GENETIC CHARACTERIZATION AND ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF *PROTEUS MIRABILIS* ISOLATED FROM DOMESTIC RATS IN ARUSHA MUNICIPALTIY, TANZANIA

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EXTENDED ABSTRACT

The aim of this study was to genetically characterize and assess antimicrobial susceptibility patterns of *Proteus mirabilis* isolates from rats cohabiting with human dwellings. A cross sectional survey was conducted where a total of 139 rats were trapped in houses and around peri domestic areas in selected wards in Arusha city. Following euthanization, rats were identified to Genus/species level using morphological and morphometric features. Deep intestinal swabs were ascetically obtained and pre enriched in buffered peptone water prior laboratory analysis conventional culture methods and biochemical methods were used for bacterial isolation. Molecular confirmation of the isolates was done using the 16s ribosomal RNA PCR identification method. Susceptibilities to Tetracycline (TE, 30µg), Trimethoprim sulfamethoxazole (SXT, 25 µg), Ciprofloxacin (CIP, 5µg), Cefotaxime (CTX,5µg), Ampicilin (AMP,10µg), Azithromycin (AZM, 15µg) and Gentamycin (CN,10µg) was performed and resistance genes (*bla TEM, tetA, tetB, mphA, SHV, bla CTX-M, sul1* and *sul2*) were traced in each isolate using PCR methods. Mixed rat species *Rattus rattus* (55.4%; n=77) *Mus musculus* (15.8%; n=22) and *Mastomys natalensis* (28.8%; n=40) were captured. *Proteus mirabilis* was isolated from 4 samples (2%) from *Rattus rattus*. All isolates were 100% similar to *P. mirabilis* strains from NCBI. Constructed phylogenetic tree showed all *P. mirabilis* isolated from this study were closely related to Tunisia isolates. Three isolates showed MDR trait against Triomethoprim- sulfamethaxole, Azithromycin, and Ampicillin., all isolates were resistance to Azithromycin, and Ampicillin, three were resistance to Triomethoprim- sulfamethaxole, and intermediate to Tetracycline and all susceptible to Ciprofloflaxcin, Gentamicin and Cefotaxime, PCR analysis showed the presence of *TetA, blaTEM, Sul1* and *Sul2* resistant genes in all isolates. The study has shown that rats are potential reservoirs of *Proteus mirabilis* with antimicrobial resistance trait that could be

transmitted to the public and it's the first study to isolate *P. mirabilis* from rats in Tanzania with antimicrobial resistance trait.

DECLARATION

I, Floramanka Patrick Ndakidemi, 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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(Candidate:MSc. Public Health and Food Safety)

The declaration is hereby confirmed by;

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Dr. Abdul A.S. Katakweba Date

(Supervisor)

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Dr. Alexander Mzula Date

(Supervisor)

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DEDICATION

To my beloved parents Professor Patrick Ndakidemi and Mrs. Esther Ndakidemi who took me to school.

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ABBREVIATIONS AND ACRONYMS

BLAST	Basic Local Alignment Search Tool
Bp	base pairs
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended Spectrum Beta Lactamases
FCSN	Female vagina closed
FPSN	Female vagina perforated nipple small
FPSY	Female perforated pregnancy lactating
gDNA	Genomic deoxyribonucleic acid
GPS	Global positioning system
IgA	Immunoglobulin alpha
IgG	Immunoglobulin gamma
LPS	Lipopolysaccharide
MDR	Multi drug resistance
M.M	*Mus musculus*
MAN	Male testes abdominal
MAST	*Mastomys natalensis*
ml	Microlitre
WHO	World health organization
SUA	Sokoine university of Agriculture
CVMBS	College of veterinary medicine and biomedical sciences
XLD	Xylose Lysine Deoxycholate agar
GIS	Geographic information system

USA	United states of America
LTD	Limited
mm	Millimeter
MSV	Male scrotal visible
NCBI	National Center of Biotechnology Information
PCR	Polymerase chain reaction
R.R	*Rattus rattus*
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SGI	Salmonella Genomic Island
TAE	Tris acetate EDTA buffer
URT	United Republic of Tanzania
UTI	Urinary Tract Infection
UV	Ultraviolet
XDR	Extensive drug resistance

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Rodents are a successful mammalian group reported as pests that cause momentous loss in agricultural crops in many tropical regions (Singleton *et al.,* 2003). They are also a significant threat to humans and animal health as they compromise food security and act as reservoirs of pathogens and vectors such as bacteria, rickettsia, viruses, fungi, parasites (protozoan, helminthes) and fleas that cause diseases (Meerburg *et al.,* 2007; Ribas *et al.,* 2016; Eisen *et al.,* 2018). Several findings report on the direct and indirect consequences imposed when rat populations in some regions are not controlled resulting to transmission of zoonotic diseases to humans and domestic animals that can either have remedy or not. This may include, infectious diseases such as plague, listeriosis, salmonellosis, Trypanosomiasis, toxoplasmosis, leshmanisasis, leptospirosis, rat bite fever, Tularemia, Hanta virus pulmonary syndrome and gastroenteritis (Battersby *et al.,* 2002; Meerburg *et al.,* 2007; Cummings *et al.,* 2012; Ayyal *et al.,* 2019; Stanaway *et al.*, 2019).

*Proteus mirabili*s are opportunistic pathogens belonging to the family *Morganellaceae* of major medical and veterinary significance (O'Hara *et al.,* 2000; Jemilehin *et al.,* 2016). These pathogens are zoonotic (Morand *et al.,* 2015; Ogunleye *et al.,* 2016; Ayyal *et al.,* 2019). Particularly they are normal flora to humans, avian and other mammals like rodent's gastrointestinal tracts and kidneys. In addition, *Proteus mirabilis* are also reported as environmental microbes and play an important role in decomposing organic matter of animal origin (Robertson, 1986; O'Hara *et al.,* 2000). Further, scientific evidence show that they are of clinical significance as they have contributed to opportunistic infections, nosocomial outbreaks and mostly hematogenous and ascending urinary tract infections (Mirzaei *et al*., 2019). *Proteus mirabilis* species are reported as the third most prevalent

(12%) pathogen after *Escherichia coli* and *Klebsiella pneumoniae* as etiology of human urinary tract infections (UTI) and second after *Providencia stuartii* as etiological agent of catheter-associated bacteriuria in the group of long- term catheterized patients (Warren, 1996). Literature reveals that these pathogens have been underrated as gastrointestinal pathogens (Hamiliton *et al.,* 2018; Zhang *et al.,* 2021). Apart from being isolated from a wide range of environments like soil, polluted water sources, sewage systems, humans, poultry meat, banknotes, bats and rodents (Nagano *et al.,* 2003; Mukhtar *et al.,* 2018; Ayyal *et al.,* 2019; Lakshmi *et al.,* 2020), poultry farms infested with rodents are also reported to harbor these organisms (Jemilehin *et al*., 2016; Ogunleye *et al.,* 2016; Ayyal *et al*., 2019). This implicates that these pathogens can be transmitted inherently to humans, and other domesticated animals thus imposing clinical threat.

The above pathogens are normally treated with commercial drugs. However, resistance to antimicrobials is an emerging issue to both veterinary and human medicine (Knodler *et al.,* 2019; Tack *et al*., 2020). It has been established that effects imposed by antimicrobial resistance have a great impact on animals and human health, where animals and humans share the same environment (Katakweba *et al.,* 2012a; Jemilehin *et al.,* 2016; Sonola *et al.,* 2021). To-date efforts to reduce antimicrobials resistance rely on bacterial populations as a result of exposure to antimicrobials and that restricting the use of antimicrobials (Wakawa *et al.,* 2015). This, however may not conclusively apply as antimicrobials resistance has been found to be prevalent in populations of rodents due to passage of resistant microorganism from humans to animals, between humans, and from animal to humans (Stanaway *et al.,* 2019). In view of the above, this highlights the public health importance of rodents in transmitting resistant *Proteus mirabilis* to other organisms and hence the urgency control and management of these pests (Osman *et al.,* 2014; Jemilehin *et al.,* 2016; Ogunleye *et al.,* 2016; Ayyal *et al.,* 2019). Therefore, screening of

rodents in close association with human homes for their carriage of resistant *Proteus mirabilis* could be helpful to determine their role in disease transmission and antimicrobial resistance patterns in not only humans but also animals due to sharing of the ecosystem (Doublet *et al.,* 2010; Wakawa *et al.,* 2015).

In Tanzania, *Proteus* spp have been documented in rodents and other wildlife (Mununa, 1996). However, to date little is known on the direct or indirect epidemiological role played by rodents as carriers and transmitters of *Proteus mirabilis* especially those with antimicrobial resistance trait (Doublet *et al.,* 2010; Ayyal *et al.,* 2019). Therefore, considering the potential public health threat posed by rodents due to their presence and closeness to human and animal's habitations patterns, the current study focused at characterizing and investigating the antimicrobial susceptibility of *Proteus mirabilis* isolates from house rats in Arusha city, in northern Tanzania.

1.1 Rodents

Rodents are among the most abundant and successful mammalian group in the world consisting of about 5000 species. This is because of their high reproduction rates and ability to survive in various habitats such as those found in (terrestrial, fossorial, aquatic and arboreal area (Singleton *et al*., 2003). Rodents are native to all continents of the world except in the Antarctica. The order Rodentiais comprised of over 30 families including that of rodents and mice (Family Muridae). Other families are those of squirrels, rabbits, hamsters, guinea pigs, marmots, and porcupines (Aplin *et al*., 2003). Animals in this order are characterized based on body morphology, having diastema (gap between incisors and pre molar teeth) that enables chewing and gnawing (Figure. 1.1). Other features include possession of rooted or rootless ever growing pair of incisors (Aplin *et al*., 2003).

