, Ethiopia to the north, Isiolo County to the south east. Its size is 70,961km^2 and has human population of about 459,785. Marsabit camel population is approximately 132,215. The camels are highly valued by the community as their main source of food i.e. meat and mixture of milk and blood. Marsabit county is dry, with cold months ranging between 10.1° C (June and July) and 30.2° C during the hot months (January-March and September-October)(Census, 2019). Turkana County (3° 09′N 35 ° 21′E) is located in northern Kenya bordering Samburu to the South-east, Marsabit to the East, Baringo and West Pokot to the South-west, Uganda to the west, and South Sudan to the north and Ethiopia to the north-east. It covers 68,680 km^2 with human population of about 926,976. Turkana camel population is approximately 456,826. Livestock especially camels are important as a source milk, meat and blood (Census, 2019).

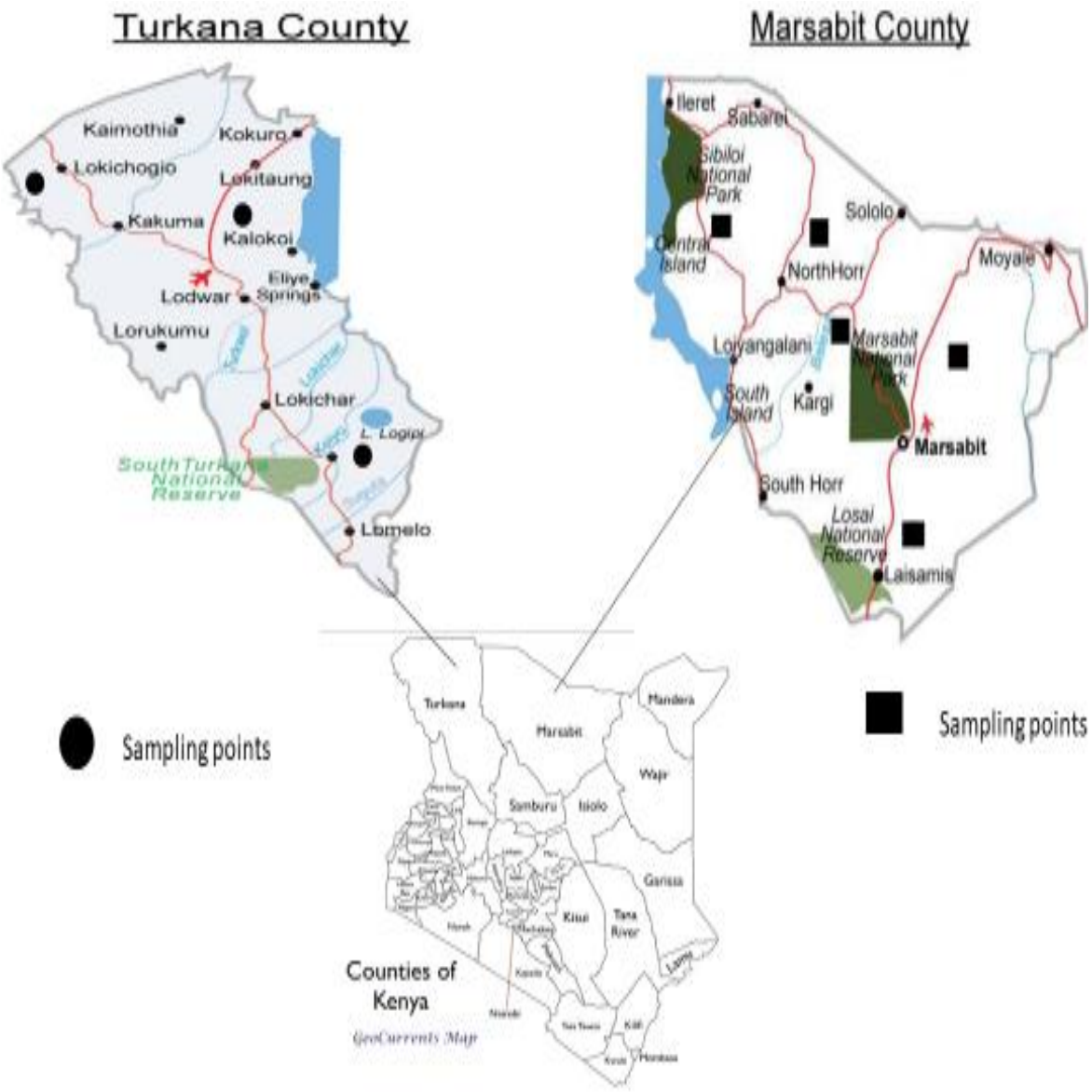


Figure 3.1: Location of Marsabit and Turkana Counties in Kenya

Source: Kenya Bureau of Statistics (2015)

3.2 Study design

The study was a retrospective study design, the study used samples collected for diagnosis purpose for other respiratory infection but none of the diseases intended to be diagnosed was detected.

3.3 Sample collection

A total of 132 samples (n=132) were collected. 61 EDTA blood in November 2018 at five locations namely; Laisamis-moile, Nairibu, Molobot, El burumagado and Galas in Marsabit County, 41 EDTA blood and 30 nasal swabs in April 2019 at three location namely Nadapal, Lokolia and Lokore in Turkana County from randomly selected clinically sick camels population that had been affected by Pasteurellosis like disease. Nasal swabs were collected in phosphate buffered saline (PBS) and transported to the laboratory in a cool box. The nasal swabs were put in tryptic soy broth (Carramore International limited, Holmfirth, United Kingdom) and incubated at 37°C for 18 hours. The blood and nasal swabs were stored at -80°C freezer. The blood and nasal swabs were used for DNA extraction for subsequent molecular analysis for *P. multocida* KMT1 and capsular groups' *capA (hyaD-hyaC), capB (bcbD), capD (dcbF), capE (ecbJ) and capF (fcbD*) gene sequences.

3.4 Determination of sample size

Slovin's formula, (Galero-Tejero, 2011) for sample size determination was used

$$n = \left(\frac{N}{1 + Ne^2}\right)$$

n = number of samples

N = total population

e = error tolerance level

Applying a confidence level of 95% gives error tolerance of 0.05. The total camel population from the affected areas was 200 camels.

Plug in the data into the formula

$n= 200/ (1+200*0.05^2) =133$ camels

3.5 Consent/ Ethical statement

Institutional Animal Ethics Committee approval to conduct this study was not necessary as there was no invasive procedure applied. The authority to use the EDTA whole blood and nasal swabs was sought from State Department of Livestock, Directorate of Veterinary Services (Appendix 1)

3.6 DNA extraction

The frozen whole blood samples and swabs were retrieved from freezer and left to thaw at room temperature. The genomic DNA was extracted using Invitrogen PureLink Genomic DNA mini Kit Cat. No.k1820-01 for purification of genomic DNA by thermofisher Scientific (Invitrogen, 2012) Water bath was preset at 55°C. To a sterile Microcentrifuge tube, 200µL of thawed whole blood and nasal swab suspension was added, 20 µL of each proteinase K and RNase A was added, briefly vortexed and incubated at room temperature for 2 minutes. Homogenous solution was made by vortexing after adding 200µL of lysis/ binding buffer. Incubation of the lysate was done at 55°C for 10 minutes to activate protein digestion. DNA was precipitated using 96% ethanol and bound to a resin on a mini spin column, the column was centrifuged at 12000

rpm for 1 minute. The flow through was discarded together with the collection tube. The spin column was inserted into a new collection tube. The spin column was washed two times using 500 µL wash buffers 1 and 2 and centrifuged at room temperature at 12000 rpm for 1 minute and maximum speed for 3 minutes respectively to remove the lysis buffer. DNA was eluted using 60µl elution buffer (Invitrogen, 2012, Rice, 2019) and the DNA was kept at -20°C awaiting PCR analysis.

