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MSc. Dissertation

Virulence attributes and antimicrobial profile of *Pasteurella multocida* isolated from Pneumonic Goats in Northern Tanzania

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VIRULENCE ATTRIBUTES AND ANTIMICROBIAL PROFILE OF *PASTEURELLA MULTOCIDA* ISOLATED FROM PNEUMONIC GOATS IN NORTHERN TANZANIA

A Dissertation Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Applied Microbiology of Sokoine University of Agriculture. Morogoro, Tanzania.

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EXTENDED ABSTRACT

Pneumonic pasteurellosis, stemming from *Pasteurella multocida*, poses a significant economic threat to small-scale goat farmers in northern Tanzania. This study aimed to assess the prevalence of antimicrobial resistance and virulence genes within *Pasteurella multocida* isolates collected from goats displaying clinical symptoms of the disease. A total of 200 nasal swab samples were obtained from goats exhibiting signs of pneumonic pasteurellosis. In the laboratory, these samples underwent culture on blood agar enriched with 5% horse blood at 37°C and 5% carbon dioxide for 24-48 hours. Isolates displaying characteristics resembling *Pasteurella multocida* were subjected to gram staining and biochemical tests, including indole, oxidase, and catalase. Among the presumptive *Pasteurella multocida* isolates, 10 were initially identified, later confirmed using specific molecular primers, Pm0762 and Pm1231, with only five isolates ultimately confirming the presence of either or both genes. The confirmed isolates were then subjected to susceptibility testing against ten antibiotics. Additionally, screening for the presence of virulence genes, including *ptfhA, ptfA, fimA, exbD*, and *exbB*, was conducted. Moreover, antimicrobial resistance genes (*sul1, sul2, blaTEM, aac(3)-1, tetA,* and *acrA*) were investigated. Out of the 200 analyzed nasal swab samples, *Pasteurella multocida* was isolated in only 2.5%. This seemingly low prevalence may be attributed to the larger sample size utilized in this study compared to prior investigations or the influence of geographic and environmental factors. Serogrouping revealed that 20% of the isolates belonged to serogroup A, while a substantial 80% defied classification, posing challenges for surveillance and control efforts. Further analysis uncovered the presence of virulence genes crucial for iron acquisition (*exbD* found in 100% of isolates) and adhesion (*ptfA* in 60%), pivotal for bacterial survival and respiratory tract infection. However, despite their significance, all isolates exhibited resistance to a broad spectrum of antimicrobials, including commonly used drugs such as ampicillin, amoxicillin and erythromycin. Resistance extended to cephalosporins (cefotaxime), sulfamethoxazole/trimethoprim, and even macrolides. This widespread resistance underscores the need for judicious antibiotic use in veterinary medicine to curb the

emergence of multidrug-resistant strains. Notably, susceptibility was retained against gentamicin and ciprofloxacin, suggesting potential alternative treatment options. Nevertheless, the prevalence of multidrug resistance across all isolates (100%) emphasizes the urgency for innovative strategies to combat pneumonic pasteurellosis. Additionally, the identification of antimicrobial resistance genes like *blaTEM* and *sul1* in some isolates further accentuates the evolving threat of resistance. Moreover, the prevalence of untypable strains underscores the necessity for further research to understand their implications and devise alternative typing methodologies for effective control. In summary, this study reveals a concerning landscape of antimicrobial resistance and virulence in *Pasteurella multocida* among goats in northern Tanzania. Addressing challenges such as low isolation rates, untypable strains, and widespread resistance necessitates a multifaceted approach involving prudent antibiotic use, exploration of novel treatment modalities and diagnostics, and collaboration among public health authorities, veterinarians, and livestock producers. Only through concerted efforts can we effectively combat pneumonic pasteurellosis and safeguard the health and livelihoods of small-scale goat farmers in the region.

Keywords: *Pasteurella multocida*, Pneumonic pasteurellosis, Goat, Tanzania

IKISIRI KUU

Ugonjwa wa mapafu wa nimonia unaosababishwa na bakteria *Pasteurella multocida*, huwa tishio kubwa kiuchumi kwa wafugaji wa mbuzi wa kiwango kidogo kaskazini mwa Tanzania. Utafiti huu ulichunguza kiwango cha upinzani wa antimicrobial na jeni za ukali kwenye maumbile ya *Pasteurella multocida* kutoka kwa mbuzi wanaoonesha dalili za kliniki za ugonjwa huo.

Pasteurella multocida iligunduliwa kwenye asilimia 2.5 tu ya sampuli za uchunguzi wa pua zilizochunguzwa (5 kati ya 200). Ingawa hii inaonyesha kiwango cha chini cha kutokea, inaweza kuwa ni kutokana na ukubwa wa sampuli iliyotumiwa ikilinganishwa na utafiti uliopita au ushawishi wa sababu za kijiografia na mazingira. Uchunguzi wa serogroup ulifunua matokeo ambayo hayajapokelewa awali katika eneo hilo: asilimia 20 ya maumbile yalikuwa yanamilikiwa na serogroup A, huku asilimia 80 zikibaki bila aina, hivyo kuwa changamoto katika juhudi za ufuatiliaji na udhibiti. Uchunguzi zaidi uligundua uwepo wa jeni za ukali zinazohusiana na upatikanaji wa chuma (exbD kwa asilimia 100 ya maumbile) na kushikamana (ptfA kwa asilimia 60). Jeni hizi ni muhimu kwa kuishi na uwezo wa bakteria kuingiza ugonjwa kwenye mfumo wa upumuaji. Maumbile yote yalionyesha upinzani kwa wigo mpana wa antimicrobials, ikiwa ni pamoja na dawa zinazotumiwa kawaida kama ampicillin, amoxicillin, na erythromycin. Upinzani pia ulionekana dhidi ya cephalosporins (cefotaxime), sulfamethoxazole/trimethoprim, na hata macrolides. Upinzani huu mkubwa unasisitiza haja ya haraka ya matumizi sahihi ya antibiotics katika tiba ya mifugo ili kupunguza maendeleo ya vimelea vya upinzani. Kwa kuvutia, maumbile hayakuonyesha upinzani kwa gentamicin na ciprofloxacin, hivyo kutoa chaguzi mbadala za matibabu. Hata hivyo, uwepo wa upinzani wa wigo mpana katika maumbile yote (asilimia 100%) unasisitiza haja muhimu ya mikakati mipya kusimamia ugonjwa wa pneumonic pasteurellosis. Utafiti pia uligundua uwepo wa jeni za upinzani wa antimicrobial kama vile blaTEM na sul1 katika baadhi ya maumbile, hivyo kuonyesha tishio linaloendelea la upinzani. Aidha, uwiano mkubwa wa maumbile ambayo hayakuweza kufunguliwa unasisitiza

haja ya utafiti zaidi kuelewa umuhimu wao na kuendeleza njia mbadala za uchunguzi kwa udhibiti bora.

Kwa muhtasari, utafiti huu unaonyesha taswira ya kusisimua kuhusu upinzani wa antimicrobial na ukali katika *Pasteurella multocida* kwa mbuzi wa kaskazini mwa Tanzania. Kutatua changamoto za kiwango cha chini cha maambukizi, maumbile yasiyofunguliwa, na upinzani mkubwa kunahitaji njia mbalimbali zinazojumuisha matumizi sahihi ya antibiotics, utafiti wa mikakati mipya ya matibabu na uboreshaji wa uchunguzi, na ushirikiano kati ya maafisa wa afya ya umma, wafugaji, na wazalishaji wa mifugo. Ni kupitia juhudi za pamoja tu tunaweza kudhibiti kwa ufanisi ugonjwa wa pneumonic pasteurellosis na kulinda afya na maishaa wafugaji wa mbuzi wa kiwango kidogo katika eneo hilo.

Maneno muhimu: *Pasteurella multocida*, Pneumonic Pasteurellosis, Mbuzi, Tanzania.

DECLARATION

I, **Mwanga, Irene Frank**, do hereby declare to the senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

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(MSc. Candidate)

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(Supervisor)

_____ _____

Dr. George S. Fasha Date
(Supervisor)

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DEDICATION

In loving memory of my father, Mr. Frank Mwanga, whose unwavering love and support fueled my journey through this dissertation. Though his physical presence is gone, his spirit lives on in every page.

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LIST OF ABBREVIATIONS

CLSI	Clinical Laboratory Standard Institute
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
PCR	Polymerase chain reaction
RPM	Rotation Per Minute

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background Information

Livestock farming is the livelihood activity of many poor households in the East Africa Community. The factor behind Tanzania's large numb er of livestock keeping is due to the agro-ecological zones that make t he country suitable for animal production. Due to the increase in the world population, there has also been an increase in the demand for animal protein (Pica-ciamarra *et al.,* 2011). Small ruminants have an i mportant role in nutrition and income generation for people around th e world. Goats are among the important parcels of agro-pastoral and pastoral production systems in Tanzania as they ensure sustained food security for rural and urban inhabitants, they give income to meet various family needs, manure for good cropping and dependency on the byproduct of the goats such as hair and skin which are used for home and commercial purposes (Mbilu *et al.,* 2007). The majority of these small ruminants such as goats are kept under a traditional free-range system. Nevertheless, this small ruminant's free-range farming system is characterized by high morbidities and mortalities, caused by several factors but diseases are believed to be the main limiting factor, pneumonia being among the most prevalent.

Pneumonia in goats is an infection of the lungs characterized by fever (40-41 ºC), anorexia, painful coughing, dyspnea, mucopurulent nasal discharge, and depression. It is one of the most common respiratory il lnesses in goats throughout the world (Brogden *et al.,* 2007). Althoug h pneumonia frequently occurs in kids but it also infects adult goats. B oth infectious and non-infectious agents are responsible for lung infec tion (Mekibib *et al.,* 2019). Among the infectious agents, *Pasteurella multocida* is an opportunistic pathogen frequently associated with the outbreak of acute pneumonia and death of goats of all ages (Hashemi, 2014). This syndrome is caused by a complex interaction between the environmental stress, microorganisms, and immunity of the host (Rawat *et al*., 2019). These bacteria are commonly found in the upper respiratory tract of healthy goats. Factors such as poor management,

poor housing, transportation stress, overcrowding pens, unexpected e nvironmental changes, viral infection (e.g., parainfluenza-3 virus), lun g parasites, and other stressful factors make goats more susceptible t o pneumonia, this means that pneumonia in small ruminants is primarily caused by certain viral agents and is predisposed by the extremes of the environmental insults leading to the colonization of *Pasteurella multocida* in lung tissue hence more severe disease (Rawat *et al*., 2019). Pneumonia caused by Pasteurella *multocida* can lead to wide spread financial losses because of death, reducedlive we ight, delayed marketing, treatment cost and unthriftiness among survi vors (Daniel *et al.,* 2006). *Pasteurella multocida* contains several virulence factors including lipopolysaccharides (LPS), capsules, toxins, adhesins, outer membrane proteins, and porins all of which contribute to the pathogenicity and survival of *Pasteurella multocida*. These virulence factors are imperative during host invasion and colonization, capsule and lipopolysaccharide constitutes the bacterial cell component and play vital roles in the pathogenesis of *Pasteurella multocida* (Chen *et al*., 2021).