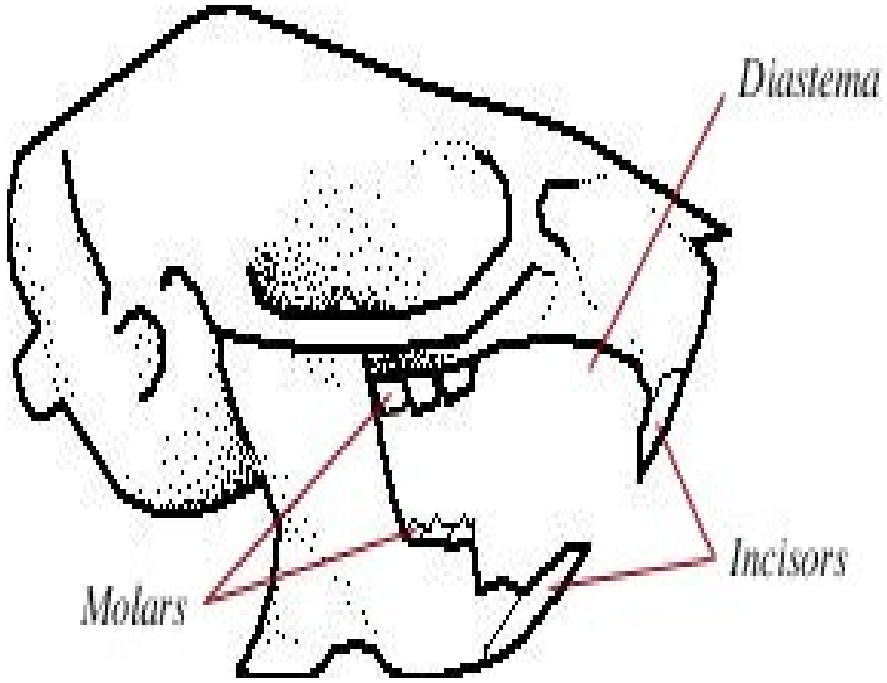

Figure 1.1: Rodent skull showing diastema, and orientation of incisors

Source: https://lh3.googleusercontent.com accessed on 24/08/2020

Human populations are likely to encounter devastating effects due to rat infestations in their localities such as infrastructural damage, distraction of stored grains and are mainly involved in causing over 20 animal and human disease (Singleton *et al.,* 2003). In India, black rats are used in cultural and religious practices, in some traditions. These rats are allowed to consume some foods before human consumption to indicate good luck upon community members (Singla *et al.,* 2008). This is a risky practice as humans may be exposed to some potential pathogens. Several authors have reported a wide range of zoonotic and non- zoonotic diseases that can place rats as definitive or intermediate agent in the process (Battersby *et al.,* 2002; Meerburg *et al.,* 2007;Cummings *et al.,* 2012; Ribas *et al.,* 2016; Eisen *et al.,* 2018; Ayyal *et al.,* 2019; Stanaway *et al.,*2019) these include plague, Lassa fever, listeriosis, salmonellosis, toxoplasmosis, leshmanisasis, rat bite fever, plague, Tularemia, Hanta virus pulmonary syndrome, gastroenteriris, murine typhus, tularemia, and leptospirosis (Eisen *et al.,* 2018). Despite of their negative implications,

rats play an important role in the ecosystem as they are food sources to diverse animals like reptiles, birds, small carnivore mammals and also humans in some parts of the world who consume rats like *Mastomys natalensis* and *Thryonomys* species (Singleton *et al.*, 2003). Furthermore, rats like African giant rats (*Cricetomys spp*) were recently deployed in biosensor technologies such as detecting Mycobacterium tuberculosis in sputum samples and landmines placed on the ground (Beyene *et al.*, 2012; Mgode *et al.*, 2012).

Species identification in rats can be done using morphological and molecular methods (Aplin *et al*., 2003). Table 1.1 indicates a summary of morphological features that can be used to identify different species of rats that can be trapped around house areas, except for *Matomys natalensis* which is easily identified. However a study by Matisoo-Smith and Allen, (2001) reported that molecular identification methods are more precise in species compared with the morphological identification methods. Based on what is presented in Table 1.1 (Cunningham, and Moors, 1983), the following should be noted:

1. Identification using morphological features is dubious therefore it is best to keep body measurements and animals head for further detailed examination
2. It is difficult to distinguish juvenile rats from mice hence body measurements should be kept, also specimen preservation methods like freezing or fixing can be used to assist for further investigation assays
3. *Rattus rattus* species exist in three color forms/ morphs
   (a) “rattus” uniformly black back (sometimes has a blueish look); uniformly grey belly.
   (b) “alexandrinus” brown back with long black guard hairs; uniformly grey belly.
   (c) “frugivorous” brown back with long black guard hairs; uniformly white or creamy-white belly.

Table 1.1: Summary of morphological features that are used in house rats identification*

	House mouse ***Mus musculus***	**Ship rat** ***Rattus rattus***	**Norway rat** ***Rattus norvigecus***
Normal adult weight	Up to 25g	Up to 225g	Up to 450g
Maximum body length (HBL)	115mm	225mm	275mm
Tail length	Slightly shorter or longer than HBL. Uniformly grey brown	Much longer than HBL, uniformly colored	Clearly shorter than HBL. Thick with pale underside
Ears	12.0- 15.0mm	19.0-26.0mm	14.0- 22.0 mm, do not cover eyes when pulled forward. Obvious hairs extend beyond edge of ear
Adult hind foot	15.0-21.0mm thin and small	28.0-38.0mm	30.0-41.5mm
Color of upper side of hind foot	Uniformly grey	Uniform coloring over whole foot, usually dark	Always completely pale
Fur on back	Dull grey- brown	Brown or black (see note 3)	Brown
Fur on belly	Uniformly grey	Uniform monotone of grey, white or creamy white (see note 3)	White- tipped grey giving irregular color
Length of droppings	3.9-7.6mm	6.8-13.8mm	13.4-19.1mm
Number of nipples	10-12mm	10-12, usually 10	12
Habitats	Agile climber, not known to burrow but digs small holes; nests mainly on ground; feeds on ground and in trees; infrequently swimmer	Very agile and frequent climber; rarely burrows; nests mainly in trees and shrubs; infrequent swimmer	Burrows extensively; climbs much less frequently than other rats; strong swimmer; nests underground; very wary

*Cunningham & Moors (1983)

Identification of sex in rats is done by observing mainly three distinct features which are urogenital distance (distance between the urethral opening and the anus), presence of testes and presence of nipples. In immature females, the vaginal is closed (covered with a translucent membrane) and the urogenital distance is small. While mature females have open/ perforated vagina and nipples are present. On the other hand, immature males have longer urogenital distance and scrotal sacs are visible (Figure 1.2) Cunningham and Moors (1983). Therefore, it is essential to study and identify different types of rats as this will provide insights of types found in the ecosystem and design management strategies to control them.

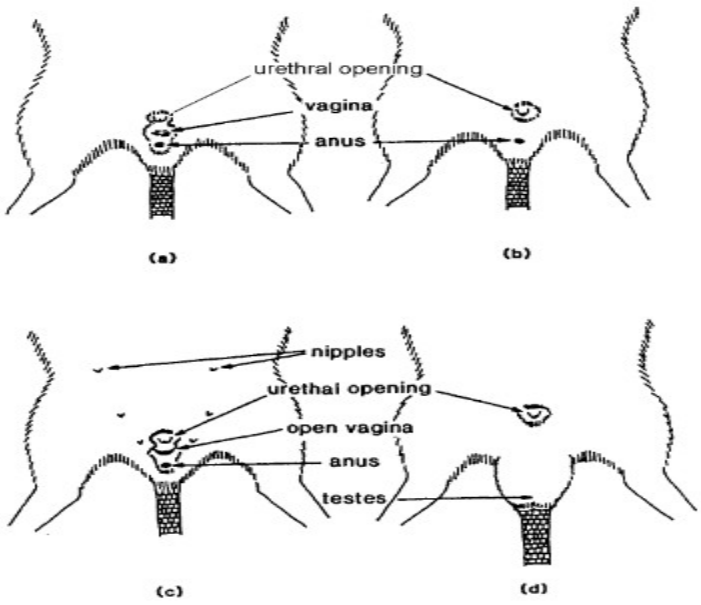

Figure 1.2: Reproductive structures in female and male rats

a=immature female, b= immature male, c= mature female and d= mature male.

Source Cunningham & Moors (1983)

1.2 *Proteus mirabilis*

1.2.1 Definition

The genus was first described in 1880's by Hauser (Hauser, 1885). The term *Proteus* means "changeability of form, as symbolized in the Homericpoems in *Proteus* and has the gift of endless transformation" (Wenner and Rettger., 1919), hence named based on their abilities to swarm on solid media. To date, the genus consists of five renowned species (*P. mirabilis*, *P. penneri*, *P. vulgaris*, *P. myxofaciens*, and *P.hauseri*) and three unnamed genomospecies (*Proteus* genomospecies 4, 5, and 6) (O'Hara *et al*., 2000). Generally, they are gram negative, facultative anaerobes, rod shaped, non-spore forming, and motile bacterium. Most strains have similar characters as they give a swarming growth pattern, decompose urea, liquefy gelatin, may form gas from glucose but in small amounts, acidify xylose, and do not do so to mannose, mannitol, adonitol, inositol with exceptions to lactose (O'Hara *et al*., 2000). Hauser (1885) named the genus *P. mirabilis* possibly based on their ability to have swarming ability with intertwined with a change of short swimmer cells into long poli-nucleated and easily excitable flagellated swammer cells. This agrees to its counterpart Latin meaning of mirabilis as amazing, splendid or marvelous character (Hauser, 1885).

1.2.2 Taxonomic classification

Members of the genus Proteus were formerly placed by Hauser under Domain Bacteria, Phylum proteobacteria, class Gammaproteobacteria, and order Enterobacteriales, Family *Enterobacteriaceae*, Genus Proteus and Species *P. mirabilis*. However, recently taxonomic classification and reconstruction of phylogenetic tree basing on shared ribosomal proteins, core proteins and multilocus protein sequences that were analyzed recommended the reclassification of the order Enterobacteriales, therefore, placing Proteus within a new *Morganellaceae* family thus being close to the genera Providencia

and Morganella as they are closely related (Adeolu *et al*., 2016). Studies on taxonomic classification are important as this will entail proper categorization of the organisms and hence their biological information Figure 1.3.