3.7 Detection of *P. multocida* by conventional PCR

Molecular detection of *P. multocida* was arrived at by amplification of the targeted *KMT1* gene using Techne TC-512 thermocycler from Bibby scientific LTD, United Kingdom Conventional PCR amplification targeting 460bp *KMT1* gene fragment using primers previously designed by Townsend *et al*. (1998) (Table 3.1) was applied to identify *P. multocida*. A *P. multocida* Kabete isolate (A73/17) was used as a positive control and nuclease free water as a negative control.

Table 3.1: Primer sequences for conventional PCR for identification of *P. multocida*

Gene	Accession number	Primer F	Primer R	Reference
KMTI	AF016259	PM KMT1T7_F ATCCGCTATTTA CCCAGTGG	PM KMT1SP6_R GCTGTAAACGAA CTCGCCAC	amplification size 460bp, Townsend *et al*.,1998

Townsend *et al*. (1998) protocol slightly modified was employed for DNA amplification. DNA amplification contained a reaction volume of 18.0 µl containing nuclease free water 12.2 µl, 2.5 µl of 5x PCR buffer, 0.5 µl of 10mM dNTPs, 0.5 µl of 10µM primer pair

each, 1.5 µl of 3mM $MgCl_2$ and 0.3 µl of Taq DNA polymerase 3unit. Amplification conditions were initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 45 sec, final extension at 72°C for 6 min, holding at 10°C until removal. PCR amplicons were resolved in 1.5g gel red stained agarose gel in tris borate EDTA buffer. Five (5) µl of PCR amplicons were mixed with loading dye and dispensed in the wells, molecular size marker 100bp (Invitrogen, Thermofisher scientific, USA) was dispensed in one well. The PCR amplicons were resolved by gel electrophoresis at 100v for one hour and visualized using a gel documentation system. Test was considered valid when all negative controls showed no band and the positive controls showed a unique 460bp size band.

3.8 Detection of *P. multocida* capsular types by multiplex PCR

P. multocida capsular typing was done by Multiplex PCR with capsular gene specific primers pairs for *capA* gene *hyaD-hyaC, capB* gene *bcbD, capD* gene *dcbF, capE* gene *ecbJ, and capF* gene *fcbD* designed by Townsend *et al*. (2001) (Table 3.2). Primer sequence with coding strand similar to capsular group E for gene ecbJ with accession number AF302466 was successfully optimized for detection of capsular group E of *P. multocida*. All 21 *P. multocida* KMT1 gene confirmed positives were subjected to multiplex PCR using distinct Primers previously designed by Townsend *et al*. (2001) for capsular group's determination (Table 3.2). The 100µM capsular type stock concentration primers were diluted to 10µM working concentration then the working concentration dilutions were pooled together and used for doing multiplex PCR.

Table 3.2: Primer sequences for conventional PCR for identification of *P. multocida* capsular types

Cap type	Gene	Primer F/R	Amplicon size (bp)	Ref.
Cap A	*hyaD-hyaC*	5'-TGCCAAAATCGCAGTGAG-3' 3'-TTGCCATCATTGTCAGTG-5'	1044	Townsend *et al*., 2001
Cap B	*bcbD*	5'- CATTTATCCAAGCTCCACC-3' 3'- GCCCGAGAGTTTCAATCC-5'	760	
Cap D	*dcbF*	5'- TTACAAAAGAAAGACTAGGAGCCC-3' 3'-CATCTACCCACTCAACCATATCAG-5'	657	
Cap E	*ecbJ*	5'- TCCGCAGAAAATTATTGACTC-3' 3'- GCTTGCTGCTTGATTTTGTC-5'	511	
Cap F	*fcbD*	5'- AATCGGAGAACGCAGAAATCAG-3' 3'- TTCCGCCGTCAATTACTCTG-5'	851	

Townsend *et al*. (2001) protocol slightly modified was employed for *P. multocida* capsular typing. DNA amplification contained reaction volume of 20.0 µl containing 4.0µl of nuclease free water 10.0µl of 5x PCR buffer, 1.25 µl of 10mM dNTPs, 1.25 µl of 10µM primer pair each, 1.5 µl of 3mM $MgCl_2$ and 0.75µl of Taq DNA polymerase 3unit. Amplification conditions were initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1min, annealing at 55°C for 1min, extension at 72°C for 1min, final extension at 72°C for 7min, holding at 10°C until removal. PCR amplicons were electrophoresed using 1.5g agarose gel dissolved in 100ml 1x Tri Borate EDTA (TBE) buffer PH 8.4 by boiling using microwave. Agarose gel was cooled then stained using Gel red, a comb was placed on the trough and the agarose gel poured and allowed to set. After setting the comb was removed and five (5) µl of PCR amplicons were mixed with loading dye and dispensed in the wells, Molecular size marker 100bp

(Invitrogen, Thermofisher scientific, USA) was dispensed in one well. The PCR amplicons were resolved by gel electrophoresis at 100v for one hour and visualized using a gel documentation system (Townsend *et al*., 2001). Test was considered valid when all negative controls show no band and the positive controls showed a distinct bands as shown in table 3.2.

3.9 Determination of genetic diversity

This study used a total of 132 samples, 102 whole blood and 30 nasal swabs to detect *P. multocida*. Only the samples that where positive for *P. multocida* were exposed to multiplex PCR to determine the presence of capsular types. Gel electrophoresis of PCR products for each capsular types show unique bands of approximate different sizes; *capA* at 1044bp, *capB* at 760bp, *capD* at 657bp, *capE* at 511bp, and *capF* at 851bp (Townsend *et al*., 2001). Genetic diversity was determined based on the number of capsular types detected.

3.10 DNA sequencing

Twenty one (21) *P. multocida* PCR amplicons were sequenced, some at International Atomic Energy Agency (IAEA) Seibersdorf Laboratories, Vienna, Austria and others at inqaba biotec Africa's genomic company, South Africa using Sanger dideoxy sequencing procedure (Gomes and Bruce, 2018). PCR products were cleaned using the ExoSAP IT (Waltham, USA), which consist of Exonuclease that digest excess primers and alkaline phosphatase that dephosphorylates nucleotides (dNTPs) in the reaction mix. The DNA products were used as a template for the generation of DNA amplicons, the incorporation of a chain terminating dideoxynucleotide in 4 different

reaction tubes makes each strand to terminate randomly, hence producing many DNA amplicons, each terminating at a different site. Sequence products ran on a gel made easy the determination of where each chain terminating dideoxynucleotide was added. The DNA was visualized on a UV light since the primer to initiate the reaction was radioactive or some of the dNTPs were radioactive (Kurnaz, 2015).

3.11 Sequence analysis

The sequences were viewed using chromas version 2.6.6 (Treves, 2010) and assembled using Geneious prime program version 2020.2.4(Kearse *et al*., 2012) to allow for editing of the assembly and creation of consensus sequence. BioEdit software version 7.2 (Hall**,** 1999; Hall *et al***.,** 2011) was used to import, align sequences, save aligned sequences and BLAST program on NCBI for sequence similarity. MEGA X (Kumar *et al*., 2018) was used to construct phylogenetic tree by Maximum Likelihood method to determine relatedness. This information were used to determine the genetic diversity of *P .multocida* based on capsular gene percentages of occurrence.

CHAPTER FOUR

RESULTS

The study was carried out to detect, characterize and determine the genetic diversity *P. multocida* in blood and nasal swabs.

4.1 Conventional PCR results for *P. multocida* detection

Detection of DNA band of 460bp in size indicate the amplification of *KMT1* gene confirming the presence of *P. multocida* (Figure 4.1) (Teleb *et al*., 2019). A total of 132 samples consisting of 61 samples from Marsabit county and 71 samples from Turkana county were analyzed for the presence of *P. multocida* using primers targeting KMT1 gene. The positive rate of amplification of KMT1 gene for Marsabit county was 15 (24.6%) and Turkana county was 6 (8.4%). The overall positive rate from amplification of KMT1 gene for both Marsabit and Turkana counties were 21 (15.9%). Marsabit County, Molobot Location had the highest positive samples of 4/16 (25%) and Laisamis-Moile had the lowest positive samples of 2/10 (20%). Turkana County, Nadapal Location had the highest positive samples of 3/11(27%) and Lokore Location had the lowest positive sample of 1/30 (3%) (Table 4.1).