According to Kgotlele (2018) *Pasteurella multocida* was among the bacterium found to be in co-infection with other viral aetiologies in small ruminant. Presence of *Pasteurella multocida* and other infections provided an insight in the severity of the outbreak in sheep and goats in the northern part of Tanzania (Ngorongoro). Despite the fact that, many studies have been conducted on pasteurellosis in chicken, ducks, dogs, cats and hummel, there is no enough information about *Pasteurella multocida* in goats in terms of most prevalent strain and molecular characterization of the prevalent strain in Tanzania (Dziva *et al*., 2010).

Vaccination is the principal method for the control of the disease but difficulties of vaccine administration led to the low vaccine coverage accompanied by presence of different strains which affects the efficiency of the vaccine (Zamri-Saad *et al.,* 2006). Additionally, the development of an effective vaccine will still be a challenge without sufficient knowledge of the most common strain and their molecular characterization. This research is conducted so as to isolate and molecular characterize *Pasteurella multocida* isolates to further

understand the circulating strain in the country for the further development of an efficient vaccine against the bacteria.

1.1.1 History of the bacteria

Ability of the *Pasteurella multocida* to cause disease in vertebrates was discovered over ten decades ago and the infections are commonly referred to as pasteurellosis. The bacteria have a wide range of animal host causing specific infections that manifest in a different way. Without question *Pasteurella multocida* has a very broad range of host but this distinctive property is still poorly understood. Research about the potential virulence factors of this bacteria have been conducted but no host specific factors have been identified yet *(*Dziva *et al*., 2010).

Pneumonic pasteurellosis is a disease that occurs mainly due to the compromised immunity in the respiratory system (pulmonary defense mechanism) of animals where the commensal *Pasteurella multocida* becomes a pathogen proliferating in the nasopharynx and spreading to lungs and other organs. In cattle the disease develops following previous exposure to the stressing experience such as shipping hence the name ‘shipping fever” was derived, showing that this disease is highly provoked by the physical and psychological stress (Mohamed & Abdelsalam, 2008).

1.1.2 Characteristics and isolation of the bacteria

Pasteurella multocida is a non-motile, gram negative, facultatively anaerobic coccobacillus bacteria of the family *Pasteurellaceae* responsible for the most of the animal disease. It is a causative agent of pasteurellosis, one of the grievous disease in the livestocks (Soriano-vargas *et al.,* 2012). The samples from patients’ goats from which the infective *Pasteurella multocida* was isolated included nasal mucosa, lung lesions, goats-hearts blood, liver and lymph nodes (Sadeghian *et al*., 2011). The most accurate epidemiological tracing of the *Pasteurella multocida* involves molecular and serological tests, isolation and accurate identification of the species Dziva (2010). Nasal tonsillar swabs (NTS), blood samples and lung samples from the small ruminants who showed the clinical signs of the disease were collected and inoculated in the blood and MacConkey agar for

the isolation purpose (Alemneh & Tewodros, 2016; Ikenna, 2008). In the blood agar Pasteurella *multocida* isolates always produce round, smooth (mucoid) and non-hemolytic colonies (Tahamtan *et al.,* 2014). It doesn't grow on MacConkey agar and microscopically the bacterium is gram negative coccobacillary. The bacterium is positive to oxidase and catalase and it is able to ferment glucose and sucrose and it can able to produce indole (Alemneh & Tewodros, 2016).

1.1.3 Occasional septicemias caused by various capsule types

Pasteurella multocida strains that are not typical type B or type E HS-associated strains have been associated with numerous reports of toxemic/septicaemic disease. These outbreaks are sporadic and most commonly affect pigs and calves, with less frequent occurrences in lambs and horses. Affected animals die from septic shock, and the clinicopathological features closely resemble those of HS. A representative example is an outbreak of septicemia in a large pig herd associated with *P. multocida* subspecies gallicida (capsule type A), as reported by Cameron *et al.* (1996). While the organism was isolated in large numbers from most cultured tissues, administering even very high doses of the recovered organisms intranasally or intravenously to healthy pigs failed to induce disease. Another outbreak of a similar clinical and pathological condition in pigs was linked to *P. multocida* capsular type D (Mackie *et al.,* 1992). Although some potential predisposing factors were identified, such as abrupt changes in ambient temperature, dietary modifications, and high levels of manure gas, the specific factor(s) responsible for predisposing the pigs to the disease outbreak remained undetermined.

Outbreaks of serositis and septicemia in calves have been reported in Germany (Catry *et al.,* 2005) and New Zealand (McFadden *et al.,* 2011), involving capsular types F and B, respectively. Outbreaks in neonatal lambs have also been associated with capsular type F (Watson and Davies, 2002) and an untyped strain (Rad *et al.,* 2011). These sporadic septicemias share the common feature of a sudden onset of fulminating toxemic disease characterized by rapid prostration and death from septic shock. Crowding, exposure to cold, wet conditions, and other aspects of poor management are suspected

as contributing factors, but the specific elements that trigger an outbreak remain unclear. It is not yet known whether the sudden virulence of the associated *Pasteurella* species results from the upregulation of virulence factors in response to environmental influences or whether host immunity is compromised, allowing these commensal organisms to proliferate unchecked. The wide range of strains involved in these sporadic septicemias suggests that changes in the host are likely more critical in initiating the syndrome. However, the possibility that these strains harbor essential virulence factors or require a specific combination of factors should also be considered.

Carriage of *Pasteurella multocida* strains in the oropharynx is common, suggesting that some individuals within a group may initially harbor these organisms as part of their commensal microflora. Two reported incidents involved strains belonging to capsular group F, which is not typically associated with mammals. In these cases, the causative organism could have been acquired from another species that had been in recent contact. The exact mechanism by which this syndrome develops is unclear. It appears that an infection site, such as the lungs, pleura, or peritoneum, becomes established and spreads either directly to other tissues or into the bloodstream, leading to dissemination to multiple sites. Clinical symptoms develop rapidly, likely when endotoxin levels reach a critical threshold. The disease course is typically very short, characterized by depression, fever, prostration, and death from septic shock.

1.1.4 Pathogenesis of the bacteria

Pasteurella multocida infections usually arise from within the body, caused by the normally resident bacteria in the upper respiratory tract (Quinn *et al.,* 2002). However, external infections can also occur through direct contact with infected animals or by inhaling contaminated aerosols. In either case, the disease is typically triggered by a sudden exposure to a stressful condition or by an initial infection with certain respiratory viruses, mycoplasma, or bacteria. Stress and/or viral infections can weaken the local pulmonary defense mechanisms by damaging the ciliating cells and mucous coating of the trachea, bronchi, and bronchioles (Lopez, 2001). This allows the causative bacteria from the nasopharynx to reach the

ventral bronchi, bronchioles, and alveoli through gravitational drainage along the tracheal floor, leading to a deep-seated infection within the lung tissue (Jones *et al.,* 1997).

The rapid growth and multiplication of bacteria in infected lobules produces endotoxins that cause extensive intravascular thrombosis of pulmonary veins, capillaries, and lymphatics (Zecchinon *et al.,* 2005). These vascular disturbances eventually lead to focal ischemic necrosis of the pulmonary parenchyma accompanied by a severe inflammatory reaction dominated by fibrinous exudate. The formation of antigen-antibody complexes may also contribute to vascular permeability and neutrophil chemotaxis, with the subsequent release of lysozyme. The severity of the lesions depends on the rate and extent of bacterial proliferation and the amount of endotoxin released, which in turn depends on the virulence of the bacterial strain and the degree to which the host's defenses are impaired (Mohamed, 2002). Research has shown that the ability of pathogenic bacteria to cause infection is significantly influenced by certain internal factors, known as virulence factors. These factors, which are typically components of the bacterial cell's surface or cellular products, enhance the organism's pathogenicity and facilitate rapid invasion and destruction of target tissues in susceptible hosts (Malazdrewich *et al.,* 2004).

1.1.5 Virulence factors of the bacterium

As it is stated by Mohamed and Abdelsalam (2008), the ability of the bacteria to cause infection is greatly accounted by certain endogenous factors known as virulent factors which enhance the pathogenicity of the organism facilitating expeditious invasion and destruction of target tissues of the susceptible host. *Pasteurella multocida* isolates can be classified into five capsular serogroups (A, B, D, E and F) and 16 liposaccharides somatic serotypes (Dziva *et al.*,2010). *Pasteurella multocida* contains several virulence factors including lipopolysaccharides (LPS), capsule, toxin, adhesins, outer membrane proteins and porins all of which contribute to the pathogenicity and survival of *Pasteurella multocida* (Ewers *et al.*, 2006). These virulence factors are imperative during host invasion and colonization, capsule and lipopolysaccharide constitutes the bacterial cell component and play vital roles in the pathogenesis *of*

Pasteurella multocida (Chen *et al.,* 2021). Serogroup A and D are mostly responsible for the pneumonic pasteurellosis in goats and sheep (Assefa & Kelkay, 2018).

1.1.6 Resistance to host immunity

One of the initial obstacles that invading bacteria encounter is the host's innate immune system, which employs various defense mechanisms, including phagocytic cells, the sequestration of free iron by the host, the bactericidal action of complement, and the activity of antimicrobial peptides. The capsule and lipopolysaccharide (LPS), the major surface components of *Pasteurella multocida*, play crucial roles in the bacteria's ability to resist these mechanisms. Studies have shown that defined acapsular mutants are less virulent because they are readily phagocytosed and/or killed by complement (Boyce and Adler, 2000; Chung *et al.,* 2001). Similarly, truncating the LPS molecule leads to decreased virulence, partly due to increased susceptibility to the bactericidal action of avian antimicrobial peptides such as fowlicidin (Boyce *et al.,* 2009).

Iron is a critical element for the growth and survival of most bacterial species. However, the concentration of free iron in mammalian and avian host tissues is extremely low due to its sequestration by host proteins like transferrin, lactoferrin and hemoglobin. To overcome this iron deficiency, *P. multocida* has developed sophisticated mechanisms to acquire iron from these host iron-binding proteins (May *et al.,* 2001). The bacterium's genome encodes over 50 proteins that are predicted to play roles in iron acquisition and transport. Whole-genome microarray studies have identified a large number of genes (up to 12%) that exhibit altered expression under iron-limiting conditions or in response to specific sole iron sources (Paustian *et al.,* 2001, 2002).. While many of these genes are not directly involved in iron metabolism, they reflect physiological responses to altered environmental and nutritional conditions. Notably, analysis of the outer membrane sub-proteome under iron-limiting conditions revealed only two proteins, OmpW and Pm803, with increased expression n (Boyce and Adler, 2006).