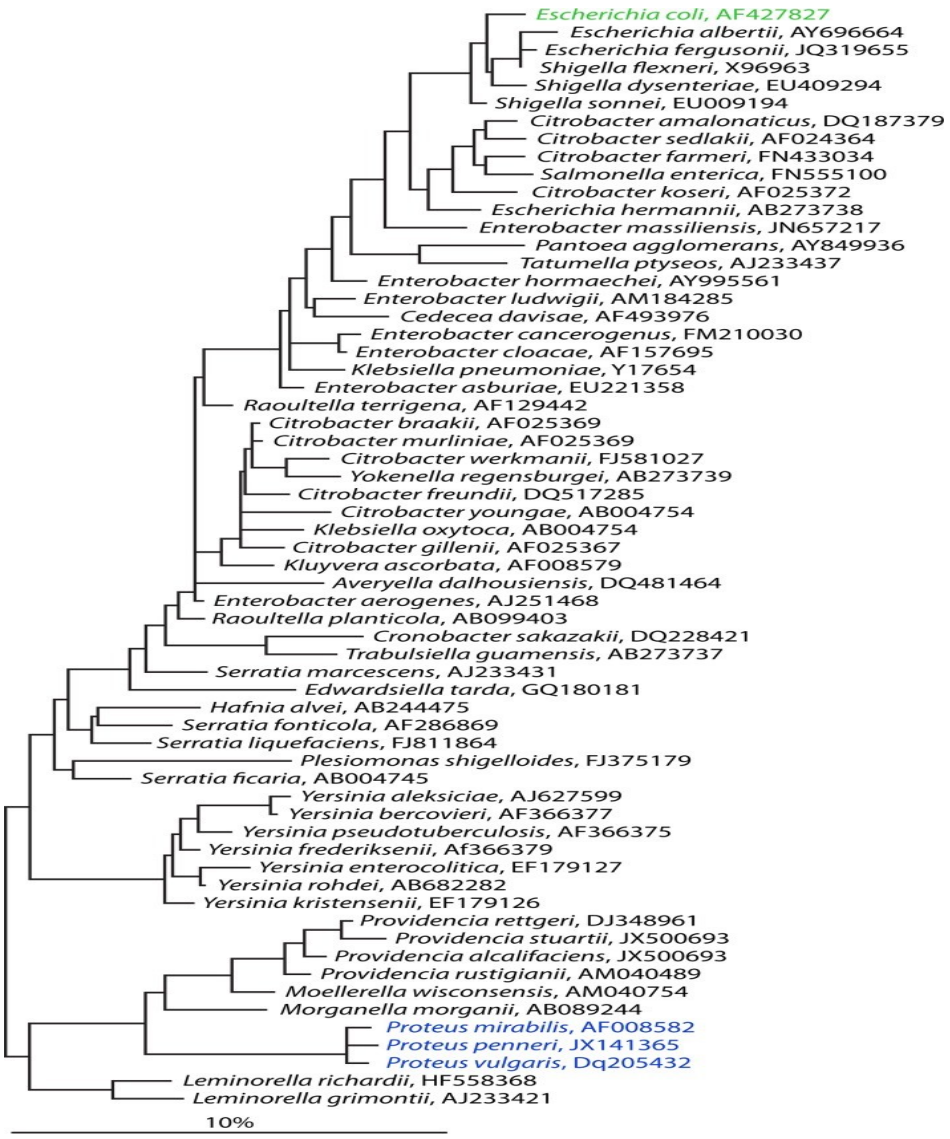

Figure 1.3: Taxonomic tree showing the species from the Enterobacteriaceae family (example *E. coli* highlighted in green) also showing members of the Morganellaceae family in one clade (*Providencia*, *Morgonella* and *Proteus* species in blue) accession numbers of the16S rRNA gene sequences are provided for each species.

Source: Rajilić-Stojanović and de Vos (2014).

1.2.3 Habitat

Organisms of this genus are spatially distributed and have successful been isolated from a wide range of environment and are generally regarded as opportunistic pathogens and intestinal commensals (O'Hara *et al.,* 2000). Their occurrence in soil indicates recent contamination with animal or human fecal wastes, hence acting as fecal indicator pathogens. For example, Hauser (1885) was able to isolate these organisms from 23 out 52 garden samples. They are frequently present in contaminated waters like sewage systems, sluggish streams, and stagnant pools. Moreover they are frequently isolated from vegetables and fecal matters of animals and humans (Hamiliton *et al*., 2018). Apart from its mesophilic and saprophytic property, members of this genus have been isolated from clinical samples like urine, pus, ear discharges, liver abscesses, and blood from septicemia and typhus fever patients (O'Hara *et al.,* 2000).

1.2.4 Culture methods

As they are abundantly found in the environment, *P. mirabilis* are easy to culture as they can grow on both complex and chemically defined media (Pearson, 2021). Routine culture from mixed indwelling sites such as from urinary tract catheters, sewage, and gastrointestinal tract can be performed on MacConkey and Blood agar although culture on the latter is difficult as swarming is excessive due to richness in the media (Pearson, 2021). To make things easy for further molecular and microbiological applications, differential media such as Xylose Lysine desoxycholate (XLD) agar is proposed to be used from which *P. mirabilis* presumptive colonies exhibit yellow, transparent, with clear edges, black center although further biochemical (Table 1.2) and serological tests should be performed to confirm isolation (Pearson, 2021).

Table 1.2: Biochemical properties of *P. mirabilis* that differentiates it from other *Proteus strains*

Test of description	*P. mirabilis*	*P. penneri*	*P. vulgaris*		*P.myxofaciens*
			Biogroup 2	Biogroup 3	
Orinithine decarboxylase	+	-	-	-	-
Maltose fermentation	-	+	+	+	+
Salicin fermentation	-	-	+	-	-
D-Xylose fermentation	+	+	+	+	-
Esculin hydrolysis	-	-	+	-	-
Indole production	-	-	+	+	-
Human pathogen	+	+	+	+	-
Insect pathogen	-	-	-	-	+

Source: Rozalski *et al*. (1997)

1.2.5 Molecular identification methods

Advancement in diagnosis of bacterial nucleic acids brought about development of highly sensitive and specific nucleic acid detection methods. 16S rRNA PCR amplification method is commonly used as conventional detection method since bacterial culture and biochemical methods used before do not exhibit distinguishable phenotypic traits (Wong *et al*., 2013). Next generation sequencing methods have been robust and reliable to characterize bacterial genome whereas PCR amplification of virulent genes in targeted bacterial genome of *P. mirabilis* genome will use primers that will amplify *UreC, ZapA, hpmA,* and *aac(6)-lb* genes (Zhang *et al*., 2021).

1.2.6 Antibiotic resistance

Proteus mirabilis have intrinsic antimicrobial resistant. For instance, polymyxin resistance is managed through modifications of LPS at lipid A site also due to acquisition of *eptC* gene which plays a role in modifying the LPS core (Olaitan *et al*., 2014). Again, they also have inherent resistance to tetracycline, colistin, and tigecycline (Zhang *et al*., 2015). Studies have demonstrated the presence of genes for a conjugal transfer pilus in *Proteus mirabilis* sequences, which allows the horizontal genetic transfer of plasmids encoding antimicrobial resistance (Andrea *et al*., 2011). Meanwhile, most species of *Proteus* remain sensitive to a range of antimicrobials, acquired antimicrobial resistance including *Proteus mirabilis* has escalated and is a global threat (Iredell *et al*., 2016). Between 2007 and 2010, *P. mirabilis* strains were reported to attain *Salmonella* genomicisland 1 (SGI1) in China, Palestine and England (Doublet *et al*., 2010; Iredell *et al*., 2016). SGI1 is a mobilegenomic element first identified in *Salmonella Typhimurium* that integrates into the recipient chromosome and carries multiple genes encoding resistance to streptomycin, trimethoprim, tetracycline, sulfonamides, chloramphenicol, fluoroquinolones, and abroad spectrum of B-lactam antibiotics (Iredell *et al.*, 2016). Recently, an SGI1-positive *P. mirabilis* strain PM58 attained a plasmid containing the New Delhi metallo--lactamase 1 gene that has extensively drug-resistant (XDR),which is resistant to all antibiotics used for *Enterobacteriaceae* except aztreonam (Qin *et al*., 2015).

1.2.7 Significance of *Proteus mirabilis* to natural environments

Proteus mirabilis as commensal and opportunistic pathogens to humans, colonize several niches in human bodies due to the virulent factors they possess that account to their pathogenesis (O'Hara *et al.*, 2000). In humans, these pathogens can cause a range of suffering from urinary tract infections also causing cross infectious and auto infectious

UTI, nosocomial infections, wound infections, bacteremia, meningitis, respiratory infections (Hamiliton *et al*., 2018). Again, they are postulated as putative intestinal pathogens as they cause food poisoning and Crohn's disease (Zhang *et al.*, 2021).

Domestic and wild animals (avian, mammals, reptiles, amphibians, seafood and insects) have been studied by several groups of researchers and reported presence of *P. mirabilis* as hosted symbiotically with these organisms and cause diseases like urinary tract infections in dogs, donkeys and cats; also they are associated with wound infections in horses. Further, they can cause swine tonsil. In birds, they highly penetrate the egg shell therefore causing embryonic mortality and further they can cause infection in turtles. They have been successfully isolated in shrimp where they act as probiotics (Rozalski *et al*., 1997). Isolation of these organisms from the environment indicates environmental fecal pollution. Their presence in the environment is advantageous as their ability to produce nitrogen is essential for plants growth and also their ability to take up some metal such as iron and zinc enables bioremediation in polluted environments. However, *Proteus mirabilis* strains carrying antimicrobial resistance genes have been successful isolated from humans, animals and from the environment, particulate land and water sources due to extensive urbanization and rapid growth of industries. Therefore pose threat in disease transmission and spread of resistance genes to pathogenic and commensal microflora (Qin *et al.*, 2015). It is important to study the relationship between *Proteus* spp found in the natural environments and those found in the human and animal guts to establish if the environmental *Proteus* spp. strains differ significantly from the clinical types.

1.2.8 Control of antimicrobial resistance in humans, animals and the environment

Control strategies such as monitoring antimicrobial use and resistance in humans, animals and environment by using initiatives like drug licensing, banning use of certain drugs like

tetracyclines, streptomycins and penicillins in *P. mirabilis* infections, altering prescriber's behaviors by keeping restrictions, educating users, improving diagnostics, and creating prudent use guidelines, improving animal health and improving personal hygiene (Aarestrup *et al*., 2008).

1.3 Problem Statement

Emergence and wide spread of multidrug resistance in *Proteus mirabilis* has been reported (Girlich *et al.,* 2020; Zhang *et al.,* 2021). This is due to acquired resistance to commonly used antimicrobials (Doumith *et al.,* 2009). The ability to detect Salmonella genome island 1 variant SGI1-0, Extended Spectrum Beta Lactamase (ESBL), AmpC-type cephalosporinase and carbapenemases producers which are responsible for multidrug resistance in *Proteus mirabilis* indicates a threat (Girlich *et al.,* 2020) as this trait can be transferred to other bacteria of medical and veterinary importance. Resistance genes are acquired through plasmids, integrons, insertion sequences and transposones mediated with antimicrobial resistance traits in their cells facilitating horizontal gene transfer of resistant plasmids as they have the conjugal transfer pilus (Doublet *et al.,* 2010; Andrea *et al.,* 2011; Iredell *et al.,* 2016).

Resistance to commonly used antimicrobials for the treatment of bacterial infections in animals and human has been studied and reported in Tanzania and many parts of the world (Adesiji *et al.,* 2014; Gaspary *et al.,* 2016; Katakweba *et al*., 2018; Sindiyo, 2019; Sonola *et al*., 2021). However, little is known on the role played by rodents as carriers and transmitters of *Proteus mirabilis* with antimicrobial resistances traits. Therefore, considering the potential public health threat posed by rodents to humans and domestic animals due to their presence in urban and peri-urban areas and their closeness to humans,

this study focused at characterizing and investigating the antimicrobial susceptibility profile of *Proteus* spp isolated from house rodents in Arusha city, in northern Tanzania.