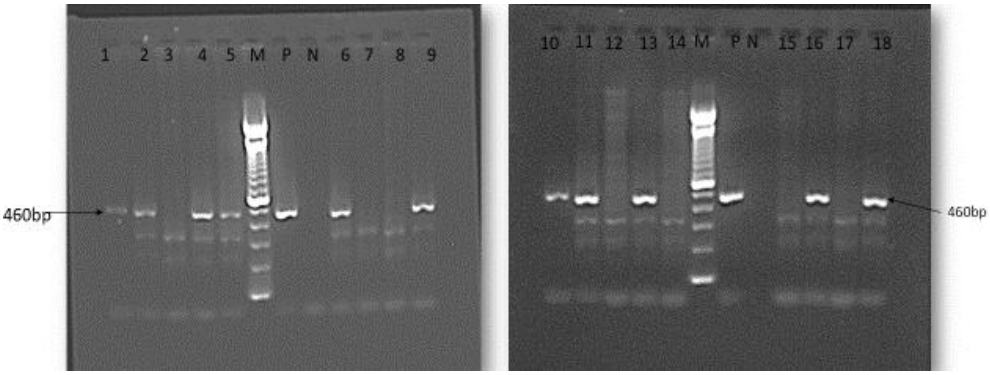


Figure 4.1: Conventional PCR amplification of *P. multocida* KMT1 gene.

The PCR amplicons were resolved on a 1.5% agarose gel and stained with gel red. Lane 1-Mar/nrb/c3, 2- mar/nrb/c7, 3-mar/nrb/c11, 4-mar/mle/c13, 5-mar/mle/c19, 6-mar/elb/c30, 7-mar/elb/c32, 8-mar/elb/c33, 9-mar/mal/c2, 10-mar/mal/c6, 11-mar/mal/c8, M-molecular size marker100bp (Invitrogen, Thermofisher scientific, USA), p- positive control, N- negative control.

Table 4.1: Results of PCR analysis of the samples collected for the study

County	Location	No. of samples	No. positive (%)
Marsabit	Laisamis-moile	10	2 (20)
	Nairibu	12	3 (25)
	Malabot	16	4 (25)
	El burumagado	8	3 (38)
	Galas	15	3 (20)
	Total	**61**	**15 (24.6)**
Turkana	Nadapal	11	3 (27)
	Lokolia	30	2 (7)
	Lokore	30	1 (3)
	Total	**71**	**6 (8.4)**
	Overall %	**132**	**21 (15.9)**

4.2 *P. Multocida* capsular typing results

P. multocida capsular typing was done by using primers shown in table 3.2 and the protocol described in section 3.8. The twenty - one (21) DNA identified as *P. multocida*, capsular type E was the only capsular polysaccharide detected by Multiplex PCR. A distinct amplicon of 511bp size on agarose gel denoted the presence of capsular type E for *ecb*J gene as shown in Figure 4.2. Other capsular types A, B, D and F were not detected

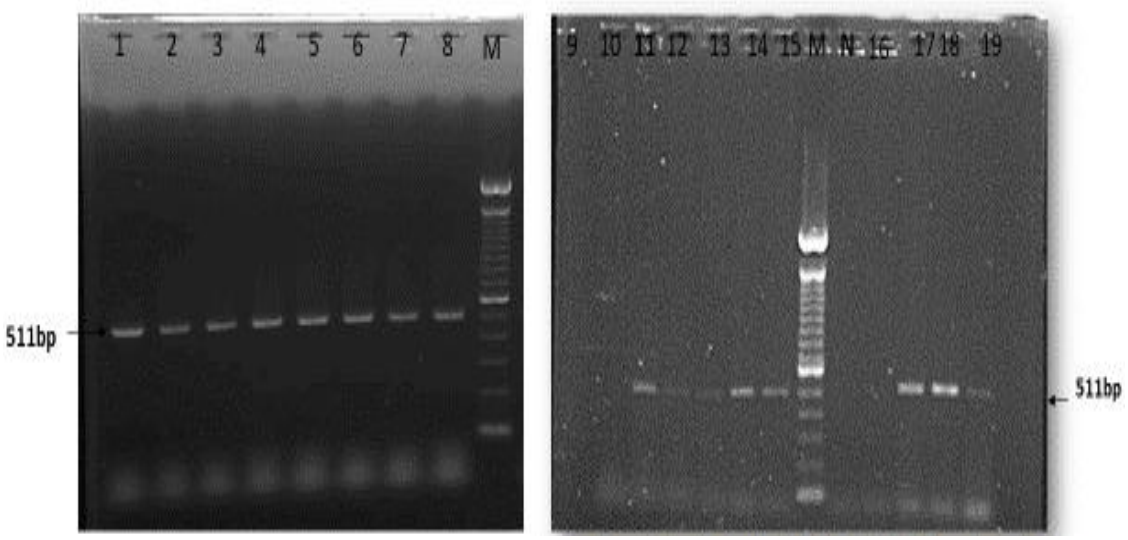


Figure 4.2: Agarose gel showing amplification of 511bp size fragments distinct for capsular type E ecbJ gene. The PCR amplicons were resolved on a 1.5% agarose gel and stained with gel red. 1-Mar/nrb/c3, 2- mar/nrb/c7, 3-mar/nrb/c11, 4-mar/mle/c13, 5-mar/mle/c19, 6-mar/elb/c30, 7-mar/elb/c32, 8-mar/elb/c33, 9-mar/mal/c2, 10-mar/mal/c6, 11-mar/mal/c8, 12-mar/mal/c10, 13- mar/gal/c18, 14-mar/gal/c20, 15-mar/gal/c24, 16-tur/lok/c14, 17-tur/nad/c1, 18-tur/nad/c2, 19-tur/nad/c11, M-molecular size marker100bp, N- negative control.

4.3 Sequence analysis results

Sequence analysis by BLASTn method exhibited a 98% to 100% nucleotide identity to nine (9) homologues related to strain NCTC10323 genome assembly chromosome 1. One (1) homologue related to serovar B2 KMT1 gene partial cds, three (3) homologues related to strain Razi Pm0001 complete genome, one (1) homologue related to strain 20N chromosome complete genome and *P. multocida* subsp. Multocida isolate CD10_34 pm_03 alpha/beta hydrolase gene partial cds respectively, two (2) homologues related to *P. multocida* strain FDAARGOS 218 chromosome complete

genome, strain 375-A/15 chromosome and strain CQ6 chromosome complete genome respectively (Table 4.2). Gene analysis by BLASTp of the protein revealed a 99% to 100% identity indicating that KMT1 gene a transmembrane protein is related to homologues Kmt1, partial *P. multocida*, hydrolase family protein partial *P. multocida*, outer membrane protein partial *P. multocida* and alpha/beta hydrolase partial *P. multocida* subsp. Multocida (Table 4.3) .

BLASTn analysis of the ecbJ gene nucleotides specific for capsular type E had 100% homologues identical to *P. multocida* P1234 region 2 capsule biosynthesis gene cluster partial sequence (Table 4.4). *ecbJ* gene is a capsule biosynthesis gene and BLASTp of capsular type E protein had homologues identical to putative glycosyltransferase EcbJ *P. multocida* (Table 4.5). Figure 4.3 show description of sequences producing significant alignment and nucleotide base pairing for *P. multocida* strain NCT10323 genome assembly chromosome 1 and Figure 4.5 indicate BLASTn description of sequences producing significant alignments to *P. multocida* P1234 region 2 capsule biosynthesis gene cluster partial sequence and nucleotide base pairing. Figure 4.4 show BLASTx description alignment showing sequences producing significant alignments and translated nucleotide to protein for *P. multocida* hydrolase family protein and Figure 4.6 indicate BLASTx alignment showing description of sequences producing significant alignments and translated nucleotide to protein for *P. multocida* putative glycostltrasferase ecbj gene.