1.1.7 Predisposing factors

The incidence of pneumonic pasteurellosis in cattle, sheep, and goats is influenced by several key predisposing factors.

1.1.7.1 Stress

Stress can be psychological, induced by fear, restraint, or handling, or physical, resulting from hunger, thirst, fatigue, or extreme temperatures (Grandin, 1997). It cannot be directly measured but can be indicated by elevated body temperature, increased heart rate, decreased body weight, and elevated levels of plasma cortisol, glucose, free fatty acids, urea, and betahydroxybutyrate (Morton *et al.,* 1995; Warriss *et al.,* 1995). Stress's role in pneumonic pasteurellosis is evident in the disease's association with sudden exposure to stressful situations like adverse physical, environmental, or climatic conditions. Examples include extreme weather, overcrowding, poor ventilation, bad management, rough handling, and distant transport (Mohamed & Abdelsalam, 2008). Transport is a common predisposing factor, leading to the term "shipping fever" in cattle. Other stressful situations include excessive dust in feedlots, high parasite loads and mixing animals from different sources. Predisposing factors can act alone or in combination and have been experimentally demonstrated in cattle, sheep and goats. Stress can also be induced artificially by drugs like dexamethasone and its effect on susceptibility is similar to that of natural stress (Radostits *et al.,* 2000). The increased susceptibility to *Pasteurella multocida* in stressed animals is attributed to the breakdown of innate pulmonary immune barriers. Stress and viral infections also increase mammalian tissue fibronectin, promoting the growth of Gram-negative bacteria (Malazdrewich *et al.,* 2004).

1.1.7.2 Respiratory viruses

Previous or concurrent infection with certain respiratory viruses can increase the susceptibility of farm animals to secondary bacterial pneumonia. Examples of these viruses include parainfluenza-3 virus (PI-3), bovine herpesvirus type 1 (BHV-1), respiratory syncytial virus (RSV), adenoviruses (ADV) and reovirus. These viruses can increase susceptibility to M. (P) haemolytica infection. Studies have shown that initial infection with BHV-1 followed by *P. haemolytica* in calves can

lead to severe febrile disease with clinical signs and pulmonary lesions indicative of pneumonic pasteurellosis (Hodgson *et al.,* 2005). Similarly, combined infection with PI-3 virus and *P. haemolytica* in lambs can cause severe fibrinopurulent broncho- interstitial pneumonia with focal necrosis. Simultaneous inoculation of lambs with RSV and *Pasteurella multocida* can also result in the development of massive pulmonary lesions closely resembling those observed in naturally occurring cases of ovine pneumonic pasteurellosis (Reffett *et al.,* 1985). Moreover, infection with ovine adenovirus-6 followed by *Pasteurella haemolytica* induced more severe lesions in lambs than those produced by either agent alone. The mechanisms by which respiratory viruses enhance secondary bacterial infections are not fully understood, but several possibilities have been proposed. Viral infections can damage the mucociliary clearance mechanism, which is responsible for removing pathogenic organisms from the lower respiratory tract (Hodgson *et al.,* 2005). Virus-induced injury to the respiratory epithelium can also enhance bacterial attachment and colonization. Additionally, respiratory viruses can impair the phagocytic function of pulmonary alveolar macrophages. More recent studies have shown that respiratory viral infection can enhance toll-like receptor (TLR) expression and increase proinflammatory responses, which contribute to the severity of *Mannheimia haemolytica* infection. TLRs are crucial for detecting bacterial infection and inducing proinflammatory responses (Aderem & Ulevitch, 2000).

1.1.7.3 Other factors

Several additional factors, unrelated to the previously mentioned predisposing factors, have also been associated with an increased risk of pneumonic pasteurellosis in susceptible animals. These factors include twin pregnancy, selenium deficiency, exposure to mycotoxins, inhalation of foreign material, and obstruction of pulmonary airways (Reffett *et al.,* 1985; Pfeffer, 1988). Experimental studies have also shown that the susceptibility to *P. (M) haemolytica* and *P. multocida* infections is significantly increased in laboratory and farm animals by the repeated administration of injectable or dietary iron compounds (Al-Sultan & Aitken, 1984; Ali, 1999; Mohamed, 2002). This increased virulence is primarily attributed to the vital role of iron as a growth-

promoting factor for these microorganisms. Parasitism is another important predisposing factor for pneumonic pasteurellosis. Concurrent or previous infection with common gastrointestinal parasites such as *Haemonchus contortus* has been shown to increase the susceptibility to pneumonic pasteurellosis in goats (Zamri *et al.,* 1994). The adverse effect of these parasites is attributed to their ability to induce immunosuppression, allowing the development of pneumonic lesions. Similar findings have been obtained with *Fasciola gigantica,* where the susceptibility to pneumonic pasteurellosis in Nubian goats was remarkably increased with concurrent infection with the liver fluke (Mohamed, 2002).

1.2 Problem Statement and Justification

The Tanzanian population keeps on increasing, which is expected to rise from the current 61.7 million to 151.3million by 2050 (Tanzania National Bureau of Statistics, 2022). This increase will lead to increase in animal protein demand. Among the animal species kept, small ruminants such as goat form a common resource for different gender groups to which they attach high socio-economic values. Majority of these small ruminants' population are kept under the traditional free-range system which is characterized by high morbidities and mortalities, caused by a number of factors, but diseases are believed to be the main limiting factor. In goats, respiratory diseases contribute greatly to the economic loss. Pneumonic Pasteurellosis cause major lose in small ruminants especially goats and sheep in developing countries including Tanzania due to emergence of resistance to commonly used drugs as well as lack of enough information on the most prevalent strain and their characterization.

Vaccination has been a practical solution in control of any disease including pneumonic pasteurellosis. Currently no any live or killed pasteurellosis vaccine locally developed available in Tanzania. Due to strain diversity, imported vaccine would also require knowledge of the circulating strain in the country. In order to implement either of the two further research on the most prevalent circulating strain of *Pasteurella multocida* is required. There is no enough information about the prevalent strain of *Pasteurella multocida* causing

pasteurellosis in goats in Tanzania. This study therefore aims to contribute to the body of knowledge on the current circulating strains of *Pasteurella multocida* in Tanzania.

The study is carried out in a sense that the isolation and molecular characterization of *Pasteurella multocida* in Tanzania will highly contribute to the further control of pasteurellosis in goats in Tanzania. The outcomes of this study are envisaged to be beneficial to goat keepers through its contribution in improved goat production and improved household food security, nutrition and finally national income.

1.3 Objectives

1.3.1 General objective

To Isolate and molecular characterization of *Pasteurella multocida associated with pneumonic pasteurellosis* in northern Tanzania.

1.3.2 Specific objectives

i. To isolate, molecularly detect, and test for antibiotic susceptibility of *Pasteurella multocida* isolates
ii. To determine virulence genes and antimicrobial resistance genes of *Pasteurella multocida* isolates

CHAPTER TWO

MANUSCRIPT I

2.0 Isolation, Identification and Antimicrobial Susceptibility Testing of *Pasterella multocida* Isolated from Goats in Northern Tanzania

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ABSTRACT

Pneumonic pasteurellosis caused by *Pasteurella multocida* can have a significant impact on animal health and productivity, leading to substantial economic losses for producers. This study aimed at isolating *Pasteurella multocida* associated with pneumonic pasteurellosis in goats. The effectiveness of different antibiotics against the *Pasteurella multocida* isolates was assessed using the disk diffusion method. A total of 200 swabs were collected from goats showing signs of pneumonic pasteurellosis (fever, coughing, weight loss etc) for bacterial isolation from Babati, Mbulu, Simanjiro, Kiteto and Hanang. After conventional methods, the results showed that 10 and 15 of 200 isolates yielded positive results of *Pasteurella multocida* and *Mannheimia hemolytica* respectively. However, under molecular detection, only five isolates were positive for *Pasteurella multocida* and none of the *Mannheimia hemolytica* isolates were confirmed. The test revealed that *Pasteurella multocida* was highly resistant to a wide range of antibiotics, including Cefotaxime (100%), Co-trimoxazole (100%), Amoxicillin (100%), Erythromycin (100%), Ampicillin (100%), Chloramphenicol (80%), Tetracycline (60%) and Pefloxacin (60%). However, the isolates remained 100% susceptible

to gentamicin and ciprofloxacin. The findings suggest that *Pasteurella multocida* is becoming increasingly difficult to treat. This study highlights the importance of implenting novel strategy towards control of pneumonic Pasteurellosis in Tanzania.

Keywords: *Pasteurella multocida*, pneumonic Pasteurellosis, Goat, Tanzania

2.1 INTRODUCTION

Goat is one of the most important livestock in Tanzania. According to Agri Census key findings 2019/20, the goat population in the country is estimated to be between 24.5 and 27 million herds. This makes Tanzania the third largest goat producing country in Africa, after Ethiopia and Sudan. Goats are an important source of skin and manure, but they are also a significant source of protein through their meat and milk. This makes them valuable for improving food security, generating income for farmers, and creating jobs in the livestock sector (Livestock, 2022). Despite, its contribution to the country and individual level, goat production in Tanzania is facing multiple challenges, but the occurrence of disease outbreaks, particularly those associated with pneumonia, has been highlighted to significantly reduce the goat population (Kusiluka *et al.,* 2000).

Pneumonic pasteurellosis being among of those diseases, it affects wide range of animals including sheep, goats, rabbits, cattle, and pigs. *Pasteurella multocida* is the most frequent cause of pneumonic pasteurellosis; however other bacteria, including *Mannheimia haemolytica* and *Bibersteinia trehalosi*, can also cause the illness (Akane *et al.*, 2022). *Pasteurella multocida* is a normal inhabitant of the upper respiratory tract of goats, but the bacterium can become pathogenic when the animal's immune system is weakened (Brogden *et al.*, 1998). This can happen due to a variety of factors, including stress from factors such as transportation, overcrowding, or extreme weather; viral infections which damage respiratory tract making it easier for the bacteria to invade the lungs, also the disease can be due to poor nutrition or direct contact with infected animals or their secretions (Hailu *et al.,* 2017: Sadeghian *et al.,* 2011).

According to Kgotlele *et al.* (2018), *Pasteurella multocida* was discovered to co-infect goats with other viral aetiologies in small ruminants. These co-infections has been reported to be leading and significantly contributing to economic losses in the northern portion of Tanzania. However, large body of literature on *Pasteurella multocida* has been skewed to cats, chickens, and rabbits (Noah *et al.,* 2014: Chota *et al.,* 2019). Limited work and lack of exclusive data on *Pasteurella multocida* in goats has led to a potential underestimation

of its true prevalence and significance in this host. This is likely due to the fact that *Pasteurella multocida* is often considered an opportunistic pathogen in goats, and may therefore be overlooked or misdiagnosed. Therefore, the study aimed at isolating and characterizing *Pasteurella multocida* from goats in Tanzania at the species level and assess on the growing concern of it antimicrobial resistance to commonly used antimicrobials.