1.4 Justification

Identifying, characterizing, and investigating antimicrobial susceptibility profile of *Proteus mirabilis* isolated from house rodents in the study area will provide an insight on the burden of resistant isolates in house rodents with conceivable cause of multidrug resistance in humans and domestic animals implicating significant public health threat. Little is known on the type of house rodent species found in Arusha together with resistant genes harbored in *Proteus mirabilis*. This information will help in setting up prevention and control strategies of rodents in Arusha and other parts with similar pests. Therefore, this study will contribute to the Tanzania Vision 2025 and Sustainable development goals by 2030 and hence accounting to high quality livelihood, good health and wellbeing. It will also contribute to improving treatment regimens for humans and other veterinary animals suffering from infections after revealing resistance patterns from the commonly used antimicrobial drugs.

1.5 Research objectives

1.5.1 General objective

To explore genetic characterization and antimicrobial susceptibility patterns of *Proteus mirabilis* isolated from domestic rats in Arusha Municipality, in northern Tanzania.

1.5.2 Specific objectives

i. To identify species of domestic rats from the study area
ii. To isolate and characterize *Proteus mirabilis* from different house rats species,
iii. To determine antimicrobial susceptibility patterns of *Proteus mirabilis* isolates using commonly used antimicrobials in Arusha.
iv. To detect resistance genes in isolated *Proteus mirabilis* strains,

CHAPTER TWO

Manuscript One

Prevalence of Proteus mirabilis isolated from domestic rats in Arusha municipality, Tanzania

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Summary

*Proteus mirabili*s is a bacterial pathogen of the major medical and veterinary significance in humans that contributes to opportunistic and urinary tract infections. Presence of rats cohabiting with humans can inherently have a zoonotic status to humans due to rats-human-environment interaction nature. It is therefore essential to assess the prevalence of *P. mirabilis* from rats cohabiting with humans for literature confers that its one of the underrated bacteria reported to associate with human and animal diseases. A total of 139 rats were trapped in houses and around peridomestic areas in different wards in Arusha city. Following euthanization, rats were identified to genus/species level using morphological and morphometric features. Deep intestinal swabs were aseptically obtained and pre-enriched in buffered peptone water before laboratory analysis. Conventional culture and biochemical methods were conducted for bacterial isolation and identification. Mixed rat species, *Rattus rattus* (55.4%) *Mus musculus* (15.8%) and *Mastomys natalensis* (28.8%) were captured. *Proteus mirabilis* was isolated from 4 samples (2%), all of which were from *Rattus rattus.* Molecular confirmation of the isolates was done using the 16sRNA PCR identification method and sanger sequencing was performed for precise confirmation. After sequencing all isolates were 100% similar to *P. mirabilis* strains from NCBI. The Constructed phylogenetic tree showed all isolates from this study were closely related to Tunisia isolates. The study has discovered the first *Proteus mirabilis* isolates from rats in Tanzania that could be transmitted to the public.

Keywords: *Proteus mirabilis*, rats, zoonotic, public health, 16S rRNA , Arusha

Introduction

Rodents are a successful mammalian group reported as pests that cause momentous loss in agricultural crops in many tropical regions (Singleton *et al.,* 2003). They are also a significant threat to humans and animal health as they compromise food security and act as reservoirs of pathogens and vectors such as bacteria, rickettsia, viruses, fungi, parasites (protozoan, helminthes) and fleas that cause diseases (Meerburg *et al.,* 2007; Ribas *et al.,* 2016; Eisen *et al.,* 2018). Several findings report on the direct and indirect consequences imposed when rat populations in some regions are not controlled resulting to transmission of zoonotic diseases to humans and domestic animals that can either have remedy or not. This may include, infections diseases such as plague, listeriosis, salmonellosis, Trypanosomiasis, toxoplasmosis, leshmanisasis, leptospirosis, rat bite fever, Tularemia, Hanta virus pulmonary syndrome and gastroenteritis (Battersby *et al.,* 2002; Meerburg *et al.,* 2007; Cummings *et al.,* 2012; Ayyal *et al.,* 2019; Stanaway *et al.,* 2019).

*Proteus mirabili*s is a zoonotic pathogen of major medical and veterinary importance (Jemilehin *et al*., 2016). It contributes largely to opportunistic infections, nosocomial outbreaks, and mostly hematogenous and ascending urinary tract infections (Nagano *et al*., 2003; Hamilton *et al*., 2018; Mirzaei *et al*., 2019). *P. mirabili*s species are reported as the third most prevalent (12%) pathogen (after *Escherichia coli* and *Klebsiella pneumoniae*) aetiology of cumbersome urinary tract infections (UTI) and second after *Providencia stuartii* etiological agent of catheter-associated bacteriuria in the group of long-term catheterized patients (Warren, 1996). Other complications include diarrheal diseases, bacteraemia, Crohn's disease, respiratory infections, neonatal meningoencephalitis, rheumatoid arthritis, and wound sepsis (O'Hara *et al*., 2000; Zhang *et al*., 2021). This bacterium can be isolated from a wide range of environments like soil, polluted water sources, sewage systems, humans, poultry meat, banknotes, bats, and

rodents (O'Hara *et al*., 2000; Nagano *et al*., 2003; Mukhtar *et al*., 2018; Mirzaei *et al*., 2019; Lakshmi *et al*., 2020) . Therefore, these pathogens can be transmitted inherently to humans and other domesticated animals (Morand *et al*., 2015; Ogunleye *et al*., 2016; Ayyal *et al*., 2019).

Rodents among other animals have been associated with transmission of bacteria including *Proteus mirabilis* (Katakweba *et al*., 2012; Sonola *et al*., 2021). It is essential therefore to determine the direct or indirect epidemiological role played by rodents as carriers and transmitters of *Proteus mirabilis* but also those with antimicrobial resistance traits. The current study focused on characterizing and investigating the antimicrobial susceptibility of *Proteus mirabilis* isolates from house and peridomestic rats in Arusha city, in northern Tanzania. We screened rodents in close association with human homes for their carriage of resistant *Proteus mirabilis* to determine their role in disease transmission and antimicrobial resistance patterns in not only humans but also animals due to sharing of the ecosystem.

Materials and Methods

Study Area

The study was conducted in the Arusha region located in northeastern Tanzania specifically in Arusha municipality which lies between 3° 23' and 12.9300" south and 36° 40' and 58.7820" longitude east. Arusha municipality has nineteen wards composed of urban, peri-urban, and rural areas with a population size of 416,442 as per the 2012 national census (DOSM, 2020). This area is surrounded by multiple national parks and game reserves (Ngorongoro conservation area, Serengeti National Park, Lake Manyara, Tarangire National Park, Arusha National Park) indicating human-animals-wildlife interactions that make it the ideal site for this study. Various agricultural practices are

done which involve the farming of vegetables, potatoes, bananas, and coffee at the Northern and Eastern parts of the city and wheat, maize, and beans at the southern and western parts which offers a conducive habitation for rodents (NBS, 2012). Eight wards were purposively selected for sampling based on high population density, and people's complaints due to rodent infestations and highly targeting slam areas. Sampling was conducted at Unga LTD, Kwawakereketwa, Mitamiambili, Kilombero, Ngaramtoni, Olasiti, Arusha airport, and Majengo-Elerai. These study wards were recorded and mapped using a global positioning system (GPS) (Figure 2.1).

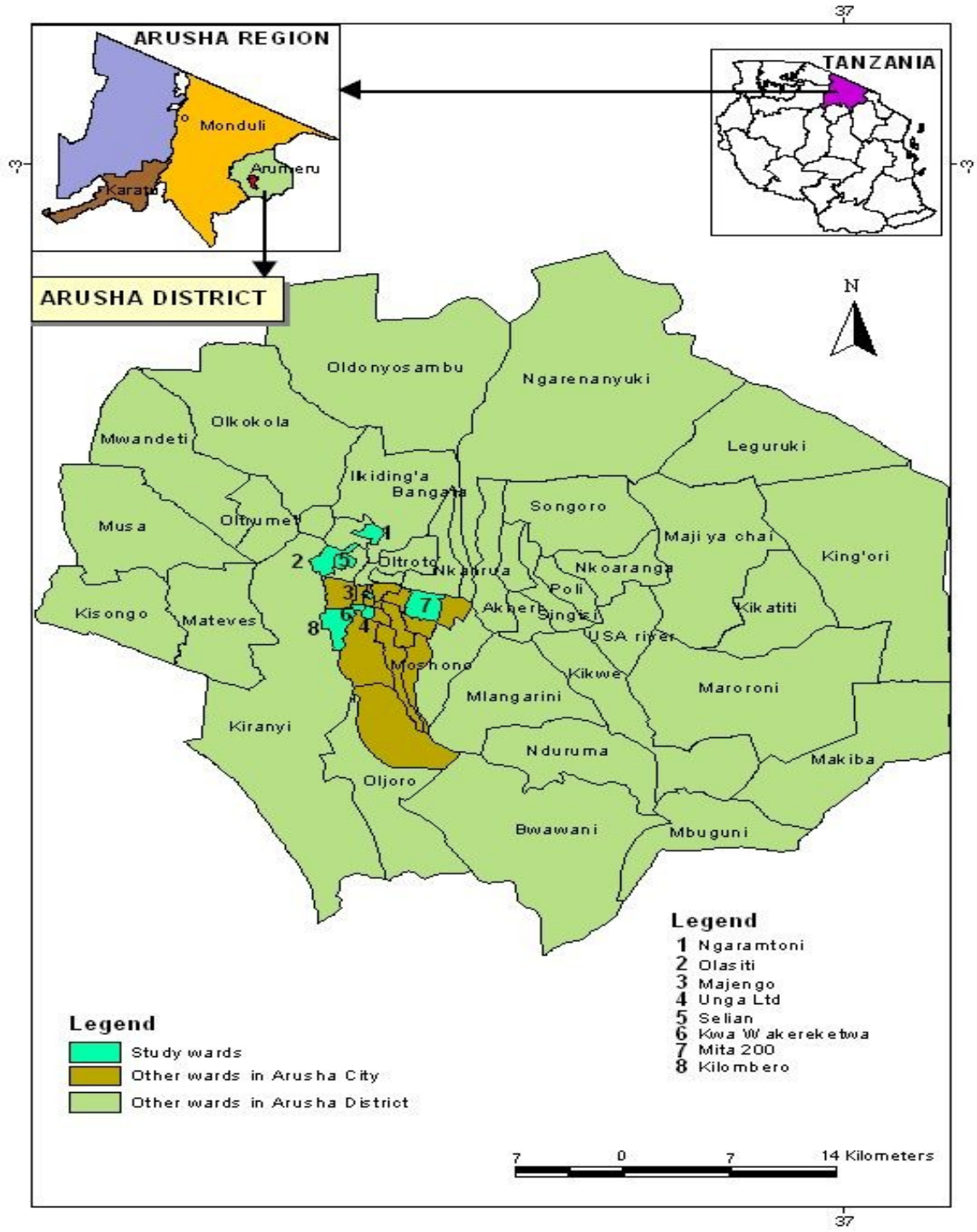


Figure 2.1: Map of Arusha municipality showing sampling wards.