Table 4.2: Homologous sequences detected by BLASTn analysis based on segment analysis of *P. multocida KMT1* gene sequences

Isolate ID	Homologous sequences	Identity %
MAR/NRB/C3/5	*p. multocida* strain NCTC10323 genome	99
MAR/NRB/C7/15	*p. multocida* strain NCTC10323 genome	99
MAR/NRB/C11/20	*P. multocida* serovar B2 KMT1 gene	99
MAR/MLE/C13/4	*p. multocida* strain NCTC10323 genome	100
MAR/MLE/C19/1	*p. multocida* strain NCTC10323 genome	100
MAR/ELB/C30/1	*P. multocida* strain Razi Pm0001 complete genome	100
MAR/ELB/C32/2	*P. multocida* strain Razi Pm0001 complete genome	99
MAR/ELB/C33/1.5	*P. multocida* strain 20N chromosome complete genome	99
MAR/MAL/C2/9	*P. multocida* CD10_34 pm_03 alpha/beta hydrolase gene	99
MAR/MAL/C6/9	*p. multocida* strain NCTC10323 genome	100
MAR/MAL/C8/1	*p. multocida* strain NCTC10323 genome	99
MAR/MAL/C10/15	*P. multocida* strain FDAARGOS 218 chromosome complete genome	99
MAR/GAL/C18/5	*P. multocida* strain FDAARGOS 218 chromosome complete genome	100
MAR/GAL/C20/7	*P. multocida* strain Razi Pm0001 complete genome	100
MAR/GAL/C24/6	*P. multocida* strain 375-A/15 chromosome	99
TUR/NAD/C1	*p. multocida* strain NCTC10323 genome	100
TUR/NAD/C2	*P. multocida* strain 375-A/15 chromosome	99
TUR/NAD/C11	*P. multocida* strain CQ6 chromosome complete genome	99
TUR/LOK/C13	*P. multocida* strain NCTC10323 genome	99
TUR/LOK/C14	*P. multocida* strain NCTC10323 genome	99
TUR/LOR/C11	*P. multocida* strain CQ6 chromosome complete genome	100

Key

NRB – Nairibu, MLE – Moile, ELB - El burumagado, MAL – Malabot, GAL – Galas,

NAD – Nadapal, LOK – Lokolia, LOR – Lokore, Mar – Marsabit, Tur – Turkana

Table 4.3: Homologous sequences obtained by BLASTp analysis of *P. multocida* KMT1 gene sequences.

Isolate ID	Homologous sequences	Identity %
TUR/LOK/C13	Kmt1	100
TUR/NAD/C2	hydrolase family protein	100
MAR/GAL/C20	hydrolase family protein	100
MAR/MAL/C8	Kmt1	99
MAR/ELB/C32	outer membrane protein	100
MAR/NRD/C7	hydrolase family protein	100
MAR/NRD/C3	hydrolase family protein	100
MAR/NRB/C11	hydrolase family protein	100
MAR/MLE/C13	Kmt1	100
MAR/MLE/C19	Kmt1	99
MAR/ELB/C30	outer membrane protein	99
MAR/ELB/C33	outer membrane protein	100
MAR/MAL/C2	alpha/beta hydrolase	100
MAR/MAL/C10	alpha/beta hydrolase	100
MAR/MAL/C6	Kmt1	100
MAR/GAL/C18	hydrolase family protein	100
MAR/GAL/C24	alpha/beta hydrolase	100
TUR/NAD/C1	outer membrane protein	100
TUR/NAD/C11	Kmt1	100
TUR/LOK/C14	alpha/beta hydrolase	100

Key

NRB – Nairibu, MLE – Moile, ELB - El burumagado, MAL – Malabot, GAL – Galas,

NAD – Nadapal, LOK – Lokolia, LOR – Lokore, Mar – Marsabit, Tur – Turkana.

Table 4.4: Homologous sequences for *P. multocida* capsular type E detected by BLASTn analysis based on *ecbJ* gene

Isolate ID	Homologous sequences	Identity %
CapMAR/MAL/C8	P. multocida P1234 region 2 capsule biosynthesis gene	100
CapTUR/LOK/C13	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/GAL/C20	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/NRB/C11	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapTUR/NAD/C1	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/MLE/C19	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/ELB/C32	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapTUR/LOK/C14	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapTUR/LOR/C11	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapTUR/NAD/C2	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/NRB/C3	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/MLE/C13	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/MAL/C6	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/GAL/C24	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/NRB/C7	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/ELB/C33	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/MAL/C2	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/MAL/C10	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
CapMAR/GAL/C18	*P. multocida* P1234 region 2 capsule biosynthesis gene	100
capMAR/ELB/C30	*P. multocida* P1234 region 2 capsule biosynthesis gene	100

Key

NRB – Nairibu, MLE – Moile, ELB - El burumagado, MAL – Malabot, GAL – Galas,

NAD – Nadapal, LOK – Lokolia, LOR – Lokore, Mar – Marsabit, Tur – Turkana

Table 4.5: Homologous sequences for *P. multocida* capsular type E detected by BLASTp analysis based on *ecbJ* gene

Isolate ID	Homologous sequences	Identity %
CapMAR/MAL/C8	putative glycosyltransferase EcbJ	99
CapTUR/LOK/C13	putative glycosyltransferase EcbJ	100
CapMAR/GAL/C20	putative glycosyltransferase EcbJ	100
CapMAR/NRB/C11	putative glycosyltransferase EcbJ	99
CapTUR/NAD/C1	putative glycosyltransferase EcbJ	99
CapMAR/MLE/C19	putative glycosyltransferase EcbJ	100
CapMAR/ELB/C32	putative glycosyltransferase EcbJ	100
CapTUR/LOK/C14	putative glycosyltransferase EcbJ	100
CapTUR/LOR/C11	putative glycosyltransferase EcbJ	95
CapTUR/NAD/C2	putative glycosyltransferase EcbJ	100
CapMAR/NRB/C3	putative glycosyltransferase EcbJ	100
CapMAR/MLE/C13	putative glycosyltransferase EcbJ	90
CapMAR/MAL/C6	putative glycosyltransferase EcbJ	97
CapMAR/GAL/C24	putative glycosyltransferase EcbJ	97
CapMAR/NRB/C7	putative glycosyltransferase EcbJ	100
CapMAR/ELB/C33	putative glycosyltransferase EcbJ	82
CapMAR/MAL/C2	putative glycosyltransferase EcbJ	100
CapMAR/MAL/C10	putative glycosyltransferase EcbJ	97
CapMAR/GAL/C18	putative glycosyltransferase EcbJ	99
capMAR/ELB/C30	putative glycosyltransferase EcbJ	100

Key

NRB – Nairibu, MLE – Moile, ELB - El burumagado, MAL – Malabot, GAL – Galas, NAD – Nadapal, LOK – Lokolia, LOR – Lokore, Mar – Marsabit, Tur – Turkana

A

Sequences producing significant alignments Download Manage Columns Show 10

select all *0 sequences selected* GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Pasteurella multocida strain NCTC10323 genome assembly, chromosome: 1	809	809	98%	0.0	99.11%	LR134532.1
Pasteurella multocida strain 375-A/15 chromosome	809	809	98%	0.0	99.11%	CP023305.1
Pasteurella multocida strain Razi_Pm0001, complete genome	809	809	98%	0.0	99.11%	CP017961.1
Pasteurella multocida strain Jhakhrana hydrolase family protein (kmt) gene, partial cds	804	804	98%	0.0	98.89%	KX348143.1
Pasteurella multocida strain NCTC10382 genome assembly, chromosome: 1	798	798	98%	0.0	98.67%	LS483473.1
Pasteurella multocida strain FDAARGOS_218 chromosome, complete genome	798	798	98%	0.0	98.67%	CP020405.2
Pasteurella multocida subsp. gallicida strain CSWRI/AH/PmAg16 hydrolase family protein (KMT1) gene, partial cds	798	798	98%	0.0	98.67%	KY825088.1
Pasteurella multocida strain CSWRI/AH/PmA16 hydrolase family protein (KMT1) gene, partial cds	798	798	98%	0.0	98.67%	KY825086.1
Pasteurella multocida OH1905, complete genome	798	798	98%	0.0	98.67%	CP004392.1