2.2 Materials and Method

2.2.1 Study area

The study was conducted in five districts showing the greatest respiratory diseases morbidity and mortality namely; Mbulu, Babati, Hanang, Kiteto and Simanjiro (Figure1) (Mellau *et al.,* 2010; Chota *et al.,*2019; Kgotlele *et al.,* 2019).

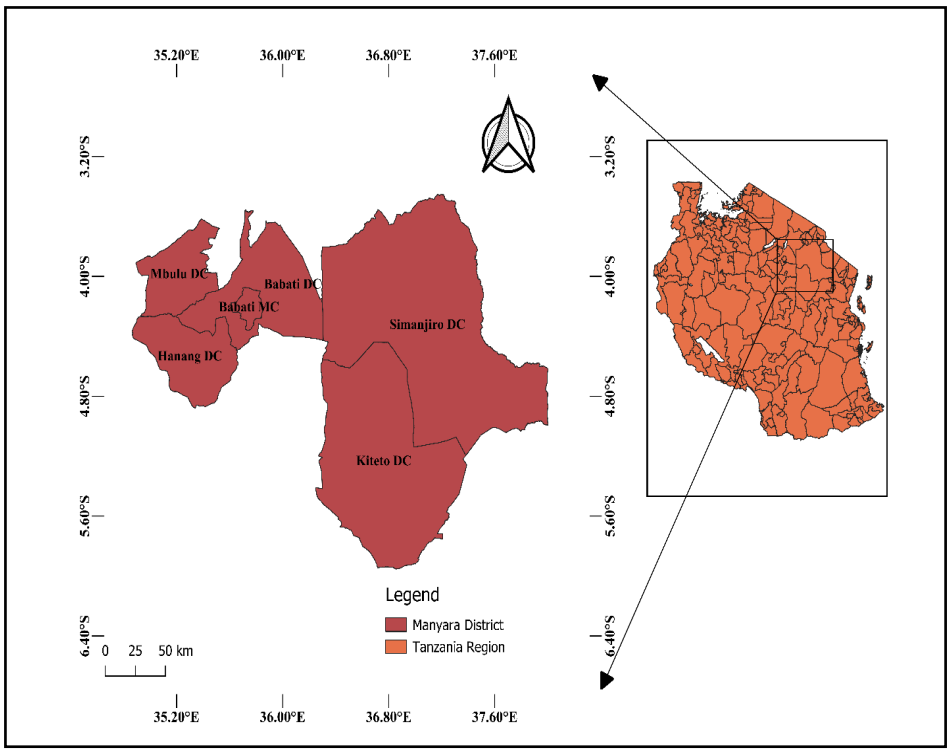

Figure 2.1: A map showing the five districts in Manyara region from which the samples were collected

2.2.2 Study population

Goats from the field manifesting pneumonic pasteurellosis-like respiratory signs were included in this study. Therefore coughing, dyspnea, lethargy, oculonasal discharges and fever were used as the criteria for including animals in the study.

2.2.3 Study design and sampling technique

A cross-sectional study design was employed in collecting samples for identifying *Pasteurella multocida* from goats suspected of having pneumonic pasteurellosis from December 2022 to March 2023. A total of 200 samples from 200 goats were collected from different study sites as follows: Mbulu (n = 36), Babati (n = 67), Kiteto (n = 25), Hanang (n = 32) and Simanjiro (n = 40).

The purposive and snowball sampling techniques were employed in identification of goats and sampling. Nasal swabs, oral-pharyngeal swabs, lungs, and pleural fluids were sampled.

For nasal and oral-pharyngeal samples, the sterile bacteriological swabs were inserted deep into the goats nostrils rotated clockwise and then reinserted back into tubes with labels that contained Stuart transport medium. All the samples were ice-packed and transported from the field to the Veterinary Microbiology laboratory at Sokoine University of Agriculture (SUA) located in the College of Veterinary Medicine and Biomedical Sciences (CVMBS) for bacteriological analysis.

2.2.4 Culture and isolation of *Pasteurella multocida*

Nasal swabs were placed in nutrient broth (oxoid)-filled universal bottles and incubated for 24 hours at 37° with 5% carbon dioxide. A loop of the broth cultures was then collected and streaked over a Petri dish that had a blood agar base enriched with 7% horse blood. The dish was then immediately incubated at 5% carbon dioxide at 37°C for 24-48 h. As a result, representative colonies were gram-stained from culture-positive colonies to ascertain staining characteristics and cellular morphology under a light microscope. Gram negative bacteria with typical cell morphology were sub cultured on both MacConkey and blood agar for further analysis. The growth of colonies on both MacConkey and blood agar were

characterized whereby in blood agar colonies were checked for the presence of hemolysis, type of hemolysis, and general appearance of the colonies.

2.2.5 Biochemical confirmation of *Pasteurella multocida*

Isolates which had colonial morphology and gram staining properties similar to that of *Pasteurella multocida* were subjected to various biochemical test which were catalase, indole and oxidase test.

2.2.6 Confirmation of *Pasteurella multocida* by polymerase chain reaction (PCR)

2.2.6.1 DNA extraction from the pure *Pasteurella multocida* colonies

DNA extraction was performed at the College of Veterinary Medicine and Biomedical Sciences (CVMBS) at the Genome Science Laboratory. A few colonies of 24 hours pure cultures of phenotypically identified *Pasteurella multocida* taken and grown for from nutrient agar were put into 1.8 mL Eppendorf tubes. Then, DNA extraction was performed using the boiling method following the procedures described by Ozgul *et al.* (2021). Whereby, From a plate with pure colonies grown overnight in nutrient agar three single colonies were picked using a sterile disposable loop and added in an eppendorf tube containing 150µl of nuclease free water. The tubes were well shaken to mix the contents and then immersed in boiling water at 95°C for five minutes followed by freezing the tubes at -21°C for ten minutes and then thawed again in 95°C, the cycle was repeated three times. After the freeze-thaw cycle the tubes were left to cool at room temperature then vortexed and centrifuged at 12 000rpm for 1 minute. 140µl of supernatant was collected and transferred to another sterile eppendorf tube for PCR process.

2.2.6.2 Polymerase chain reaction (PCR)

Using specific primer pairs that target the putative transcriptional regulator genes Pmo762 and Pm1231, isolates assumed to be *Pasteurella multocida* were subjected to a PCR assay (Liu *et al.,* 2004). Table 1 provides the expected amplified product size and primer sequence. We prepared a reaction mixture for PCR with a total volume of 25µl. The mixture contained 5µl of the Bioneer AccuPower

Taq PCR Master mix (including DNA polymerase, dNTPs, reaction buffer, and tracking dye), 1µl (10pM/µl) of each primer pair, 14µl of nuclease-free water, and 4µl of DNA template. The PCR amplification conditions involved initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds each, annealing at 55°C for 30 seconds, first extension at 72°C for 1 minute, and a final cycle of extension at 72°C for 7 minutes. We used a reaction tube without DNA template as a negative control.

Detection of the PCR products was done in 2% (*w*/*v*) agarose gel, prepared from 1X Tris-Acetate-EDTA buffer stained with Ethidium bromide. Each PCR product (5 µl) was loaded into separate well of the pre-prepared gel while 100bp DNA molecular marker was loaded onto the first and last lane and run at 80 V for 45 min on a gel electrophoresis tank(Bio-Rad, Model 200/2.0). The different band sizes were visualized under UV transilluminator and photographed in gel documentation system (UVI TEC, UK).

2.2.7 Antimicrobial susceptibility testing of *Pasteurella multocida* isolates

Using the Kirby Bauer disk diffusion method, five isolates of *Pasteurella multocida* were evaluated for antimicrobial susceptibility in Mueller-Hinton agar supplemented with 5% defibrinated blood. Ten distinct antimicrobials with varying applications in Tanzanian Veterinary practices were tested against the isolates. These antimicrobials were; ciprofloxacin (5µg), pefloxacin (5µg), ampicillin (10µg), erythromycin (15µg), gentamicin (10µl), amoxicillin (30µl), tetracycline (30µl), co-trimoxazole (25µl), and cefotaxime (30µl). The Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, CLSI (Clinical Laboratory Standard Institute) Document VET01 5th edn was followed when interpreting the data.

Table 2.1*:* Specific primers for the detection of *Pasteurella multocida*

gene	Forward (5'-3')	Reverse (5'-3')	PCR product (bp)
Pm0762	TTGTGCAGTTCC GCAAATAA	TTCACCTGCAA CAGCAAGAC	567
Pm1231	AGAAAGCACATG ACCAAAGG	GCAGCTACTCG CAGAAGGTT	601

2.2.8 Data analysis

Descriptive analysis was employed to examine the antimicrobial susceptibility test results for each *Pasteurella multocida* isolate. The data were meticulously entered into an Excel spreadsheet, allowing for systematic organization and analysis. Descriptive statistics were then computed to elucidate key aspects of the susceptibility patterns facilitating the determination of the proportions or percentages of drugs to which each isolate exhibited

2.2.9 Ethical approval

This study has obtained an ethical approval by Tanzania Livestock Research Institute, TALIRI with registration number **No. TLRI/RCC.22/004**. Animals were approached with great care according to the guidelines for ethics of animal research. Moreover, animal owners were consented, and the benefits and outcomes of the study explained for the study participants.

2.3 RESULTS

2.3.1 Isolation and biochemical test results

Of the 200 nasal swab samples from pneumonic goats 25 isolates were found to have staining characteristics and culture morphology similar to those of *Pasteurella* and *Mannheimia* species. *Mannheimia hemolytica* (10 isolates) which were β-hemolytic in blood agar were also shown to grow on MacConkey agar. *Pasteurella multocida* (15 isolates) were identified following their growth in blood agar with non-hemolytic colonies that did not grow on MacConkey agar. All of the 25 isolates were Gram-negative cocco-bacilli in short chains and exhibited bipolar staining characteristics of the isolates tested positive for catalase, oxidase and indole except *Mannheimia hemolytica* isolates which tested negative for indole test (Table 2).

Table 2.2*:* Phenotypic characteristics of isolates *Pasteurella multocida* and *Mannheimia hemolytica*

Characteristics (tests)	**species**	
	Pasteurella multocida	***Mannheimia hemolytica***
Hemolysis	-	+
Growth on MacConkey agar	-	+
Catalase	+	+
Oxidase	+	+
Indole	+	-
Total number of isolates fulfilling the identification criteria	n=10	n=15

a b c

Figure 2.2: (a) *Pasteurella multocida* colonies on blood agar showing non-hemolysis characteristics.
(b) Indole positive test
(c) *Pasteurella multocida* showing gram negative and bipolar coccobacillary cell morphology

2.3.2 Molecular confirmation of the isolates

The Pm0762 and Pm1231 genes were amplified from conventionally identified *Pasteurella multocida* and *Mannheimia hemolytica*. Five of the ten isolates (n = 10) that were first identified as *Pasteurella multocida* had one or both of the genes under investigation; two isolates (n = 2) tested positive for both genes, two isolates (n = 2) tested positive for the Pm0762 gene, and one isolate (n = 1) tested positive for the Pm1231 gene only.