Source: Arc GIS 10.3 version (2015)

Sampling Strategies

A cross-sectional study design was employed. Rat collection was done from March to May 2021. The sample size was determined by using the prevalence of 10% reported by Ogunleye *et al*. (2016) and the following equation as described by (Hilton *et al*., 2002) as; $\mathbf{n=Z^2P(1-P)/d^2}$ Where: n= sample size P= prevalence from a reported study= 10% Z = standard normal deviation at 95% confidence interval =1.96% D= absolute desired precision at 5% =0.05.

Trapping and processing of rodents

Trapping of live rodents was conducted using Sherman traps 8×9×23 cm (H.B. Sherman Traps Inc., Tallahassee, USA) in peri-domestic areas and modified wire traps 12×15×20 in houses (Anthony *et al*., 2005). Peanut butter mixed with maize bran was used to bait the traps. Traps were set in the evening for 5 consecutive nights and were checked every morning to capture rats to reduce heat stress experienced by the mammals. Trapped animals were anesthetized with cotton wool soaked into the di-ethyl ether before collection of the pre-moistened rectal swab. Sterile microbiological swabs (IMPROSWAB®) were used. Afterward collected deep intestinal swabs were placed into bijou bottles containing sterile transport media, and preserved at -20^0C for further laboratory analysis. Animals were cut open, labelled, and fixed in 70% ethanol. Morphometric and anatomical parameters such as body weight, head-body length, tail length, hindfoot length, and ear length were taken to be used in genus/species identification together with rodent identification keys (Skinner and Chimimba, 2005). Sex identification was done using morphological features like urogenital distance (distance between the genital papilla area and the anus) to determine the sexual activeness of the rodents (Cunningham and Moors, 1993). Age classification as to whether young,

juvenile, or adult, morphometric anatomical parameters such as body weight, body size, and reproductive activeness were used to judge the classification (Klevezal, 2007).

Determination of sex, age and species of rats

Sex identification was done using morphological features like urogential distance (distance between the genital papilla area and the anus). Females have a shorter distance while males have a longer distance. Further identification was done using validated unique features like presence of nipples or perforated vagina in females and visibility of scrotal sac in males. This was essential in determining sexual activeness of the rats (Cunningham and Moors, 1983).

Age classification of as to whether young, juvenile or adult, morphometric anatomical parameters such as body weight, body size and reproductive activeness were used to judge the classification (Klevezal, 2007). Species identification was done based on external morphological appearances to the lowest taxonomic level using taxonomic nomenclature (Cunningham and Moors, 1983; Wilson and Reeder, 2005).

Bacterial isolation, identification, and biochemical characterization

A swab from thawed sampled transport media was inoculated into 10mls of selective enrichment broth; Muller Hinton broth MH (OXOID Ltd., Detroit, Michigan, USA) and incubated overnight at 35 ± 2^0C. Aseptically, a loopful of MH culture was streaked onto MacConkey and Blood agar (OXOID). The latter had 15% rabbit blood and was incubated aerobically at 35^0C ± 2 for 18 to 24hrs, and differential subculture was done on XLD media (OXOID). Gram staining was performed for presumptive colonies. The gram-negative bacilli colonies with wavy concentric circles (swarming pattern) on blood agar, colourless, smooth non-lactose fermenter's colonies on MacConkey agar and

yellow colonies with dark centres (H_2S production) on XLD media were sub-cultured on nutrient agar for further tests protocol as demonstrated by (Pearson, 2021). Biochemical tests (Urease, indole, catalase, triple sugar iron test, motility test, and oxidase tests) were carried out to confirm the isolates. Lastly, the isolates were preserved in nutrient broth with 15% glycerol at -80^{0}C for further analysis.

Bacterial DNA extraction

Bacterial colonies were obtained from an overnight culture on nutrient agar at 37±2^{0}C. Genomic DNA (400µl) was extracted using the Quick-gDNA miniprep extraction kit (Zymo Research, USA) protocol as per manufactures instructions. The supernatant of gDNA was transferred into a clean Eppendorf tube and stored at -20^{0}C for further uses. To evaluate the quantity and quality of isolated gDNA, NanoDrop ND- 1000 system (NanoDrop technologies, Inc., Wilmington, DE, USA) was used at spectrophotometer wavelength 260nm (A_{260}/A_{280}). A ratio between 1.8 and 2 indicated a high-quality gDNA.

Polymerase chain reaction (PCR) amplification to determine 16S rRNA gene

Before PCR amplification, the concentration of the primer pair and annealing temperature was optimized. Primer sequences of 16s rRNA, expected band size, and primer annealing temperature are listed on (Table 2.1). The optimized PCR was conducted using a total reaction volume of 25µl containing 12.5µl of Quick load *Taq* 2× master mix, 0.5µl of reverse and forward primer, 6.5 of nuclease-free water, and 3µl of DNA template. PCR protocol used for amplification was 95^{0}C for 5min for initial denaturation, 94^{0}C for 30 secs for denaturation, annealing temperature as allocated in (Table 2.2) for 30 secs, extension for 72^{0}C for 2min and final extension for 10 min. This was done in 35 cycles.

Uniplex PCR to detect 16s rDNA was conducted at a total reaction volume of 20µl (Table 2.3) with *AccuPower*® PCR Premix. The experimental protocol involved 0.5µl of

reverse and forward primer (one pair of primer for uniplex for the genes *16S rDNA,* nuclease-free water and 3µl of DNA template. PCR procedure for amplification was 95^0C for 5min for initial denaturation, 94^0C for 30 secs for denaturation, annealing temperature as allocated in Table 2.1 for 30 secs, extension for 72^0C for 2min and final extension for 10 min. This was done in 35 cycles. Nuclease-free water was added in PCR tubes as a negative control, GeneAmp® PCR system 9700 (Applied Biosystems, USA) was used as a thermocycler to perform the thermal profiles.

Table 2.1: 16s rDNA oligonucleotide sequence and annealing temperature used for PCR amplification

Primer name	Gene	Oligonucleotide sequence (5'-3')	Size (bp)	A/temp (^{0}C)	Reference
Universal primer	*16s rDNA*	27F AGAGTTTGATCATGGCTCAG 1492R TACGGYTACCTTGTTACGACTT	1500	58	(Lakshmi *et al*., 2020)

Step	Temperature (^{0}C)	Time
Pre- denaturation	95	5 min
Denaturation	95	30sec
Annealing	Varying as indicated on Table 5	30sec
Extension	72	2min
Final extension	72	10min

Table 2.2: PCR amplification conditions

Table 2.3: AccuPower® PCR Premix contents

Components	20µl reaction volume
Template DNA	2µl
Forward primer	0.5 µl

Reverse primer	0.5 µl
Nuclease free water	Variable (16 -17) µl
Total reaction volume	20 µl

Detection of post PCR products by agarose gel electrophoresis

PCR products were then separated using agarose gel with 1.5% tris EDTA (TBE) buffer with 5µl of PCR product and ladder at 120Volts for 45 minutes and visualized using gel red staining in ultraviolet trans-illuminator. This was performed using a gel imaging and documentation system as per manufactures instructions (EZ GelDoc, Bio Rad, USA).

Sequencing

The 16s rDNA post PCR obtained fragments were sent to Macrogen Company (Seoul, South Korea) for sequencing using the sanger sequencing technique for further confirmation and characterization.

Statistical Analysis

Descriptive statistics were used to get the proportions of rat species, sex, age, and physiological status of the rodents captured. Outputs of Sanger sequencing were viewed, edited, and trimmed using Sequence scanner software 2.0 and BioEdit software 7.2 (Hall and Carlsbad, 2011). Consensus sequences obtained were compared in the GenBank using Basic Local Alignment Search Tool (BLASTn) whereas comparison was made between sequences obtained from this study with sequences available at the (NCBI) database. Multiple alignments between *Proteus mirabilis* sequences obtained from this study and similar sequences retrieved from NCBI were done using MEGA X software (Tamura and Nei, 2007). A phylogenetic tree was constructed using the maximum likelihood model (Hall, 2013). Phylogeny test to analyze the support strength for each

clade was done using bootstrap confidence analysis at 1000 replications. Ten sequences from GenBank and the ones from this study were used in evolutionary tree construction

Results

Rodents trapped from peri-urban and urban wards in Arusha municipality

A total of 139 rats were captured in the course of 5 consecutive nights. Of the 139 rats three species were identified. These included *Rattus rattus* (55.4%), *Mus musculus* (15.8%), and *Mastomys natalensis* (28.8%) as presented in (Table 2.4). Among them, 54.7% were females, whereby 28.1% were sexually active, 2.9% pregnant/ lactating and 23.7% were not sexually active. Of the rats captured, 45.3% were males. About 25.9% were sexually active and 19.4% weren't. Generally, in all genders, 56.9% were sexually mature.