B

Pasteurella multocida strain NCTC10323 genome assembly, chromosome: 1

Sequence ID: LR134532.1 Length: 2330363 Number of Matches: 1

Range 1: 1687195 to 1687645 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
809 bits(438)	0.0	447/451(99%)	1/451(0%)	Plus/Plus

```
Query  7        AAACG-ACTCGCCCCTTTTTGTTTCATTTGGACTGACACGATTAAACCGTTGAACACGAA  65
               ||||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687195  AAACGAACTCGCCACTTTTTGTTTCATTTGGACTGACACGATTAAACCGTTGAACACGAA  1687254

Query  66       GAAAAAGACCAAAATAGGTAACCAATACACGATAAATAAATTAAACCGCTCTGTCGTTAA  125
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687255  GAAAAAGACCAAAATAGGTAACCAATACACGATAAATAAATTAAACCGCTCTGTCGTTAA  1687314

Query  126      TGGCTTCAATAATGGCCATAAGAAACGTAACTCAACATGGAAATATTGATAAATCAGACT  185
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687315  TGGCTTCAATAATGGCCATAAGAAACGTAACTCAACATGGAAATATTGATAAATCAGACT  1687374

Query  186      GACAAGGAAATATAAACCGGCAAATAACAATAAGCTGAGTAATAAATAACGTCCAATCAG  245
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1687375  GACAAGGAAATATAAACCGGCAAATAACAATAAGCTGAGTAATAAATAACGTCCAATCAG  1687434
```

Figure 4.3: BLASTn description of sequences producing significant alignments and nucleotide base pairing.

A

Sequences producing significant alignments Download Manage Columns Show 100

select all *98 sequences selected* GenPept Graphics

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
hydrolase family protein [Pasteurella multocida]	196	196	98%	1e-61	98.00%	AOO85324.1
Kmt1 [Pasteurella multocida]	196	196	98%	1e-61	97.33%	ARK19646.1
Kmt1 [Pasteurella multocida]	196	196	98%	1e-61	97.33%	AJO54374.1
alpha/beta hydrolase family protein [Pasteurella multocida]	196	196	98%	1e-61	97.33%	AKA60237.1
Kmt1 [Pasteurella multocida]	195	195	98%	4e-61	97.33%	AJO54370.1
Kmt1 [uncultured Pasteurella sp.]	195	195	98%	4e-61	97.33%	ACG56673.1
Kmt1 protein [Pasteurella multocida]	194	194	98%	6e-61	96.67%	AJB29544.1
Kmt1 [Pasteurella multocida]	195	195	98%	1e-60	97.33%	ACT10301.1
hydrolase family protein [Pasteurella multocida]	192	192	98%	5e-60	96.67%	ART92622.1

B

hydrolase family protein, partial [Pasteurella multocida]

Sequence ID: AOO85324.1 Length: 151 Number of Matches: 1

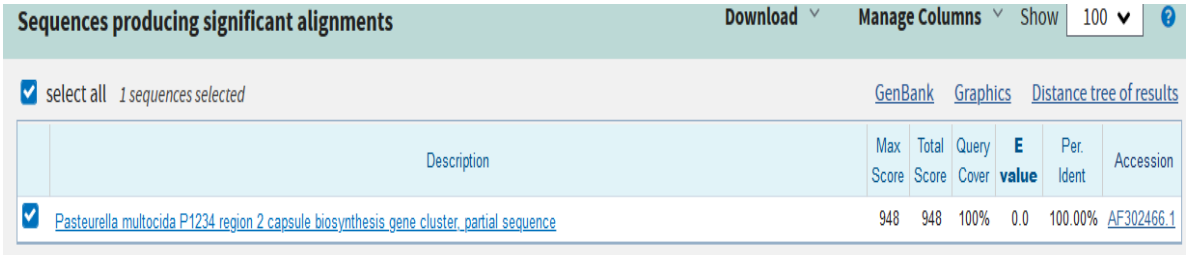
Range 1: 1 to 150 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
196 bits(499)	1e-61	Compositional matrix adjust.	147/150(98%)	147/150(98%)	0/150(0%)	-2

```
Query  455  PLITQWGGANEPIAAKLSFMPLEMGNgiilwlvvsglvgsllfglWQRKAQFCWAEFGVL  276
            PL TQWGGANEPIAAKLSFMPLEMGNGIILWLVVSGLVGSLLFGLWQRKAQFCWAEFGVL
Sbjct  1    PLFTQWGGANEPIAAKLSFMPLEMGNGIILWLVVSGLVGSLLFGLWQRKAQFCWAEFGVL  60

Query  275  SQSASLTTAQligrylllslllfaglyflvsLIYQYFHVELRFLWPLLKPLTTERFNLFI  96
            SQSASLTTAQLIGRYLLLSLLLFAGLYFLVSLIYQYFHVELRFLWPLLKPLTTERFNLFI
Sbjct  61   SQSASLTTAQLIGRYLLLSLLLFAGLYFLVSLIYQYFHVELRFLWPLLKPLTTERFNLFI  120

Query  95   VYWLPILVFFFVFNGLIVSVQMKQKGASRF  6
            VYWLPILVFFFVFNGLIVSVQMKQK AS F
Sbjct  121  VYWLPILVFFFVFNGLIVSVQMKQKVASSF  150
```

Figure 4.4: BLASTx description alignment showing sequences producing significant alignments and translated nucleotide to protein

A



B

Pasteurella multocida P1234 region 2 capsule biosynthesis gene cluster, partial sequence

Sequence ID: AF302466.1 **Length:** 11745 **Number of Matches:** 1

Range 1: 4389 to 4901 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
948 bits(513)	0.0	513/513(100%)	0/513(0%)	Plus/Plus

```
Query  1     CCGCAGAAAATTATTGACTCTCTAGTATCAGGCGTACCTGTAATTTCTACAAGGAATACA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4389  CCGCAGAAAATTATTGACTCTCTAGTATCAGGCGTACCTGTAATTTCTACAAGGAATACA  4448

Query  61    TATTTAGAAGAAAAATTTAAAGACATCATATTATTTATTGATTCCCCAACTCAACTTCAT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4449  TATTTAGAAGAAAAATTTAAAGACATCATATTATTTATTGATTCCCCAACTCAACTTCAT  4508

Query  121   GAAATTGTAGAAAAATATGAAAATGATCGTTGGGCACATGCTCGCTTAGCGCACAAAGGA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4509  GAAATTGTAGAAAAATATGAAAATGATCGTTGGGCACATGCTCGCTTAGCGCACAAAGGA  4568

Query  181   TATCGATTTGTAATGAATAATTTTTCCACACATAGTCTCAAAGCTAACTTACAAAGAGAA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4569  TATCGATTTGTAATGAATAATTTTTCCACACATAGTCTCAAAGCTAACTTACAAAGAGAA  4628
```

Figure 4.5: Blastn description of sequences producing significant alignments and nucleotide base pairing for capsular EcbJ gene

A

Sequences producing significant alignments Download Manage Columns Show 100

select all *0 sequences selected* GenPept Graphics

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
putative glycosyltransferase EcbJ [Pasteurella multocida]	348	348	100%	5e-114	100.00%	AAK17912.1
glycosyltransferase [Acinetobacter sp. Ac_877]	127	127	99%	2e-30	40.35%	WP_152873934.1
glycosyltransferase [Acinetobacter equi]	122	122	99%	6e-29	40.12%	WP_054580589.1
glycosyltransferase [Mannheimia haemolytica]	104	104	98%	1e-22	34.52%	WP_061888082.1
hypothetical protein C4O91_00325 [Mannheimia haemolytica]	103	103	98%	3e-22	34.52%	AWW65499.1
glycosyltransferase [Mannheimia haemolytica]	103	103	98%	3e-22	34.52%	WP_006253521.1
MULTISPECIES: glycosyltransferase [unclassified Alcaligenes]	102	102	99%	7e-22	33.92%	WP_009463505.1

B

putative glycosyltransferase EcbJ [Pasteurella multocida]