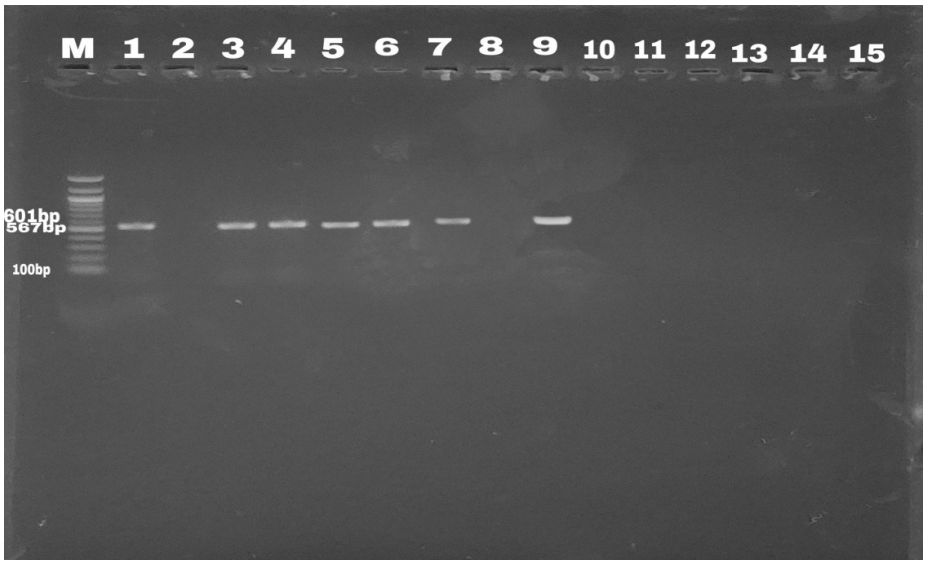

Figure 2.3: Detection of *Pasteurella multocida* by PCR using specie specific genes (*Pm0762 and Pm1231*) M; 100bp of DNA molecular marker, Lane 1 positive control: lane 3,4,5 and 6 positive for *Pm0762* gene: lane 7 and 9 positive for *Pm1231* gene

2.3.4 Antimicrobial susceptibility

The results for antimicrobial susceptibility revealed that *Pasteurella multocida* became resistant to Cefotaxime (100%), Co-trimoxazole (100%), Amoxicillin (100%), Erythromycin (100%), Ampicillin (100%), Chloramphenicol (80%), Tetracycline (60%) and Pefloxacin (60%), but very susceptible to gentamicin (100%) and ciprofloxacin (100%) (Table 3). Also all isolates had multiple drug resistance to 5 or more antimicrobial agent tested (Table4).

Table 2.3*:* Antimicrobial Susceptibility test results of 5 *P. multocida* isolated from goats

Antimicrobial agent	Susceptible	Intermediate	Resistance
Ciprofloxacin	5 (100%)	-	-
Chloramphenicol	-	1(20%)	4(80%)
Pefloxacin	-	2(40%)	3(60%)
Ampicillin	-	-	5(100%)
Erythromycin	-	-	5(100%)
Gentamicin	5 (100%)	-	-
Amoxicillin	-	-	5 (100%)
Tetracycline	-	2(40%)	5(60%)
Co-trimoxazole	-	-	5(100%)
Cefotaxime	-	-	5(100%)

Table 2.4: Antimicrobial profile pattern and multiple drug resistance (MDR) of the five *Pasteurella multocida* isolates

Isolate	Antimicrobial agent										Number of antimicrobial agents resisted
	CIP	CHLO	PEF	AMP	ERY	GEN	AMX	TE	SXT	CTX	
1	S	R	I	R	R	S	R	R	R	R	7/10
2	S	I	I	R	R	S	R	I	R	R	5/10
3	S	R	R	R	R	S	R	I	R	R	7/10
4	S	R	R	R	R	S	R	R	R	R	8/10
5	S	R	R	R	R	S	R	R	R	R	8/10

2.4 Discussion

Pneumonia is a major infectious disease of goats in Tanzania, causing significant economic losses due to death. To effectively treat this disease and prevent the development of antibiotic resistance, it is important to understand the antimicrobial susceptibility of the bacteria involved.

In the current study, microbiological and molecular assays identified 2.5% of isolates as *Pasteurella multocida*. This recovery rate is significantly lower than previously reported in the literature: 29.98% by Valadan *et al.* (2014), 20.50% by Tewodros & Annania (2016) and

50% by Rawat *et al.* (2019). Several factors may explain this discrepancy. Firstly, the sample size in our study (200 goats) was considerably larger than those used by Tewodros & Annania (2016) (10 goats) and Rawat *et al.* (2019) (14 goats). This increased sample size may have led to a more accurate representation of the true prevalence within the population, revealing a lower overall rate. Secondly, geographical variations in environmental factors might play a role. While studies by Valadan *et al.* (2014) and Tewodros & Annania (2016) were conducted in different regions, Rawat *et al.* (2019) and our study were conducted in geographically similar areas. This suggests that environmental stressors specific to these regions, such as heat, drought, and feed and water shortages, may contribute to a higher prevalence of *Pasteurella multocida.*

Interestingly, our findings align with those of Ikenna (2008) and bAhr *et al.* (2021), which also reported a low prevalence of *Pasteurella multocida* in goats. This further supports the notion that geographical variations and environmental factors significantly impact the prevalence of this pathogen in goat populations.

Furthermore, findings from the present study demonstrated the high antimicrobial resistance rate of *Pasteurella multocida* isolates against the tested antimicrobials, with all isolates being resistant to at least five or more antibiotics (Table 2.4). In vitro susceptibility testing showed that 100% of isolates were resistant to ampicillin, amoxicillin, erythromycin, co-trimoxazole and cefotaxime, and had high resistance to pefloxacin, chloramphenicol, and tetracycline (Table 2.3). The widespread use of antibiotic additives in animal feed and the extensive application of antimicrobial agents in veterinary medicine have been implicated as potential contributing factors to the emergence of multidrug resistance (MDR) in bacteria. Selective pressure exerted by antimicrobial agents, transposon-mediated drug resistance mechanisms (Kehrenberg *et al.,* 2001), and associations with specific genes may also play roles in inducing mutations within the 16S rRNA gene, thereby contributing to MDR development (Kehrenberg and Schwarz, 2007).

The present investigation's conclusions about β-lactam resistance in isolates of *Pasteurella multocida* align with those of El-Seedy *et al*. (2019) and bAhr *et al.* (2021) in Egypt. According to earlier reports (Livrelli *et al*., 1988: Schwarz *et al*., 1989), β-lactam resistance plasmids are the main mediators of this resistance.

In accordance with the report of Guo *et al*. (2020), tetracycline resistance was high in this study along with the 100% resistance against chloramphenicol which disagrees with the report of De Jong *et al.* (2014); this may be due to increased administration of the drugs for the treatment of respiratory diseases in goats. This study indicated resistance to inexpensive, readily available antibiotics (amoxicillin, telacycline), which can be purchased off the shelf without a valid prescription from a veterinarian in practice. This widespread use of antibiotics in food-producing animals leads to the emergence of antibiotic-resistant strains. According to Rodríguez-Martínez *et al*. (2011), plasmids can also mediate fluoroquinolone resistance.

High resistance to chloramphenicol in this study was found to be in contrast with the findings of Choudhary *et al*. (2019), who showed that all isolates of *Pasteurella multocida* were susceptible to the drug. Chloramphenicol resistance is generally linked to plasmids that encode the enzymes known as chloramphenicol acetyltransferases, which are known to be inactivating. This was reported by Vassort-Bruneau *et al*., 1996.

All *Pasteurella multocida* isolates were sensitive to ciprofloxacin and gentamicin likely due to decreased or controlled use of gentamicin and ciprofloxacin in the treatment of respiratory infection in the flock, results similar to those obtained by bAhr *et al*. (2021) in Egypt.

It is important to note that the prevalence of pneumonic pasteurellosis is consistently underestimated because the illness is frequently linked to other stressors or misdiagnosed as another respiratory illness due to its broad range of clinical symptoms, which include fever and coughing. Furthermore, the diagnosis is challenging and usually depends on a combination of gross and histopathological lesions,

clinical signs, and bacterial culture. Bacterial culture is not always accurate, and it can be challenging and time-consuming.

2.5 Conclusion and Recommendations

The findings of this study indicate that *Pasteurella multocida* is growing more resistant to a variety of antibiotics, such as tetracyclines, cephalosporins, penicillin, and macrolides. These results emphasize the necessity of novel control strategy for *Pasteurella multocida* such as vaccine development.

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CHAPTER THREE

MANUSCRIPT TWO

3.0 Detection of Antimicrobial Resistance and Virulence Genes in *Pasteurella multocida* isolated from Goats with Pneumonic Pasteurellosis in Northern Tanzania

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ABSTRACT

Pasteurellosis in small ruminants is a major economic burden for small-scale farmers in Tanzania. This study aimed to investigate the antibiotic resistance and virulence genes of *Pasteurella multocida* isolated from goats with pneumonic pasteurellosis so as to understand the prevalence of these genes in *Pasteurella multocida*, which can certain the development of resistance to antimicrobials and virulence level. Of the 5 (2.5%) isolates recovered from the nasal swabs collected from goats showing signs of pneumonic pasteurellosis in northern Tanzania (Mbulu, Hanang, Babati, Kiteto and Simanjiro)and screened for the six antimicrobial resistance genes (*sul1, sul2, blaTEM, aac(3)-1, tetA* and *acrA*) , capsule type (*capA, capB, capD, capE* and *capF)* and five virulence genes (*ptfhA, ptfA, fimA, exbB* and *exbD*)40%were resistant to at least one antimicrobial agent. The most common resistance gene detected was *blaTEM* and *sul1* followed by *TetA*. Capsular serogrouping revealed that 20% of the isolates belonged to serogroup A, while the other 80% of isolates were untypable. PCR analysis of virulence genes showed that all isolates harbored the iron acquisition gene *exbB*, while only 60% of

the isolates harbored the adhesin gene *ptfA.* This study highlights the presence of antimicrobial resistance and virulence genes in *Pasteurella multocida* isolates from goats in northern Tanzania. The presence of untypable strains is a particular concern, as it suggests that they may be evading traditional typing methods, which could make it more difficult to track their spread and develop effective vaccines and treatments. Further research is needed to understand the significance of untypable *Pasteurella* multocida and to develop new methods for typing these strains.