Table 2.4: Proportions of species, gender, gender status, and age of various domestic rodents trapped in Arusha Municipality

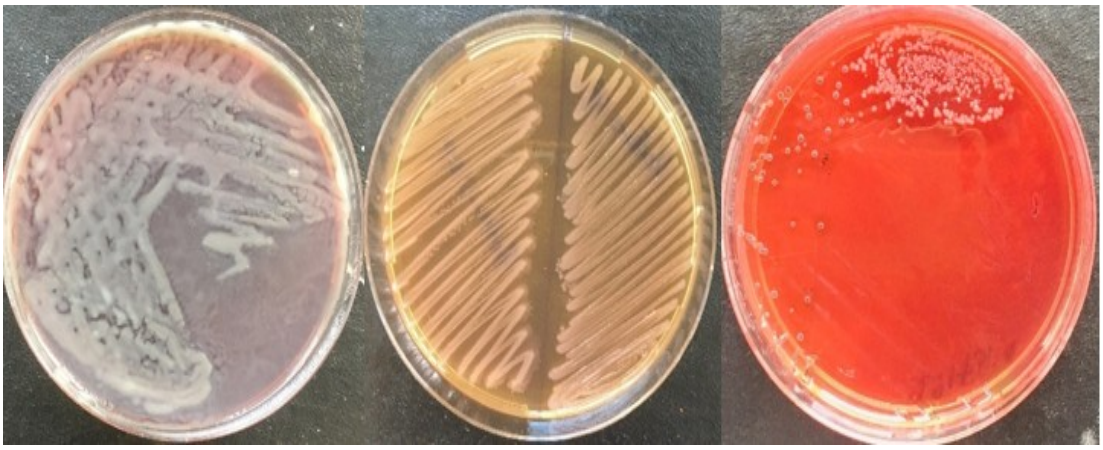
		Number of rats	Proportion N%
Species	R.R	77	55.4
	M.M	22	15.8
	MAST	40	28.8
Gender Status	FCSN	33	23.7
	FPSN	39	28.1
	FPSY	4	2.9
	MAN	27	19.4
	MSV	36	25.9
Age	JUVENILE	61	43.9
	ADULT	78	56.1
Gender	MALE	63	45.3
	FEMALE	76	54.7
Trap Site	HOUSES	95	68.3
	PERI D	44	31.7
TOTAL		**139**	100

Key: Abbreviations: R. R= *Rattus rattus*, M.M= *Mus musculus*, MAST= *Mastomys* species, FCSN= Female vagina closed, FPSN= Female vagina perforated nipple small, FPSY= Female perforated pregnancy/ lactating, MAN= Male testes abdominal, MSV= Male scrotal visible

Isolation of *Proteus mirabilis*

Of a total of 139 trapped rats, positive proportions for *Proteus mirabilis* were 4(2. 0%). The isolates were collected in both males (50%) and females (50%) rats, three isolates from Ngaramtoni and one isolate from Kilombero wards, and all isolates were recovered from *Rattus rattus* species. The microscopic gram staining test revealed gram-negative bacilli while their colonies on blood agar had a swarming pattern, colourless and smooth. The colonies appeared to be non-lactose fermenters on MacConkey agar, and yellow

colonies with dark centers (H_2S production) on XLD media (Figure 2.2). The biochemical test results are provided in Table 2.5.



A **B** **C**

Figure 2.2: *Proteus* spp on blood agar (A), MacConkey agar (B), and XLD agar (C) respectively.

Seven biochemical tests were performed and all the four culture positive samples gave similar results (Table 2.5).

Table 2.5: Biochemical properties of *Proteus mirabilis*

SN	Biochemical test	Property
1	Catalase	+
2	Gram staining	-
3	H_2S production on TSI	+
4	Indole	-
5	Motility	+
6	Oxidase	-
7	Urease	+

PCR results of 16S rRNA gene of *P. mirabilis*

All the four culture and biochemical positive samples gave similar results for 16S rRNA molecular detection as Figure 2.3 shows

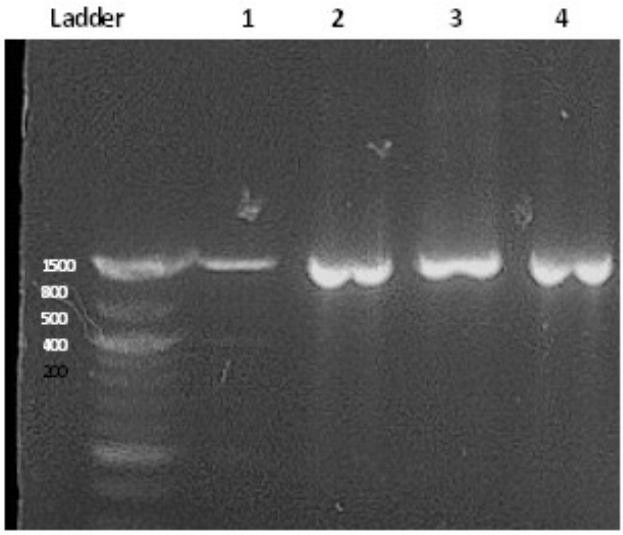


Figure 2.3: Gel picture of PCR amplification of 16s rRNA and *P. mirabilis.*
Note; *Lane 1-4 represents that of 16s rRNA at an expected size of 1500bp.*

Phylogenetic analysis

All sequences had 100% similarity and query cover to those of *P. mirabilis* in the database. The accession number of the sequences was also obtained (Table 2.6). For alignment creation, 10 databases 16S rRNA genes with 4 16S rRNA gene of *P. mirabilis* isolates from this study were aligned pairwise and multiplewise using ClustalW (Chenna *et al*., 2003) Afterwards MEGA X software was used to create an evolutionary tree using the Maximum Likelihood method and Tamura-Nei model. Corresponding to the evolutionary tree depicted in (Figure 2.4), all Tanzanian isolates had similar evolutionary relationship with isolates from Tunisia and Venezuela. They are also closely related to isolates from Iraq and Pakistan, Italy and the United Kingdom and far different from isolates from the USA, India, and Sudan.

Table 2.6: BLAST results of databases 16S rRNA genes with 16S rRNA gene of *P. mirabilis* isolates from this study

Query sequence	Sequence from database producing significant alignments with a query sequence	Accession number from NCBI	% Similarity	Query cover
KR64RR	*Proteus mirabilis* strain P8 16S ribosomal RNA gene, partial sequence	MT276305.1	100	100
NR69RR	*Proteus mirabilis* strain IGM6-13 16S ribosomal RNA gene, partial sequence	MT197283.1	100	100
NR70RR	*Proteus mirabilis* strain M17_J16 16S ribosomal RNA gene, partial sequence	MK426652.1	100	100
NR71RR	*Proteus mirabilis* strain TL3 16S ribosomal RNA gene, partial sequence	KF051775.1	100	100

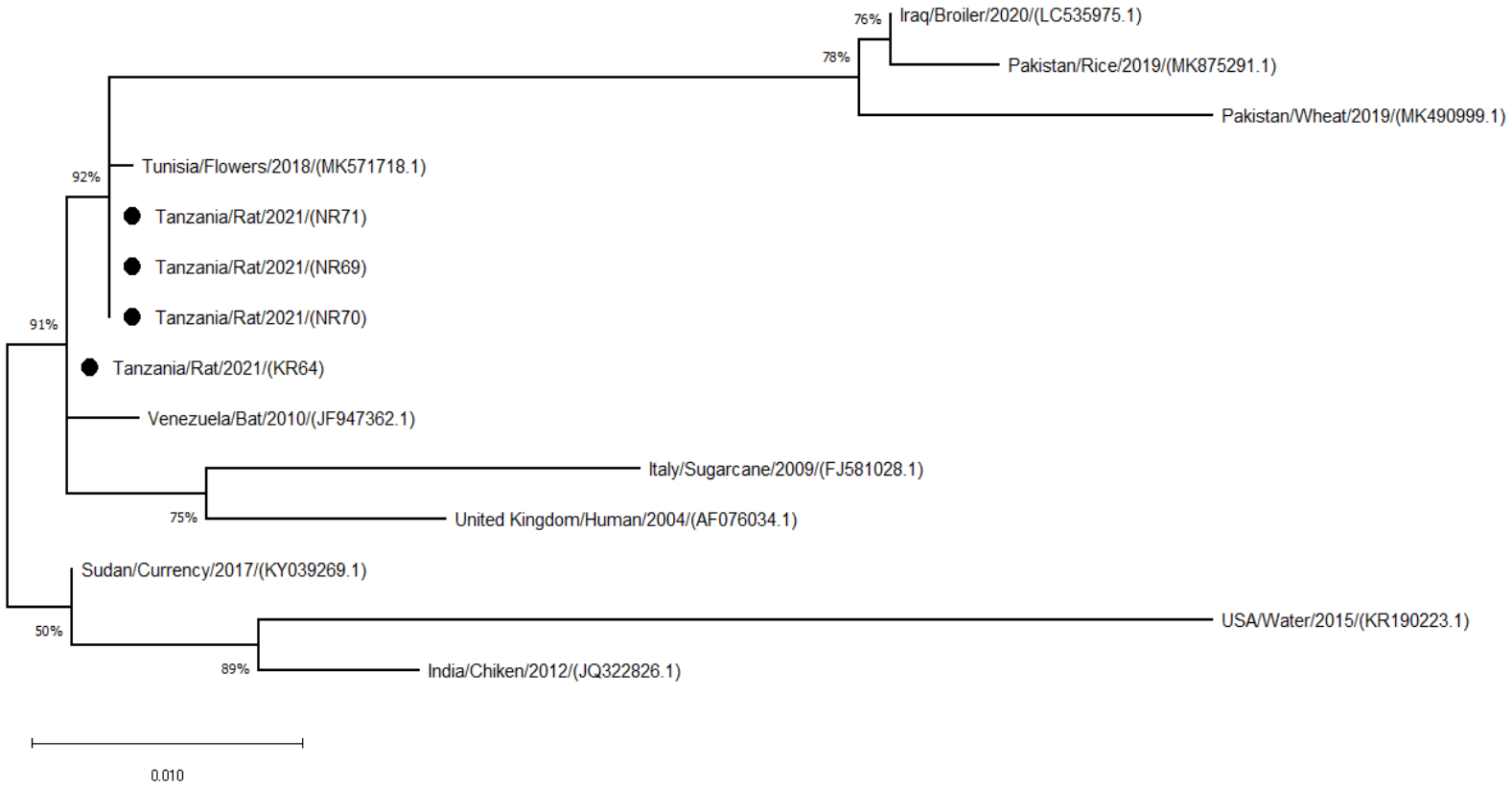


Figure 2.4: Phylogenetic tree of P. mirabilis from this study.

The sequences used, indicates he country, source, isolation year, and accession number. The phylogeny was inferred with 1000 bootstrap support values (numbers with %).

Discussion

Our study was able to show three species of rodents commonly found in the study area. *Rattus rattus* (Black rats) was a dominant species in houses than in peri domestic areas. These results are concurrent with most studies conducted worldwide. For example, studies conducted in Nigeria, South Africa, United kingdom, Canada, Trinidad and Tobago revealed that *Rattus rattus* was a dominating species in houses (Hilton *et al.,* 2002; Meerburg and Kijlstra, 2007; Nkogwe *et al.,* 2011;Wakawa *et al*., 2015; Ramatla *et al*., 2019). Black rats are commonly associated with shedding of pathogens and parasites through their fecal, urine, and fur droppings to the surrounding, foodstuffs, grain stores and other consumable materials (Singleton *et al*., 2003). These pathogens and parasites mostly are etiological agents for diseases in domestic animals and humans (Ramatla *et al.,* 2019). Several studies have reported an occurrence of pathogenic bacteria in black rats that are listed on the WHO list of preferential pathogens such as of the family *Enterobacteriaceae* like *Escherichia coli*, *Salmonella* spp, *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp and *Proteus* spp (WHO, 2017; Ayyal *et al*., 2019). Generally, species of the genus *Rattus* have conceivably accounted to more agony to humans since far back in the centuries through their role played in infrastructural damage, disease transmission and destructive impression on agricultural crops and stored grains.