Sequence ID: AAK17912.1 Length: 705 Number of Matches: 1

Range 1: 382 to 552 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
348 bits(894)	5e-114	Compositional matrix adjust.	171/171(100%)	171/171(100%)	0/171(0%)	+1

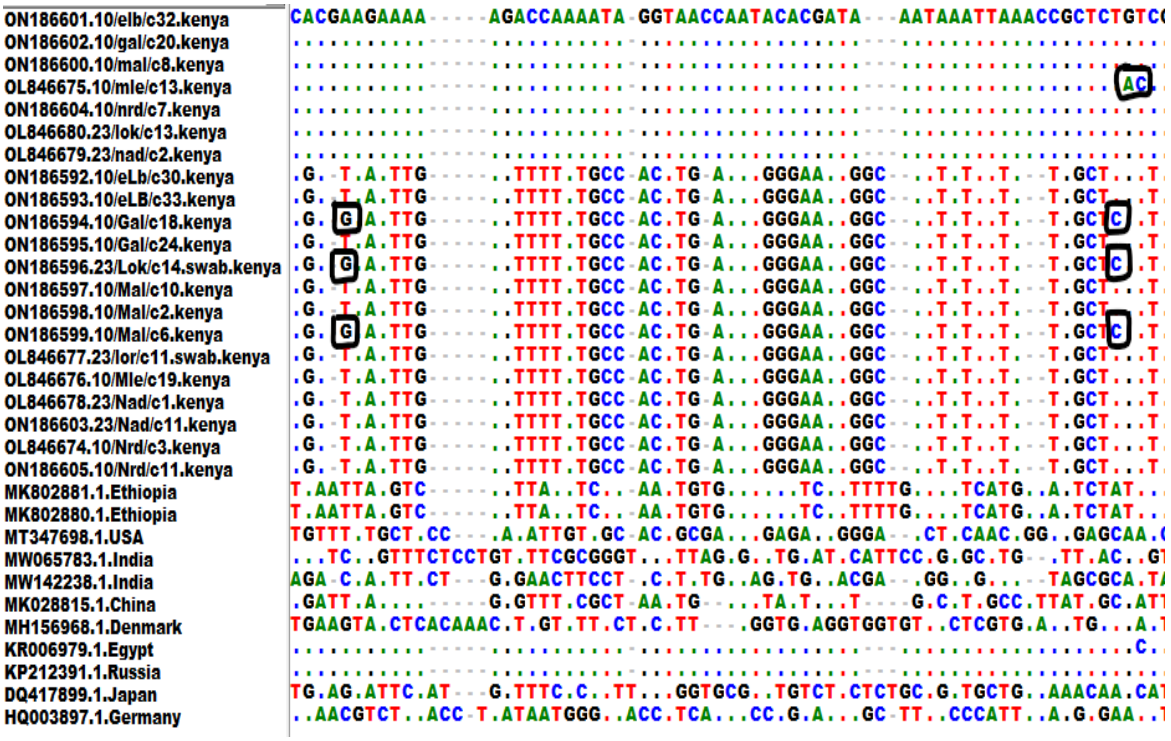
```
Query  1    PQKIIDSLVSGVPVISTRNTYLEEKFKDIILFIDSPTQLHEIVEKYENDRWAHARLAHKG  180
            PQKIIDSLVSGVPVISTRNTYLEEKFKDIILFIDSPTQLHEIVEKYENDRWAHARLAHKG
Sbjct  382  PQKIIDSLVSGVPVISTRNTYLEEKFKDIILFIDSPTQLHEIVEKYENDRWAHARLAHKG  441

Query  181  YRFVMNNFSTHSLKANLQREICGKAINSDNNPLISIIMASMREFYIDRIITNISRQTYKN  360
            YRFVMNNFSTHSLKANLQREICGKAINSDNNPLISIIMASMREFYIDRIITNISRQTYKN
Sbjct  442  YRFVMNNFSTHSLKANLQREICGKAINSDNNPLISIIMASMREFYIDRIITNISRQTYKN  501

Query  361  KELIIVTQNFSEIGLKKLQEKLEKIDDLVNFKIIVNNSEDTLGERQNQAAS  513
            KELIIVTQNFSEIGLKKLQEKLEKIDDLVNFKIIVNNSEDTLGERQNQAAS
Sbjct  502  KELIIVTQNFSEIGLKKLQEKLEKIDDLVNFKIIVNNSEDTLGERQNQAAS  552
```

Figure 4.6: Blastx alignment showing description of sequences producing significant alignments and translated nucleotide to protein for Ecbj gene

4.3.1 Multiple sequence alignment of *P. multocida* isolates with strains from other regions

Multiple sequence alignment of Kenyan isolates with genebank retrieved sequences revealed that most Kenyan isolates were conserved except five isolates. Four (4) Kenyan isolates had base differences from the other isolates as indicated in table 4.6. Ethiopian isolates were closely related to Kenyan isolates. USA, India, Denmark, Egypt, Russia, Japan and Germany isolates were different from Kenyan isolates by showing nucleotide variation indicating that they are genetically diverse (Table 4.6).

Table 4.6: Sequences aligned by clustalw showing regions with base differences. The dots represent the conserved regions.



4.3.2 Phylogenetic analysis

4.3.2.1 Phylogenetic analysis of Kenyan *P. multocida* isolates

The aligned *P. multocida* and GeneBank retrieved sequences were run on MEGA X version 10.1 (Kumar *et al*., 2018) for the generation of phylogenetic tree. A phylogenetic tree inferred using maximum likelihood method and based on the KMT1 gene was used to determine whether the 21 *P. multocida* sequences detected in this study were genetically related to those strains from different regions of the world (Fig. 4.7). Eleven Kenyan strains formed clade 1 and another three strains formed cade 2. A strain from the USA formed an outgroup to clades 1 and 2. Ethiopian strains formed clade 4. Strains from Egypt, Russia and Kenya (all single strains) formed clade 5. Another six Kenyan strains formed cade 6 and one Indian strain formed clade 7 followed by clade 8 formed by strains from Denmark, Japan, Germany, India and China. The results of the phylogenetic analysis indicate that the Kenyan *P. multocida* strains are diverse.