3.1 INTRODUCTION

Pasteurella multocida is a Gram negative, non-motile bacterium that is commonly found in the respiratory tract of healthy animals (Griffin, 2010). It possesses the conventional traits of an opportunistic pathogen, depending on predisposing factors to cause respiratory diseases, such as abrupt changes in weather, malnourishment, overwork, lengthy and stressful travels, injuries, or other microbial infections (Bisgaard, 1993; Dziva *et al.,* 2008).

Pasteurella multocida strains are categorized into five capsular types (*A, B, D, E,* and *F*) (Harper *et al.,* 2006). Each capsular type is typically, though not always, limited to a particular host and the disease predilection (Harper *et al.,* 2007). The various virulence factors of *Pasteurella multocida* are linked to its pathogenicity. The capsule protein and lipopolysaccharide have been identified as the two main virulence factors of Pasteurella multocida (Boyce *et al.,*2002). However, Fimbriae, adherence and colonization factors (*ptfA, fimA, hsf-1, hsf-2, pfhA*, and *tadD*), iron-regulated and acquisition proteins (*exbB, exbD, tonB, hgbA, hgbB, tbpA*, and *Fur*), extracellular enzymes like neuraminidase (*nanB* and *nanH*), hyaluronidase (*pmHAS*) and superoxide dismutase (*sodA* and *sodC*), dermonecrotoxin (*toxA*), and a variety of outer membrane proteins (OMPs) like protectins (*ompA, ompH, oma87*, and *plpB)* are among the many other virulence genes that are significant in the pathogenesis of *Pasteurella multocida* (Fuller *et al.,* 2000; Boyce *et al.,* 2002; Ewers *et al.,* 2006). These virulence factors enable *Pasteurella multocida* to colonize and invade by interfering with the host's defence systems, destroying host tissue, and/or inducing an

unpleasant inflammatory response in the host (Harper *et al.,* 2006b). Moreover, certain studies reported a clear relationship between capsular type and some virulence factors (Ewers *et al.,* 2006).

Despite the fact that antimicrobial therapy is a widely accessible tool for the prevention and control of clinical infections ((Kehrenberg *et al.,* 2001; Lion *et al.,* 2006; Brogden *et al.,* 2007), antimicrobial resistance in pathogenic bacteria from food-producing animals and environmental sources is acknowledged as a global public health concern. Concern has grown over the last ten years over the high level of resistance to common antimicrobials and the global emergence of multidrug-resistant phenotypes (Maillard *et al.,* 2020; Walsh & Fanning, 2008). According to earlier research, using antimicrobials carelessly increases the likelihood that resistant bacteria will emerge and that resistance genes on integrons, transposons, and plasmids will proliferate (Hunt *et al.,* 2000). Consequently, the effectiveness of the antimicrobial agents currently in use to treat infections in animals raised for food production has decreased (Garch *et al.,* 2016). The widespread use of antimicrobial classes in humans for treating infections may in fact be overdone in animals, either as a form of therapy or as a means of disease prevention, with serious implications for public health (Caprioli *et al.,* 2000).

Despite the abundance of global research on the antibiotic resistance and virulence genes of *Pasteurella multocida* from various animal hosts, there is a paucity of data on this bacterium in Tanzania. This study aims to provide information on antimicrobial resistance and virulence genes in *Pasteurella multocida* bacterium isolated from goats in northern Tanzania, the information will be helpful in the identification of desirable candidate for development of a vaccine against the bacterium in future.

3.2 Materials and Methods

Study area, study Animals, study design and sampling technique

The study location, design and sampling procedures are the same as described in the previous manuscript with the title “Isolation,

Identification and Antimicrobial Susceptibility Testing of *Pasteurella multocida* Isolated from Goats in Northern Tanzania".

3.2.1 Bacterial identification

Bacterial identification was done using conventional and molecular methods and has been described in the previous manuscript.

Table 3.1: Primers Sequences Used in the Amplification of various virulent genes

gene	function	Forward (5'-3')	Reverse (5'-3')	PCR product (bp)	Reference
ptfhA	Filamentous hemagglutinin	TTCAGAGGGATCAATCTTCG	AACTCCAGTTGGTTTGTCG	286	(Tang *et al.,* 2009)
ptfA	Adhesins	TGTGGAATTCAGCATTTTAGTGTGTC	TCATGAATTCTTATGCGCAAAATCCTGCTG	468	(Tang *et al.,* 2009)
fimA	Adhesins	CCATCGGATCTAAACGACCTA	AGTATTAGTTCCTGCGGGTG	866	(Tang *et al.,* 2009)
exbB	Iron acquisition	TTGGCTTGTGATTGAACGC	TGCAGGAATGGCGACTAA A	283	(Tang *et al.,* 2009)
exbD	Iron acquisition	CGTTCTGATTACAGCCTCTT	AACGAAATCTTGGAAACTGG	247	(Tang *et al.,* 2009)
hyaD-hyaC	Serogroup capA	GATGCCAAAATCGCAGTCAG	TGTTGCCATCATTGTCAGTG	1044	(Townsend *et al.*, 2001)
bcbD	Serogroup capB	CATTTATCCAAGCTCCACC	GCCCGAGAGTTTCAATCC	760	(Townsend *et al.,* 2001)
dcbF	Serogroup capD	TTACAAAAGAAAGACTAGGAGCCC	CATCTACCCACTCAACCATATCAG	657	(Townsend *et al.,* 2001)
ecbJ	Serogroup capE	TCCGCAGAAAATTATTGACTC	GCTTGCTGCTTGATTTTGTC	511	(Townsend *et al*., 2001)
fcbD	Serogroup capF	AATCGGAGAACGCAGAAATCAG	TTCCGCCGTCAATTACTCTG	851	(Townsend *et al.,* 2001)

3.2.2 Detection of virulence and antibiotic genes

Polymerase chain reaction (PCR) was used to amplify virulence and antibiotic resistance genes in *Pasteurella multocida* isolates. Primer pairs specific for each gene were used, as listed in Tables 3.1 and 3.2, respectively. The PCR reaction conditions for virulence genes were identical to those used to detect *Pasteurella multocida*, as described in the previous manuscript. The PCR reaction conditions for antibiotic resistance genes were the same, except for the annealing temperature, which was optimized for each primer pair, as listed in Table 3.2.

Table 3.2: Primer sequence for the detection of antibiotic resistance genes

Family of the antimicrobial agent	Targeted gene	Primer sequence (5'-3')	Amplified product size (bp)	Annealing temperature (°C)	Reference
Sulfonamides	*Sul1*	F-CGGCGTGGGCTACCTGAACG R-GCCGATCGCGTGAAGTTCCG	450	55	(Dayao et al., 2016)
	Sul2	F-GCGCTCAAGGCAGATGGCATT R-GCGTTTGATACCGGCACCCGT	625	58	(Dayao et al., 2016)
Tetracycline	*tetA*	F-GGTTCACTCGAACGACGTCA R-CTGTCCGACAAGTTGCATGA	576	58	(Dayao et al., 2016)
β lactam	*blaTEM*	F-GAGTATTCAACATTTTCGT R -ACCAATGCTTAATCAGTGA	882	58	(Dayao et al., 2016)
Aminoglycoside	*Aac(3)-1*	F-ACCTACTCCCAACATCAGCC R-ATATAGATCTCACTACGCGC	169	60	(Dayao et al., 2016)
Quinolones	*acrA*	F-CTCTCAGGCAGCTTAGCCCTAA R-TGCAGAGGTTCAGTTTTGAC	106	58	(Dayao et al., 2016)

TGTT

3.2.3 Capsular typing

The capsular type of the isolates was determined using uniplex capsular PCR where capsule specific primer pairs including *capA, capB, capD, capE* and *capF* were used as employed by Townsend *et al*. (2001). The primer sequences used in the capsule PCR are listed in Table 3.1.

Each 25µl of uniplex reaction contained 12.5µl of Quick-Load Taq 2x Master Mix (DNA polymerase, dNTPs and reaction buffer). 0.51 µl (0.2µM) of each primer pair, 7.5µl of nuclease-free water, and 4µl of DNA template. All amplifications were performed by ProFlex™ 3x32-well PCR System. The following conventional cycling approach was employed for products with an estimated size of less than 1 kb: initial denaturation at 95°C for 5 min, then 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The same cycle process was performed for the capA-amplified product, which had an anticipated size greater than 1kb (1044 bp), with the exception that the extension duration was extended by 30 s. A DNA-free PCR mixture was used as the negative control. The amplified products were separated by electrophoresis in 2% agarose gel and observed with Ethidium bromide staining under UV transilluminator.

3.3 Results

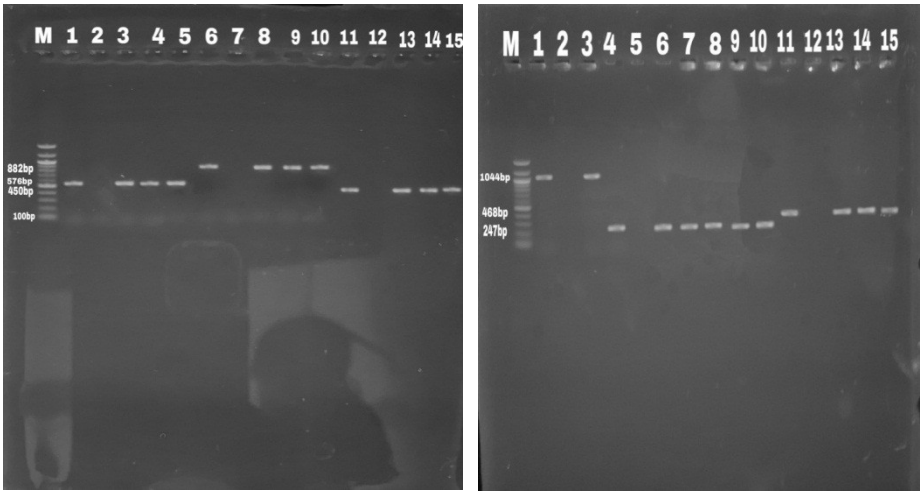
3.3.1 Occurrence of resistance gene

Of the six antibiotic resistance genes screened, only three were detected in the *Pasteurella multocida* isolates: *TetA, blaTEM*, and *sul1. Sul1* and *blaTEM* were detected in three isolates each, while *tetA* was detected in only two isolates. Forty percent of the isolates harbored two different resistance genes, while the remaining 60% harbored only one (Table 5). Moreover, three of the five isolates resistant to ampicillin conferred the blaTEM gene, only two of the five isolates phenotypically resistant to tetracycline carried the *TetA* gene,

and only three of the five isolates phenotypically resistant to co-trimoxazole conferred the *sul1* gene (Figure 3.1a).