The second abundant species from the study area was the multimammate mouse; *Mastomys natalensis*. The abundance of these species was relatively higher compared with other studies reported elsewhere. For example, a study conducted by Agbonlahor *et al*., (2017), presented low abundance of 2% and Katakweba *et al*. (2012), reported 14.3%. This could be attributed by features like high reproductive rates in the study area due to enough food availability and appropriate geographical location (Demby *et al*., 2001, Odhiambo and Oguge, 2003). *M. natalensis* are spatially distributed species and are

known to appear in cultivated lands, fallow lands, woodlands, forest clearances, savannahs and houses (Agbonlahor *et al*., 2017). These findings coincide with the current study where majority of *M. natalensis* were trapped on peri-domestic fields especially in the agricultural fields interfaces, this could be attributed to variations due to season of the year particularly after rainy seasons where *M. natalensis* populations are pronounced to grow, plenty availability of feeds in the field crops and fallow lands and the distance from the house to the farms, crops and fallows (Agbonlahor *et al*., 2017). In this study, the lowest abundant species was *M. musculus* 22 (15.8%).The occurrence of this species in Arusha was relatively low contrary to Agbonlahor *et al*. (2017) who found the abundance of 39.4% southern part of Nigeria..

Rats are reported to be carriers of pathogenic microbes since the early centuries (Singleton *et al*., 2003). Our findings showed a prevalence of 2% (4/139) for *P. mirabilis.* These findings are consistent with results from Iraq and Nigeria where *P. mirabilis* isolates from rats had a prevalence of 2% (Ogunleye *et al*., 2016 Ayyal *et al*., 2019) and almost similar to a study in Nigeria which reported a prevalence of 3% (Jemilehin *et al*., 2015). However, our findings were lower compared with results from Sudan 22.2% (Mukhtar *et al*., 2018). This variation could be due to different sanitary conditions, variation based on difference on occurrence of *P. mirabilis* on rat species and different climatic conditions concerning temperature and humidity that affect bacterial growth and multiplication.

Molecular detection methods for microbial identification and characterization outrun conventional methods like biochemical tests which rely on the expression of phenotypic traits (Wang *et al*., 2021). The 16s rRNA is a powerful molecular detection tool as it is accurate and sensitive in the detection of bacterial isolate. In this study, all isolates were

sequenced after DNA extraction and with help of BLAST and multiple sequence alignment of sequenced isolates showed all isolates were 100% similar to *P. mirabilis* in the gene bank. Using 16s rRNA partial sequences, the evolutionary tree was constructed and showed that all isolates from the study were almost similar as they clustered together. This could be due to the presence of conserved and variable regions in their genomes (Mukhtar *et al*., 2018), and also due to geographical and ecological similarities as all the isolate were obtained from rats of the same species and the same environment (Macrae, 2000). These isolates were closely related to isolates from Tunisia (Figure 4).

Findings of the study indicate presence of *P. mirabilis* from house rats implicates the possibility of widespread transmission of the bacteria in the studied area, with the possibility of causing infections, hence a serious public health concern. Therefore, comprehensive interventions, using a one-health approach, would be required to control the situation. Such measures should include improving (1) awareness of the community on the proper use of antimicrobial drugs in humans and animals, (2) good house conditions and hygienic waste management (3) integrated rodent control measures (4) improving treatment regimens for humans and other veterinary animals by doing microbial susceptibility testing before drug prescription.

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Conflicts of interest we declare no conflict of interest

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CHAPTER THREE

Manuscript Two

Antimicrobial susceptibility patterns of Proteus mirabilis isolated from domestic rats in Arusha municipality, Tanzania

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Abstract

*Proteus mirabili*s is a bacterial pathogen of the major medical and veterinary significance in humans that contributes to opportunistic and urinary tract infections. Presence of rats cohabiting with humans can inherently be zoonotic to humans due to rats-human interaction nature and especially due to the recent emergence and widespread of multidrug resistance in the bacteria. It is therefore essential to genetically characterize and assess antimicrobial susceptibility patterns of *Proteus mirabilis* isolates from rats cohabiting with humans for intervention. A total of 139 rats were trapped in houses and around peridomestic areas in different wards in Arusha city. Following euthanization, rats were identified to genus/species level using morphological and morphometric features. Deep intestinal swabs were aseptically obtained and pre-enriched in buffered peptone water before laboratory analysis. Conventional culture and biochemical methods were conducted for bacterial isolation. Antimicrobial susceptibilities were carried out to 7 commonly used antimicrobial agents in the study area. Resistance genes (*bla TEM, tetA, tetB, mphA, SHV, bla CTX-M, sul1,* and *sul2)* were traced in each isolate using PCR methods. Mixed rat species, *Rattus rattus* (55.4%) *Mus musculus* (15.8%) and *Mastomys natalensis* (28.8%) were captured. After sequencing all isolates were 100% similar to *P. mirabilis* strains from NCBI *Proteus mirabilis* was isolated from only 4 samples (2%), all of which were from *Rattus rattus*. All isolates showed Multiple Drug Resistance traits. PCR analysis showed the presence of *TetA, bla TEM, Sul1,* and *Sul2* resistant genes. The study has discovered the first *Proteus mirabilis* isolates from rats in Tanzania with pathogenic and antimicrobial resistance traits that could be transmitted to the public.

Keywords: *Proteus mirabilis*, rats, antimicrobial resistance, resistance genes, 16S rRNA, Arusha

Introduction

Antibiotic resistance has recently become a serious problem worldwide that threatens human, animal, and environmental health (Sonola *et al*., 2021). If the situation remains unmanaged by 2050, higher human mortalities (WHO, 2017) severe economic losses, and a significant drop in livestock production are expected (Orubu *et al*., 2020). The emergence and widespread of multidrug resistance in *Proteus mirabilis* have been recently reported (Girlich *et al*., 2020; Zhang *et al*., 2021). This is due to acquired resistance to commonly used antimicrobials (Doumith *et al*., 2009). The ability to detect Salmonella genome island 1 variant SGI1-0, Extended Spectrum Beta-*Lactamase* (ESBL), AmpC-type *cephalosporinase,* and *carbapenemases* producers which are responsible for multidrug resistance in *Proteus mirabilis* indicates a threat (Girlich *et al*., 2020)as this trait can be transferred to other bacteria of medical and veterinary importance. This is due to the bacterial acquisition of plasmids, integrons, insertion sequences, and transposons mediated with antimicrobial resistance traits in their cells facilitating horizontal gene transfer of resistant plasmids as they have the conjugal transfer pilus (Doublet *et al.,* 2010; D'Andrea *et al*., 2011; Iredell *et al*., 2016)**.**

Resistance to commonly used antimicrobials for the treatment of bacterial infections in animals and humans has been studied and reported in many parts of the world (Adesiji *et al*., 2014; Gaspary *et al*., 2016; Sindiyo *et al*., 2019). It has been established that effects imposed by antimicrobial resistance have a great impact on animals and human health, where animals and humans share the same environment (Jemilehin *et al*., 2016; Katakweba *et al*., 2012). To date efforts to reduce antimicrobial resistance rely on human bacterial populations as a result of exposure to antimicrobials and that restricting the use of antimicrobials, this however may not conclusively apply as antimicrobial resistance is prevalent in populations of rodents and other animals and birds due to the passage of

resistant microorganisms from humans to animals, between humans, and from animal to humans (Stanaway *et al.*, 2019; Sonola *et al*., 2021; Islam *et al*., 2021)

Rodents among other animals have been associated with transmission of resistant strains of bacteria (Sonola *et al*., 2021). It is essential therefore to determine the direct or indirect epidemiological role played by rodents as carriers and transmitters of *Proteus mirabilis* but also those with antimicrobial resistance traits. The current study focused on characterizing and investigating the antimicrobial susceptibility patterns of *Proteus mirabilis* isolates from house and peridomestic rats in Arusha city, in northern Tanzania. We screened rodents in close association with human homes for their carriage of resistant *Proteus mirabilis* to determine their role in disease transmission and antimicrobial resistance patterns in not only humans but also animals due to sharing of the ecosystem.

Materials and Methods

Study Area

The study was conducted in the Arusha region located in northeastern Tanzania specifically in Arusha municipality which lies between 3° 23' and 12.9300" south and 36° 40' and 58.7820" longitude east. Arusha municipality has nineteen wards composed of urban, peri-urban, and rural areas with a population size of 416 442 as per the 2012 national census (URT, 2013). This area is surrounded by multiple national parks and game reserves (Ngorongoro conservation area, Serengeti National Park, Lake Manyara, Tarangire National Park, Arusha National Park) indicating human-animals-wildlife interactions that make it the ideal site for this study. Various agricultural practices are done which involve the farming of vegetables, potatoes, bananas, and coffee at the Northern and Eastern parts of the city and wheat, maize, and beans at the southern and western parts which offers a conducive habitation for rodents (DOMS, 2020). Eight wards

were purposively selected for sampling based on high population density, and people's complaints due to rodent infestations and highly targeting slam areas. Sampling was conducted at Unga LTD, Kwawakereketwa, Mitamiambili, Kilombero, Ngaramtoni, Olasiti, Arusha airport, and Majengo-Elerai. These study wards were recorded and mapped using a global positioning system (GPS)

Sampling Strategies

A cross-sectional study design was employed. Rat collection was done from March to May 2021. The sample size was determined by using the prevalence of 10% reported by (Ogunleye *et al.*, 2016) and the following equation as described by (Hilton *et al*., 2002) as; $\mathbf{n=Z^2P\ (1-P)/d^2}$ Where: n= sample size P= prevalence from a reported study= 10% Z = standard normal deviation at 95% confidence interval =1.96% D= absolute desired precision at 5% =0.05.