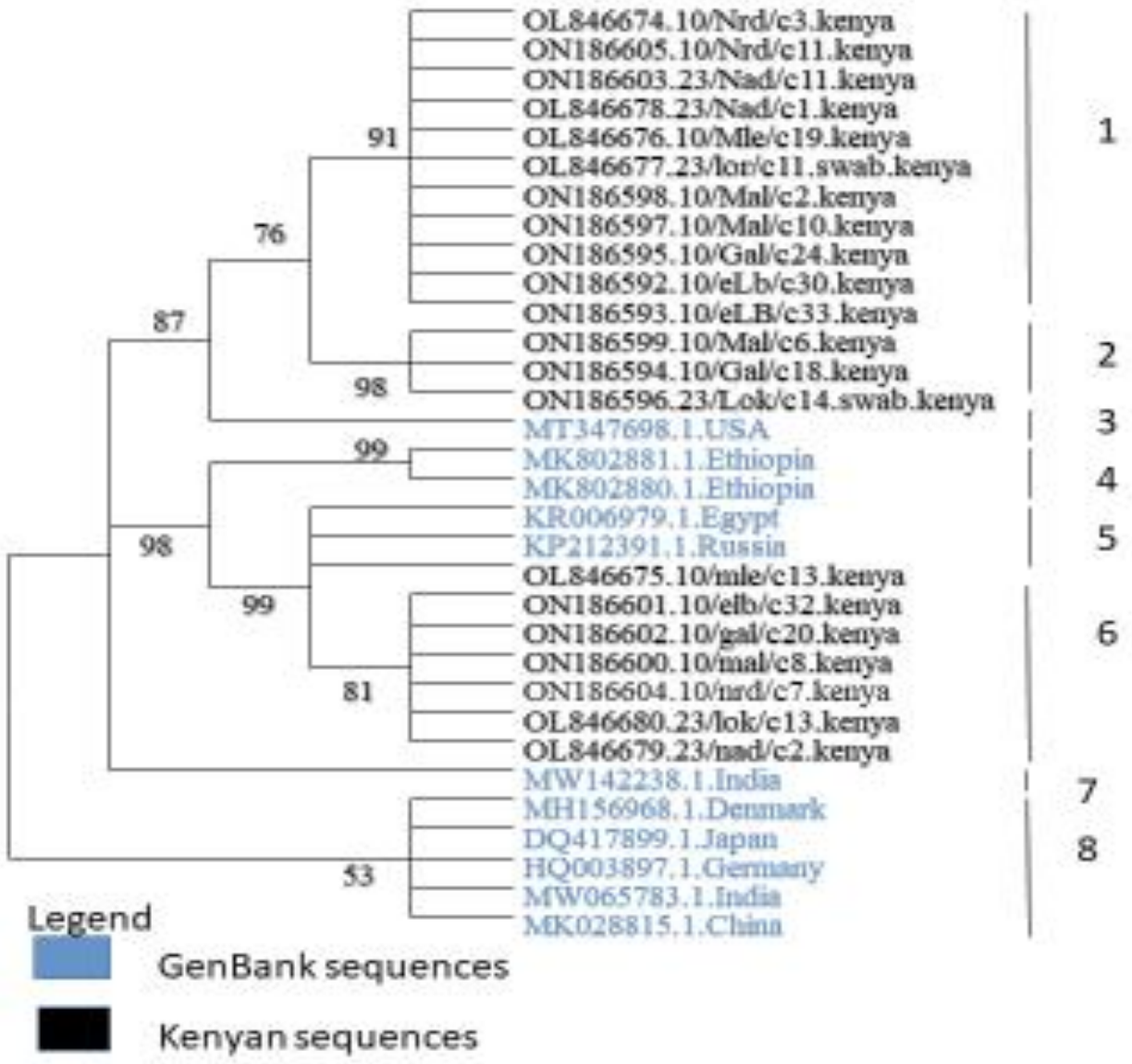


Figure 4.7: Phylogenetic tree based on kmt1 gene was inferred by using maximum likelihood method and Tamura-Nei model. The tree obtained by Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model. Phylogeny was tested with 1000 bootstrap replications. Evolutionary analyses were conducted in MEGA X version 10.1 software. Numbers indicates clades. Bootstraps are shown at the nodes.

4.4 Determination of genetic diversity

Phylogenetic analysis of the *P. multocida* sequences formed different clusters (Figure 4.7) revealing that there were different strain of *P. multocida* circulating in Marsabit and Turkana counties. Capsular group analysis by Multiplex PCR detected capsular group E which could be the cause of camel Pasteurellosis in Marsabit and Turkana counties.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

A number of fungi, bacteria, viruses, and parasites may possibly cause respiratory infection in camels, but the definitive etiology has not yet been determined (Tahamtan *et al.*, 2016). *P. multocida* cause septicemic disease attributed to high morbidity and mortality in sheep, goat, cattle, poultry and eventual economic losses (OIE, 2012; Elsayed *et al*., 2021). *P. multocida* strains are classified into five capsular serogroups A, B, D, E and F (Carter, 1955, Carter, 1961, Rimler and Rhoades, 1987). The biosynthetic loci of the polysaccharide capsule of *P. multocida* have been fully characterized allowing the creation of a PCR-based system that assigns isolates to the five capsule serovars (Townsend *et al*., 2001). This typing system involves the hyaD-hyaC gene (involved with hyaluronic acid synthesis) for capsular serogroup A, the dcbF gene (involved in heparin synthesis) for capsular serogroup D, the fcbD gene (involved in chondroitin production) for capsular serogroup F and the bcbD and ecbJ genes (both involved in glycosyltransferase synthesis) for capsular serogroups B and E respectively (Townsend *et al*., 2001).

This study was done to investigate the active blood and nasopharyngeal carrier state for *P. multocida* in camels in Marsabit and Turkana counties in Kenya. The study applied PCR assay build on primers pair targeting *KMT1* gene which is a transmembrane protein mostly used for initial *P. multocida* species identification of field strains regardless of capsular group (Townsend *et al*., 1998). The amplification of positive

control and field strains was very specific to *P. multocida* species. The specific primer pair targeting *KMT1* gene was employed to overcome tediousness together with the challenges of conventional methods used for detection of *P. multocida* and to rapidly confirm the results (Townsend *et al*., 1998). The PCR assay certified the specificity and sensitivity with correct results.

PCR assay by Townsend *et al.* (1998) was slightly modified and successfully optimized to detect *P. multocida* and its capsular types circulating in Marsabit and Turkana counties. PCR products run on agarose gel produced a distinct 460bp size corresponding to *P. multocida* strain (Townsend *et al.,* 1998, Vidhya *et al*., 2007, Tahamtan *et al.,* 2016). In the current study, *P. multocida* species were detected from the blood and nasal swabs of clinically sick camels. It was confirmed that the primer pair targeting *KMT1* gene amplified 18.6% (19/102) of the total whole blood samples and 6.7% (2/30) of nasal swabs indicating that nasal carriage was not abundant. The difference could have been due to the way the samples were handle from the field to the laboratory. These study results confirm the findings obtained in another study that reported amplicon product of 460bp size supporting the application of PCR for rapid and specific amplification of *KMT1* gene for confirmation of *P. multocida* (Townsend *et al.,* 2001, Jabbari *et al*., 2014, Jakeen *et al*., 2015, Khalid *et al*., 2016, Tahamtan *et al*., 2016). Marsabit County had high *P. multocida* infection than Turkana. The difference in *P. multocida* infection in the counties could have been due to failure to provide camels with appropriate nutrition, proper treatment and vaccination and minimizing herding of camels in crowded areas especially in wet conditions to slow the fast spread of *P. multocida* infection.

Clarification of genes for capsule biosynthesis has made the design of Multiplex PCR for laboratory typing of *P. multocida* possible based on specific gene sequences (Teleb *et al*., 2019). Multiplex PCR applied for capsular typing revealed that all positive *P. multocida* field strains belonged to capsular group E confirming that the *P. multocida* capsular groups are not diversified in both counties. This finding is in disagreement with previous studies which report that capsular group B is the characteristic capsular group detected in camels (Vidhya *et al*., 2007, Kebede and Gelaye 2010, Tahamtan *et al.*, 2016,) while another study done by Gluecks *et al*. (2017) attribute capsular group A and D as the cause of Pasteurellosis in camels.

This finding demonstrates that it is not clear which capsular group is responsible for Pasteurellosis in camels. It further suggest that different capsular groups can effectively cause Pasteurellosis depending on some prevailing factors. Furthermore, the organization of capsular type B capsule remains elusive (Townsend *et al.*, 1998) and the role it plays as the agent causing Pasteurellosis in camels remain unknown and need further study.

Blast search of the *P. multocida* field strain sequences matched with high similarity to accession number LR134532.1 and for capsular group E accession number AF302466. Blastx search of *P. multocida* sequences for protein matched with high score of 98% to accession number A0085324.1 hydrolase family protein, partial *p. multocida,* Kmt1, partial *P. multocida,* outer membrane protein, partial *P. multocida* and alpha/beta hydrolase, partial *P. multocida* subsp. multocida (figure 4.3) and 100% to accession number AAK17912.1 putative glycosyltransferase *EcbJ P. multocida* for capsular group E (figure 4.5) (Stephen *et al*., 1997). Upon alignment of the Kenyan isolates sequences

with eleven (11) sequences retrieved from GenBank using BioEdit software version 7.2 (Hall**,** 1999, Hall *et al***.,** 2011) revealed that the Kenyan isolates had regions that were highly conserved except five isolates. There were few regions with base differences indicating mutations. Marsabit County had three isolates with base variation while Turkana County had one isolate with base differences (Table 4.6). This indicated that the Kenyan isolates had not mutated a lot in different camels. Kenyan trains from Marsabit and Turkana counties formed cluster 1, 2 and 6 indicating that there was different *P. multocida* strain circulating in Marsabit and Turkana Counties in Kenya. Strains from other regions formed cluster 3, 4, 5, 7, and 8. Only one capsular group E was detected.