Table 3.3: Amplified genes

Gene amplified	isolates				
	1	2	3	4	5
Pm1231	+	+	–	+	–
Pm0762	-	+	+	+	+
CapA	-	-	+	–	–
ExbD	+	+	+	+	+
PtfA	+	+	–	–	+
BlaTEM	+	–	–	+	+
TetA	–	–	+	+	–
Sul1	+	+	-	-	+



a b

Figure 3.1. (a) PCR amplification of antibiotic resistance genes (*blaTEM, sul1*, and *tetA*), (b) PCR amplification of virulence genes (*capA, ptfA*, and *exbD*)

(a) M; 100bp DNA molecular marker, lane 1, 6, and 11 positive controls; lane 3,4, and 5 positive samples for *tetA*; lane 8,9, and 10 positive samples for *blaTEM* gene: lane 13, 14, and 15 positive samples for *sul1* gene: lane 2,7, and 12 negative controls.

(b) M; 100bp DNA molecular marker, lane 1, 4, and 11 positive control samples. Lane 3 positive positive for *capA* gene: lane 6,7,8,9, and 10 positives for *exbD* gene: lane 13,14, and 15 positives for *ptfA* gene: lane 2, 5, and 12 negative controls.

3.3.2 Capsular serogrouping and virulence genes of *Pasteurella multocida*

Capsular serogrouping of the five isolates revealed that one isolate belonged to serogroup A with an approximate molecular size of 1044bp. The isolation percentage of serogroup capA was estimated to be 20%. The other 4 (80%) isolates of *Pasteurella multocida* were non-typable (figure1b). Therefore, isolates that were negative for capsular typing were confirmed as untypable *Pasteurella multocida* in separate PCR assays with the *Pasteurella multocida*-specific primers (Townsend *et al.,* 2001).

Table 3.4: Antimicrobial resistance pattern of capA and untypable strains

ISOLATES	Antimicrobial susceptibility	Positive strain percentage (%) to individual antibiotic agent									
		CIP	CHLO	PEF	AMP	ERY	GEN	AMX	TE	COT	CTR
Untypable (n=4)	S	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
	I	0.0	0.0	25.0	0.0	0.0	0.0	0.0	25.0	0.0	0.0
	R	0.0	100.0	75.0	100.0	100.0	0.0	100.0	75.0	100.0	100.0
capA (n=1)	S	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
	I	0.0	100.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
	R	0.0	0.0	0.0	100.0	100.0	0.0	100.0	0.0	100.0	100.0

CIP: ciprofloxacin, CHLO: chloramphenicol, PEF: pefloxacin, AMP: ampicillin, ERY: erythromycin, GEN: gentamicin, AMX: amoxicillin, TE: tetracycline, COT: co-trimoxazole, and CTR: ceftriaxone.

PCR analysis of five *Pasteurella multocida* virulence genes revealed that all five isolates (100%) carried the iron acquisition gene *exbB*, while only three isolates carried the adhesin gene *ptfA*. Sixty percent of the isolates harbored more than one virulence gene (*exbD* and *ptfA*), while the remaining 40% harbored only a single virulence gene (*exbD*). The isolate carrying the *capA* gene also harbored both *exbB* and *ptfA*.

3.4 Discussion

Pasteurellosis is a disease affecting goats worldwide with specific serotype and pathotype associated with the respiratory disease. The capsular serotype of *Pasteurella multocida* is a critical factor in its ability to evade the host immune system and cause disease, and certain serotypes are more commonly associated with respiratory disease in goats. According to Townsend *et al*. (2001), the identification and sequence analysis of the biosynthetic locus of the capsule of an organism can lead to a greater understanding of its capsular polysaccharide composition and can provide a genetic basis for the serological differences observed between strains.

This study identified serogroup A as the most prevalent capsular serotype of Pasteurella multocida isolated from goats with pneumonic pasteurellosis, accounting for 20% of all isolates. Notably, this finding aligns with previous research conducted in diverse regions, including Germany (Ewers *et al.,* 2006), Brazil (Ferreira *et al.,* 2015), and Iran (Tahmtan *et al.,* 2014), which reported prevalences of serogroup A ranging from 76.3% to 100%. Therefore, these consistent observations highlight the global significance of serogroup A in the etiology of pneumonic pasteurellosis in goats.

Furthermore, genome sequencing of serogroup A isolates confirmed the presence of genes involved in hyaluronic acid synthesis, corroborating its role as the primary component of their capsule. As reported by Chung *et al.* (1998), the serogroup A capsule plays a pivotal role in bacterial virulence by facilitating adhesion to host cells, evading the immune system, and protecting against desiccation. Consequently, this potent capsule likely contributes to the dominance of serogroup A among goats with pneumonic pasteurellosis.

The prevalence of *Pasteurella multocida* serotypes can exhibit significant geographic variation and temporal fluctuations, even within the same region. In this study, a strikingly high proportion of isolates (80%) remained untypable, aligning with the findings of Sahoo *et al.* (2020) in India who reported an untypable prevalence of 43.58%. This observation also finds support in studies conducted by Davies *et al.* (2003) in the United Kingdom (1% untypable strains from pigs), Tang *et al.* (2009) in China (5.2% untypable strains from swine), and Güler *et al.* (2013) in Turkey (55.5% untypable isolates from goats).

Christensen *et al*. (2022) further identified a potential explanation for the observed untypable strains. Their whole-genome sequencing (WGS) analysis of reference *Pasteurella multocida* strains revealed mutations or deletions in genes encoding capsular and outer core LPS components. This suggests that further investigation is needed to document the phenotypic changes associated with these genetic alterations.

Furthermore, Sahoo *et al.* (2023) provide evidence suggesting a potential link between untypable strains and increased virulence. This raises the intriguing possibility that the untypable phenotype may enhance the ability of these bacteria to evade the host immune system, thereby contributing to their heightened virulence. There are two possible explanations for why capsule typing might fail: either the emergence of novel, yet-undiscovered capsule types: This scenario suggests the existence of previously unknown capsular antigens that current typing methods are unable to identify or mutations affecting capsule formation.

Interestingly, Wilson *et al.* (1993) reported a higher prevalence of untypable strains in birds. Given the cohabitation of chickens and goats often observed in Tanzanian livestock farming practices, it is plausible that the untypable strains identified in this study may have originated from avian sources, necessitating further investigation into potential interspecies transmission dynamics.

While scientists do not fully understand how *Pasteurella multocida* causes disease or why it is specific to certain hosts, several studies have found that a number of virulence factors (VFs) are associated with its pathogenic mechanisms (Hunt *et al.,* 2000; Harper *et al.,* 2006). Iron acquisition is an important virulence factor for *Pasteurella multocida*, as it allows the bacterium to adapt to different iron environments in different hosts and at different stages of infection (Sarangi *et al.,* 2015). Among the two iron acquisitions genes (*exbB* and *exbD*) which were screened in this study, 100% of the isolates were detected to confer exbD gene while none of the isolates conferred exbB gene. The fact that most isolates possess the *exbD* gene suggests that the gene is important for the survival and fitness of *Pasteurella multocida* isolates in a variety of environments. It is possible that the *exbD* gene is being transferred between *Pasteurella multocida* isolates through gene exchange since most virulence genes are linked to plasmids which allows them to be easily shared among bacteria. This could explain why all of the isolates possess the *exbD* gene. In this work, the high prevalence of an adhesion-related gene ptfA was noted (60%), a finding that was reported in some previous studies (Sarangi *et al.*, 2015; Aski & Tabatabaei, 2016). It has a major role in initial colonization of the bacterium in the upper respiratory tract (Nguyen *et al.,* 2023).

Pasteurella multocida infections are usually treated with broad spectrum antibiotics (Brogden *et al*., 2007c). Antimicrobial agent resistance is primarily mediated by the presence of antimicrobial resistance genes in the bacteria (Ujvvári *et al.,* 2018). In this study, the β-lactam resistance gene *blaTEM* was detected in 60% of the isolates, similar to the findings of Petrocchi-Rilo *et al.* (2019) in Spain. Isolates that harbored *blaTEM* were resistant to ampicillin in vitro, confirming the role of this gene in ampicillin resistance. 60% of the isolates harbored the *sul1* gene, which is associated with resistance to sulfonamide antibiotics. Additionally, all isolates were resistant to co-trimoxazole in vitro, which is a combination of sulfonamide and trimethoprim. Therefore, the *sul1* gene may also be linked to co-trimoxazole resistance. A total of 40% isolates amplified *TetA* gene

which is linked to tetracycline resistance, results similar to Petrocchirilo *et al.* (2020) in Spain where 12.5% of the isolates amplified this gene. While *tetA* was only found in two tetracycline-resistant *Pasteurella multocida* isolates, it is important to note that tetracycline resistance genes are often associated with mobile genetic elements (MGEs) such as plasmids and transposons. MGEs can facilitate the horizontal transfer of tetracycline resistance genes between different bacterial strains (Michael *et al.,* 2018). Interestingly, 2 isolates were positive for both *blaTEM* and *sul1* gene and one isolate harbored both *blaTEM* and *tetA* gene. This coexistence of ARG's has been noted in *Pasteurella multocida* isolated from Australia where *blaROB1* and *tetB* has been observed in the same isolate (Dayao *et al.,* 2016). However, the high prevalence of β-lactam and tetracycline resistance in Tanzania is likely due to the widespread use of these antibiotics for the treatment of respiratory infections.

In regard to the amplified genes *blaTEM, sul1,* and t*etA*, 40%, 40% and 60% isolates respectively did not amplify those genes. The absence of resistance genes in the isolates tested but being resistant in-vitro suggests that resistance may be mediated by genes other than those screened for in this study, or by other mechanisms such as point mutations in specific target regions of the bacterial ribosome (Dayao *et al.,* 2016). In this study, we found that *Pasteurella multocida* strains isolated have become rapidly resistant to a wide range of antibiotics. This indicates that most *Pasteurella multocida* strains are now resistant to multiple antibiotics. Multi drug resistance indicates that there is an active gene exchange of antibiotic resistance gene between *Pasteurella multocida* bacteria or between *Pasteurella multocida* with other bacteria. It is noteworthy to say that, if this situation continues, there will be no effective antibiotic therapeutic reserve for this bacterial infection. If many bacteria become resistant to many antibiotics, especially *Pasteurella multocida*, which can infect many different animals, and can spread this resistance to other bacteria in livestock, this could be a serious problem (Hunt *et al.,* 2001; Tang *et al.*, 2009).

3.5 Conclusion and Recommendations

Because *Pasteurella multocida* is a pathogenic microorganism that can infect a wide range of animal hosts, its presence in food-producing animals should not be overlooked. The widespread occurrence of multidrug-resistant *Pasteurella multocida* strains and their association with serious disease strongly indicate the need for more judicious use of antimicrobials. Furthermore, it is important to use antimicrobial agents in food animals in a way that minimizes the development of resistance in both the target pathogens and zoonotic bacteria, in order to protect public health.