Blast search of capsular group E sequences matched with accession number AF302466 for P.m. P1234 region 2 capsule biosynthesis gene cluster. Furian *et al.* (2014) noted that there is definite closeness between *P. multocida* capsular group, specific diseases and species which is also influenced by distribution of capsular groups. Furian *et al.* (2014) further reported that there has been deviation from these relationships that have been exhibited in recent years. Example is the pig pneumonia brought about by capsular type D but initially was linked to capsular group A. Dziva *et al*. (2000) reported that there is a likelihood of one capsular group to transmit infection to different species and cause different disease manifestation. Dziva *et al*. (2000) gives an example of capsular group A that causes fowl cholera, calf pneumonia, pneumonia in pigs and infections in upper respiratory tract in dogs and cats.

Further Dziva *et al*. (2000) report that Pasteurellosis has been associated with capsular group E in Africa. The variation rate of *P. multocida* in camel may result from different sample size, method of sample collection and differences amongst geographical

areas of Marsabit and Turkana. Close herding, overwork, limited food supply, and wet climatic conditions are stresses that seem to accelerate the spread of the infection thus there is an urgent need to identify the source of respiratory diseases in camels to organize better control strategies. In this study 21/132 field strains were found to be *P .multocida* and in capsular typing were found to be capsular type E. Multiplex PCR applied provided a quick replacement to available techniques for the identification of capsulated *P. multocida* because it provides the concomitant, quick detection of genes and strain typing (Dziva *et al*., 2000; Furian *et al.*, 2014).

5.2 Conclusions

i There is *P. multocida* strain circulating in Marsabit and Turkana Counties which is of capsular polysaccharide group E *ecbJ* gene.

ii Multiplex PCR provided a simple, rapid, sensitive and reliable method for molecular typing of *P. multocida* capsular polysaccharides.

iii The *P. multocida* capsular type detected in Marsabit and Turkana Counties is of capsular group E.

5.3 Recommendations

i Further characterization of *P. multocida* and capsular groups is necessary to provide definitive answers to the real cause of camel Pasteurellosis.

ii The research recommend that a large sample size covering large geographical area be used to study the capsular groups present in endemic areas of northern Kenya.

iii Monitoring the capsular types of *P. multocida* strains circulating in a specific geographical region may be important in the formulation of vaccines.

iv PCR assay targeting *KMT1* gene is recommended for large-scale surveillance of *P. multocida.*

5.4 Suggestions for further studies

i Presence of *P. multocida* should be evaluated in others counties.

ii Determine the *P. multocida* capsular types circulating in other counties

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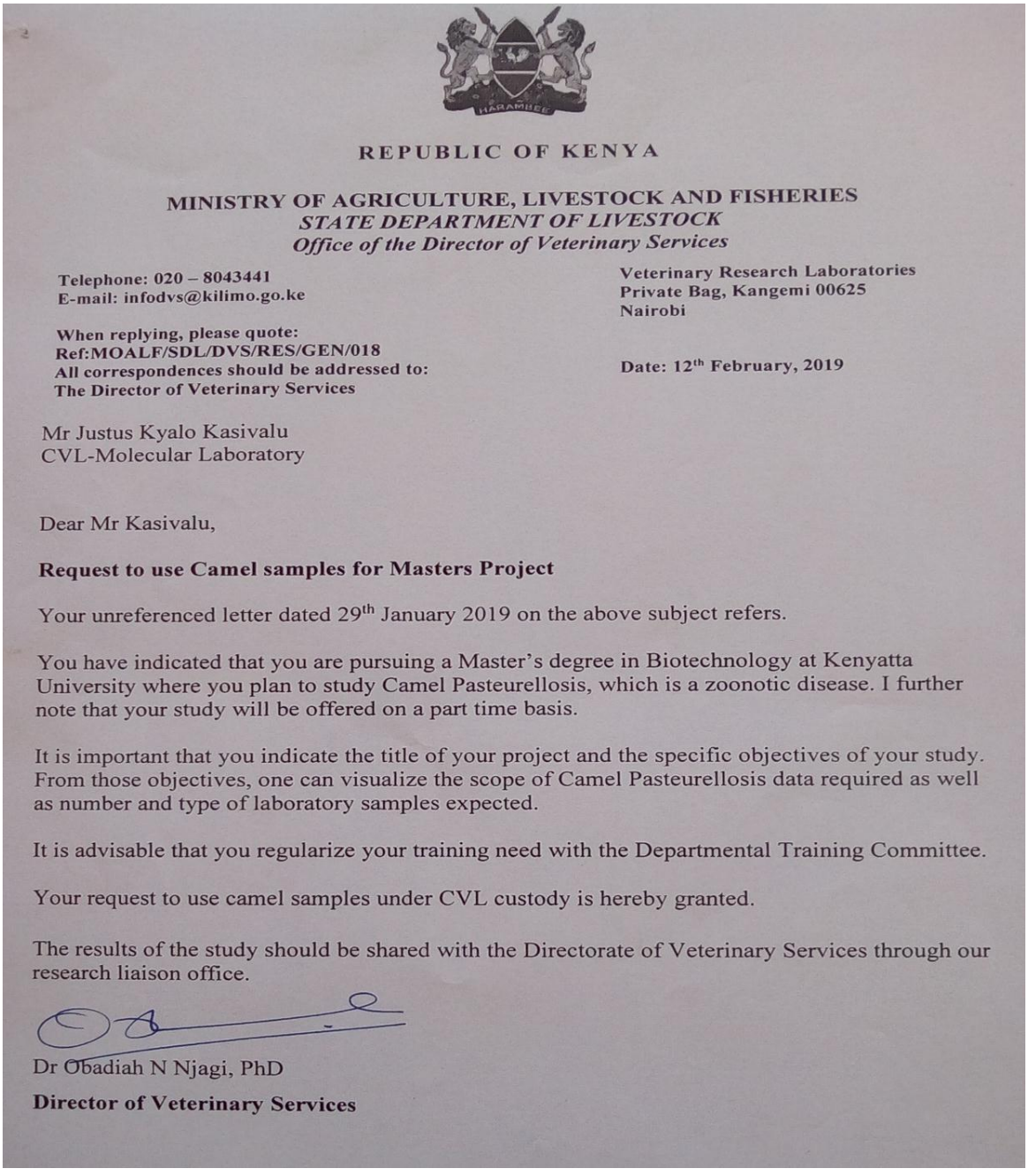
Appendix I:

Relevant research authorization form to carry out the research

Authority from Director of Veterinary services (DVS)



REPUBLIC OF KENYA

MINISTRY OF AGRICULTURE, LIVESTOCK AND FISHERIES
STATE DEPARTMENT OF LIVESTOCK
Office of the Director of Veterinary Services

Telephone: 020 – 8043441
E-mail: infodvs@kilimo.go.ke

Veterinary Research Laboratories
Private Bag, Kangemi 00625
Nairobi

When replying, please quote:
Ref:MOALF/SDL/DVS/RES/GEN/018
All correspondences should be addressed to:
The Director of Veterinary Services

Date: 12th February, 2019

Mr Justus Kyalo Kasivalu
CVL-Molecular Laboratory

Dear Mr Kasivalu,

Request to use Camel samples for Masters Project

Your unreferenced letter dated 29th January 2019 on the above subject refers.

You have indicated that you are pursuing a Master's degree in Biotechnology at Kenyatta University where you plan to study Camel Pasteurellosis, which is a zoonotic disease. I further note that your study will be offered on a part time basis.

It is important that you indicate the title of your project and the specific objectives of your study. From those objectives, one can visualize the scope of Camel Pasteurellosis data required as well as number and type of laboratory samples expected.

It is advisable that you regularize your training need with the Departmental Training Committee.

Your request to use camel samples under CVL custody is hereby granted.

The results of the study should be shared with the Directorate of Veterinary Services through our research liaison office.

Dr Obadiah N Njagi, PhD
Director of Veterinary Services

Appendix II

Sequences used for the study

P. multocida Field strains sequences

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P. multocida genebank sequences

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