Moreover, the high prevalence of untypable *Pasteurella multocida* is a serious concern because it suggests that these strains may be evading traditional typing methods, which could make it more difficult to track their spread and develop effective vaccines and treatments. Further research is needed to understand the significance of untypable *Pasteurella multocida* and to develop new methods for typing these strains. It is important to raise awareness of this issue and to encourage more research in this area, as untypable *Pasteurella multocida* poses a potential threat to public health.

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CHAPTER FOUR

4.0 GENERAL DISCUSSION, CONCLUSION AND RECCOMMENDATIONS

4.1 General Discussion

Pneumonia is a major infectious disease of goats in Tanzania, causing significant economic losses due to death. To effectively treat and control this disease, it is important to understand the antimicrobial susceptibility of the bacteria involved and its molecular characteristics.

In the current study, microbiological and molecular assays identified 2.5% of isolates as *Pasteurella multocida*. This recovery rate is significantly lower than previously reported in the literature: 29.98% by Valadan *et al.* (2014), 20.50% by Tewodros & Annania (2016) and 50% by Rawat *et al*. (2019). Several factors may explain this discrepancy. Either the sample size in our study (200 goats) was considerably larger than those used by Tewodros & Annania (2016) (10 goats) and Rawat *et al.* (2019) (14 goats). This increased sample size may have led to a more accurate representation of the true prevalence within the population, revealing a lower overall rate. Also, geographical variations in environmental factors might play a role. While, studies by Valadan *et al.* (2014) and Tewodros & Annania (2016) were conducted in different regions, Rawat *et al.* (2019) and our study were conducted in geographically similar areas. This suggests that environmental stressors specific to these regions, such as heat, drought, and feed and water shortages, may contribute to a higher prevalence of *Pasteurella multocida*.

Interestingly, our findings align with those of Ikenna (2008) and bAhr *et al.* (2021), which also reported a low prevalence of *Pasteurella multocida* in goats. This further supports the notion that geographical variations and environmental factors significantly impact the prevalence of this pathogen in goat populations.

The capsular serotype of *Pasteurella multocida* is a critical factor in its ability to evade the host immune system and cause disease, and certain serotypes are more commonly associated with respiratory disease in goats. According to Townsend *et al*. (2001), the identification and sequence analysis of the biosynthetic locus of the capsule of an organism can lead to a greater understanding of its capsular polysaccharide composition and can provide a genetic basis for the serological differences observed between strains. This study identified serogroup A as the most prevalent capsular serotype of *Pasteurella multocida* isolated from goats with pneumonic pasteurellosis, accounting for 20% of all isolates. Notably, this finding aligns with previous research conducted in diverse regions, including Germany (Ewers *et al.,* 2006), Brazil (Ferreira *et al.,* 2015) and Iran (Tahmtan *et al.,* 2014), which reported prevalences of serogroup A ranging from 76.3% to 100%. Therefore, these consistent observations highlight the global significance of serogroup A in the etiology of pneumonic pasteurellosis in goats.

Furthermore, genome sequencing of serogroup A isolates confirmed the presence of genes involved in hyaluronic acid synthesis, corroborating its role as the primary component of their capsule. As reported by Chung *et al.* (1998), the serogroup A capsule plays a pivotal role in bacterial virulence by facilitating adhesion to host cells, evading the immune system, and protecting against desiccation. Consequently, this potent capsule likely contributes to the dominance of serogroup A among goats with pneumonic pasteurellosis.

The prevalence of *Pasteurella multocida* serotypes can exhibit significant geographic variation and temporal fluctuations, even within the same region. In this study, a strikingly high proportion of isolates (80%) remained untypable, aligning with the findings of Sahoo *et al.* (2020) in India who reported an untypable prevalence of 43.58%. This observation also finds support in studies conducted by Davies *et al.* (2003) in the United Kingdom (1% untypable strains from pigs), Tang *et al.* (2009) in China (5.2% untypable strains from swine), and Güler *et al.* (2013) in Turkey (55.5% untypable isolates from goats).

Christensen *et al.* (2022) further identified a potential explanation for the observed untypable strains. Their whole-genome sequencing (WGS) analysis of reference *Pasteurella multocida* strains revealed mutations or deletions in genes encoding capsular and outer core LPS components. This suggests that further investigation is needed to document the phenotypic changes associated with these genetic alterations.

Furthermore, Sahoo *et al.* (2023) provide evidence suggesting a potential link between untypable strains and increased virulence. This raises the intriguing possibility that the untypable phenotype may enhance the ability of these bacteria to evade the host immune system, thereby contributing to their heightened virulence. There are two possible explanations for why capsule typing might fail: either the emergence of novel, yet-undiscovered capsule types: This scenario suggests the existence of previously unknown capsular antigens that current typing methods are unable to identify or mutations affecting capsule formation.

While scientists do not fully understand how *Pasteurella multocida* causes disease or why it is specific to certain hosts, several studies have found that a number of virulence factors (VFs) are associated with its pathogenic mechanisms (Hunt *et al.,* 2000; Harper *et al.*, 2006). Iron acquisition is an important virulence factor for *Pasteurella multocida*, as it allows the bacterium to adapt to different iron environments in different hosts and at different stages of infection (Sarangi *et al.,* 2015). Among the two iron acquisitions genes (*exbB* and *exbD*) which were screened in this study, 100% of the isolates were detected to confer *exbD* gene while none of the isolates conferred *exbB* gene. The fact that most isolates possess the *exbD* gene suggests that the gene is important for the survival and fitness of *Pasteurella multocida* isolates in a variety of environments. It is possible that the *exbD* gene is being transferred between *Pasteurella multocida* isolates through gene exchange since most virulence genes are linked to plasmids which allows them to be easily shared among bacteria. This could explain why all of the isolates possess the *exbD* gene.

In this work, the high prevalence of an adhesion-related gene *ptfA* was noted (60%), a finding that was reported in some previous studies (Sarangi *et al.,* 2015; Aski & Tabatabaei, 2016). It has a major role in initial colonization of the bacterium in the upper respiratory tract (Nguyen *et al.*, 2023).

This study reveals a worrying trend of high antimicrobial resistance among *Pasteurella multocida* isolates, with all isolates showing resistance to at least five antibiotics. Notably, 100% resistance was observed against commonly used antibiotics like ampicillin, amoxicillin, erythromycin, co-trimoxazole and cefotaxime. Additionally, high resistance was detected for pefloxacin, chloramphenicol, and tetracycline. This widespread resistance is likely driven by multiple factors, including the overuse of antibiotic additives in animal feed and the extensive application of antimicrobial agents in veterinary medicine (Kehrenberg and Schwarz, 2007; Rodríguez-Martínez *et al.,* 2011).

Analysis of specific antibiotic resistance genes revealed the presence of *blaTEM* (60%) associated with ampicillin resistance, *sul1* (60%) linked to sulfonamide and co-trimoxazole resistance, and *TetA* (40%) responsible for tetracycline resistance. Interestingly, some isolates harbored multiple genes, highlighting the complex interplay between various resistance mechanisms. Despite the presence of these resistance genes, a significant proportion of isolates did not amplify any of the tested genes. This suggests that alternative mechanisms, such as point mutations in specific target regions of the bacterial ribosome, might contribute to the observed resistance in vitro (Dayao *et al.,* 2016).

Our findings reveal a disturbing trend of rapidly evolving resistance to a broad spectrum of antibiotics in *Pasteurella multocida* strains. This signifies that the majority of these strains now exhibit multidrug resistance, suggestive of active exchange of resistance genes between *Pasteurella multocida* bacteria and/or other bacterial species. Such unchecked evolution poses a significant threat, potentially leading to a future where effective antibiotic therapies for *Pasteurella multocida* infections are nonexistent. This scenario would

be particularly alarming given *Pasteurella multocida's* ability to infect diverse animal species and propagate resistance further among livestock bacteria. Urgent action is required, including prudent antibiotic use, robust biosecurity measures, and exploration of alternative treatment strategies, to combat this growing problem and protect animal health.

4.2 General Conclusion and Recommendation

Though the study reveals a concerning rise in antibiotic-resistant *Pasteurella multocida,* particularly with increasing resistance to tetracyclines, cephalosporins, penicillin, and macrolides, the low isolation rate of the bacterium presents a double-edged sword. While suggesting a lower prevalence, it might also underestimate the true threat, leading to missed infections and delayed treatment, especially concerning given its opportunistic nature in worsening existing infections. The difficulty in isolating and diagnosing *Pasteurella multocida* due to its competition with other bacteria in culture media further complicates matters, potentially leading to misdiagnosis or delayed diagnosis and impacting treatment efficacy. Additionally, the emergence of untypable isolates presents a significant hurdle in surveillance and control efforts, as traditional typing methods might not be effective for these isolates. This is particularly worrisome for food-producing animals, where the presence of resistant *Pasteurella multocida* raises concerns about zoonotic transmission and potential public health impacts. Therefore, judicious antibiotic use in veterinary medicine is crucial to minimize resistance development, while increased research funding for novel control strategies like vaccines, alternative treatments, and improved diagnostics for untypable isolates is urgently needed. Moreover, collaboration between public health officials, veterinarians, and livestock producers is essential to implement effective mitigation strategies and protect both animal and public health. In conclusion, while the study highlights a concerning trend, addressing the challenges of underestimation, diagnosis and untypable isolates through improved diagnostics, research and collaboration is crucial to effectively control this potentially zoonotic threat.

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APPENDIX

Appendix 1: Plagiarism report

DISSERTATION IRENE

ORIGINALITY REPORT

13%	13%	11%	1%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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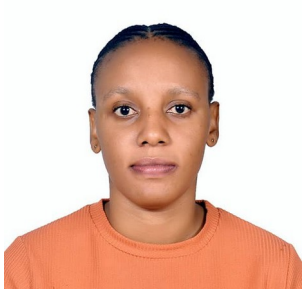

Kuhusu Tasnifu Hii

Ugonjwa wa mapafu wa nimonia unaosababishwa na bakteria *Pasteurella multocida* ni tishio kiuchumi kwa wafugaji wa mbuzi kaskazini mwa Tanzania. Utafiti huu ulichunguza upinzani wa antimicrobial na jeni za ukali kwenye bakteria hizi. *Pasteurella multocida* ilitambuliwa kwa asilimia 2.5 ya sampuli, ingawa asilimia 20 ilikuwa serogroup A. Maumbile yalionyesha upinzani kwa dawa nyingi, lakini gentamicin na ciprofloxacin hawakuonyesha upinzani. Uwepo wa upinzani wa wigo mpana unahitaji mikakati mipya ya kudhibiti ugonjwa huu. Utafiti pia uligundua jeni za upinzani kama vile *blaTEM* na *sul1*. Changamoto za upinzani na maumbile yasiyofunguliwa zinahitaji utafiti zaidi na ushirikiano wa kina kati ya wadau. Kwa muhtasari, kudhibiti pneumonic pasteurellosis inahitaji juhudi za pamoja za kuzuia na kutibu, pamoja na ushirikiano wa jamii zinazohusika.