Trapping and processing of rodents

Trapping of live rodents was conducted using Sherman traps 8×9×23 cm (H.B. Sherman Traps Inc., Tallahassee, USA) in peri-domestic areas and modified wire traps 12×15×20 in houses (Anthony *et al*., 2005), food stores, and shops. Peanut butter mixed with maize bran was used to bait the traps. Traps were set in the evening for 5 consecutive nights and were checked every morning to capture rats to reduce heat stress experienced by the mammals. Trapped animals were anesthetized with cotton wool soaked into the di-ethyl ether before collection of the pre-moistened rectal swab. Sterile microbiological swabs (IMPROSWAB®**)** were used. Afterward collected deep intestinal swabs were placed into bijou bottles containing sterile transport media, and preserved at -20^{0}C for further laboratory analysis. Animals were cut open, labelled, and fixed in 70% ethanol. Morphometric and anatomical parameters such as body weight, head-body length, tail

length, hindfoot length, and ear length were taken to be used in genus/species identification together with rodent identification keys (Skinner and Chimimba, 2005).Sex identification was done using morphological features like urogenital distance (distance between the genital papilla area and the anus) to determine the sexual activeness of the rodents. (Cunningham and Moors, 1993). Age classification as to whether young, juvenile, or adult, morphometric anatomical parameters such as body weight, body size, and reproductive activeness were used to judge the classification (Klevezal, 2007)

Bacterial isolation, identification, and biochemical characterization

A swab from thawed sampled transport media was inoculated into 10mls of selective enrichment broth; Muller Hinton broth MH (OXOID Ltd., Detroit, Michigan, USA) and incubated overnight at 35 ± 2^0C. Aseptically, a loopful of MH culture was streaked onto MacConkey and Blood agar (OXOID). The latter had 15% rabbit blood and was incubated aerobically at 35^0C ± 2 for 18 to 24hrs, and differential subculture was done on XLD media (OXOID). Gram staining was performed for presumptive colonies. The gram-negative bacilli colonies with wavy concentric circles (swarming pattern) on blood agar, colourless, smooth non-lactose fermenter's colonies on MacConkey agar and yellow colonies with dark centres (H_2S production) on XLD media were sub-cultured on nutrient agar for further tests protocol as demonstrated by (Pearson., 2021). Biochemical tests (Urease, indole, catalase, triple sugar iron test, motility test, and oxidase tests) were carried out to confirm the isolates. Lastly, the isolates were preserved in nutrient broth with 15% glycerol at -80^0C for further analysis.

Antimicrobial susceptibility testing

Disk diffusion method was used to determine resistant patterns of 7 antimicrobial agents commonly used in the study area according to Clinical and Laboratory Standards Institute

guidelines (CLSI, 2021) and performance standards for antimicrobial disks and dilution susceptibility tests for bacteria isolated from animals (CLSI VET0IS, 2020). The selection of the antimicrobials was based on common and frequent use in treating infections in humans and animals, whilst considering each antimicrobial disc belongs to a specific category of the eight groups of drugs classification. The following antimicrobial discs were used (Liofilchem®, Italy); Tetracycline (TE, 5µg), Trimethoprim sulfamethoxazole (SXT, 25 µg), Ciprofloxacin (CIP, 5µg), Cefotaxime (CTX,5µg), Ampicillin (AMP,10µg), Azithromycin (AZM, 15µg), Gentamycin (CN,10µg). *Escherichia coli* ATCC 25922 strain was used for quality control for it's a recognized control strain for international susceptibility testing.

Bacterial DNA extraction

Bacterial colonies were obtained from an overnight culture on nutrient agar at 37 ± 2^0C. Genomic DNA (400µl) was extracted using the Quick-gDNAminiprep extraction kit (Zymo Research, USA) protocol as per manufactures instructions. The supernatant ofgDNA was transferred into a clean Eppendorf tube and stored at -20^0C for further uses.To evaluate the quantity and quality of isolated gDNA, NanoDrop ND- 1000 system (NanoDrop technologies, Inc., Wilmington, DE, USA) was used at spectrophotometer wavelength 260nm (A_{260}/A_{280}). A ratio between 1.8 and 2 indicated a high-quality gDNA.

Polymerase chain reaction (PCR) amplification to determine 16S rRNA and resistant genes

Before PCR amplification, the concentration of each primer pair and annealing temperature was optimized. Primer sequences of selected gene from each group of antimicrobials tested, gene name, expected band size of the resistant gene, and primer annealing temperature are listed (Table 3.1). The optimized PCR was conducted using a

total reaction volume of 25µl containing 12.5µl of Quick load *Taq* 2× master mix, 0.5µl of reverse and forward primer, 6.5 of nuclease-free water, and 3µl of DNA template. PCR protocol used for amplification was 95^0C for 5min for initial denaturation, 94^0C for 30 secs for denaturation, annealing temperature as allocated in (Table 3.2) for 30 secs, extension for 72^0C for 2min and final extension for 10 min. This was done in 35 cycles. Uniplex and multiplex PCR to detect and resistant genes were all conducted at a total reaction volume of 20µl (Table 3) with *AccuPower*® PCR Premix. The experimental protocol involved 0.5µl of reverse and forward primer (one pair of primer for uniplex for the genes *16S rDNA, bla TEM, tetA, tetB, mphA,* and 4 pairs of multiplex primers for *bla SHV*, *bla CTX-M*, *sul1,* and *sul2)*, nuclease-free water and 3µl of DNA template. PCR procedure for amplification was 95^0C for 5min for initial denaturation, 94^0C for 30 secs for denaturation, annealing temperature as allocated in Table 2 for 30 secs, extension for 72^0C for 2min and final extension for 10 min. This was done in 35 cycles. Nuclease-free water was added in PCR tubes as a negative control, GeneAmp® PCR system 9700 (Applied Biosystems, USA) was used as a thermocycler to perform the thermal profiles.

Table 3.1: Antimicrobial agent, resistant gene, oligonucleotide sequence and annealing temperature used for PCR amplification

Antimicrobial agent	Gene	Oligonucleotide sequence (5'-3')	Size (bp)	A/temp (^{0}C)	Reference
Cephalosporins	*bla TEM*	F-ATG AGT ATT CAA CAT TTC CG R-CCA ATG CTT AAT CAG TGA GG	858	50	Gharrah *et al.,* 2017
	bla SHV	F-ATG CGT TAT ATT CGC CTG TG R-AGC GTT GCC AGT GCT CGA TC	862	58	Gharrah *et al.,* 2017
	Universal *blaCTX-M*	F-5'-SCS ATG TGC AGY ACC AGT AA R-5'-CCG CRA TAT GRT TGG TGG TG	554	58	Gharrah *et al.,* 2017
Cefotaxime	*mph(A)*	F-GTGAGGAGGAGCTTCGCGAG R-TGCCGCAGGACTCGGAGGTC	403	60	Kim *et al*., 2004
Ciprofloxacin	*acr(A)*	F-CTCTCAGGCAGCTTAGCCCTAA R-TGCAGAGGTTCAGTTTTGACTGTT	107		Kim *et al*., 2004
Gentamicin	*aac(3)-I*	F-ACCTACTCCCAACATCAGCC R-ATATAGATCTCACTACGCGC	169	60	Kim *et al*., 2004
Tetracycline	*tet(A)*	F-GGTTCACTCGAACGACGTCA R-CTGTCCGACAAGTTGCATGA	577	57	Kernn *et al.*, 2002
	tet(B)	F-CCTCAGCTTCTCAACGCGTG R-GCACCTTGCTGATGACTCTT	634	56	Kernn *et al.*, 2002
Sulfonamides	*Sul1*	F-CGGCGTGGGCTACCTGAACG R-GCCGATCGCGTGAAGTTCCG	433	69	Kernn *et al.*, 2002
	Sul2	F-GCGCTCAAGGCAGATGGCATT R-GCGTTTGATACCGGCACCCGT	293	69	Kernn *et al.*, 2002

Step	Temperature (^{0}C)	Time
Pre- denaturation	95	5 min
Denaturation	95	30sec
Annealing	Varying as indicated on Table 5	30sec
Extension	72	2min
Final extension	72	10min

Table 3.2: PCR amplification conditions

Table 3.3: AccuPower® PCR Premix contents

Components	20µl reaction volume
Template DNA	2µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Nuclease free water	Variable (16 -17) µl
Total reaction volume	20 µl

Detection of post PCR products by agarose gel electrophoresis

PCR products were then separated using agarose gel with 1.5% tris EDTA (TBE) buffer with 5µl of PCR product and ladder at 120Volts for 45 minutes and visualized using gel red staining in ultraviolet trans-illuminator. This was performed using a gel imaging and documentation system as per manufactures instructions (EZ GelDoc, Bio Rad and USA).

Statistical Analysis

Descriptive statistics were used to get the proportions of rat species, sex, age, and physiological status of the rodents captured. Antimicrobials were assessed based on comparison of minimum zone of inhibition of each tested drug and CLSI references on each respective antimicrobial and presented as whether resistant (R), intermediate (I) or susceptible (S).

Results

Results on isolation are found on the previous paper

Antimicrobial susceptibility patterns of *Proteus mirabilis* isolates

All the three strains obtained from Ngaramtoni, strain (2, 3 and 4) portrayed multidrug resistance (MDR) against Triomethoprim- sulfamethaxole, Azithromycin, and Ampicillin (Table 3.4). All isolates were resistance to Azithromycin, and Ampicillin, but susceptible to Ciprofloflaxcin, Gentamicin and Cefotaxime, three were resistance to Triomethoprim-sulfamethaxole and three were intermediate to Tetracycline.

Table 3.4: Antimicrobial resistance patterns of Proteus Mirabilis isolates from domestic rats in Arusha Municipality

	Tetracycline[b]	Folate pathway inhibitors[b]	Quinolones[b]	Cephalosporins[b]	Penicillins[b]	Macrolides[b]	Aminoglycosides[b]
Strain ID	**TET**	**SXT**	**CIP**	**CTX**	**AMP**	**AZM**	**CN**
1	S	S	S	S	R	R	S
2	I	R	S	S	R	R	S
3	I	R	S	S	R	R	S
4	I	R	S	S	R	R	S

Key: TET= Tetracycline; SXT= Triomethoprim- Sulfamethoxazole; CIP= Ciprofloxacin; CTX= Cefotaxime; AMP= Ampicillin; AZM= Azithromycin; CN=Gentamicin; R= Resistance; I= Intermediate; S=Susceptible; b= Antimicrobial class

PCR results of resistance genes

All tested isolates amplified *TetA, bla$_{TEM}$, Sul1*, and *Sul2* with the prevalence of 100% as shown in (Figure 3.3). Isolate KR64 (strain 1) shown to be phenotypically sensitive to Tetracycline and Sulfamethaxzole Trimethoprim. However, it respectively carried *TetA*, and *Sul1*, *Sul2* resistant genes in its genome (Figure 3.3), and the remaining isolates were intrinsically resistant to Tetracycline, and Trimethoprim -Sulfamethaxzole. Nevertheless, all isolates were intrinsically resistant to Ampicillin, however, it has genetically held the *bla*$_{TEM}$ gene. Whilst all isolates were sensitive to Cefotaxime (Cephalosporin III/ extended-spectrum cephalosporin), Gentamicin, and Ciprofloxacin their genes weren't amplified.

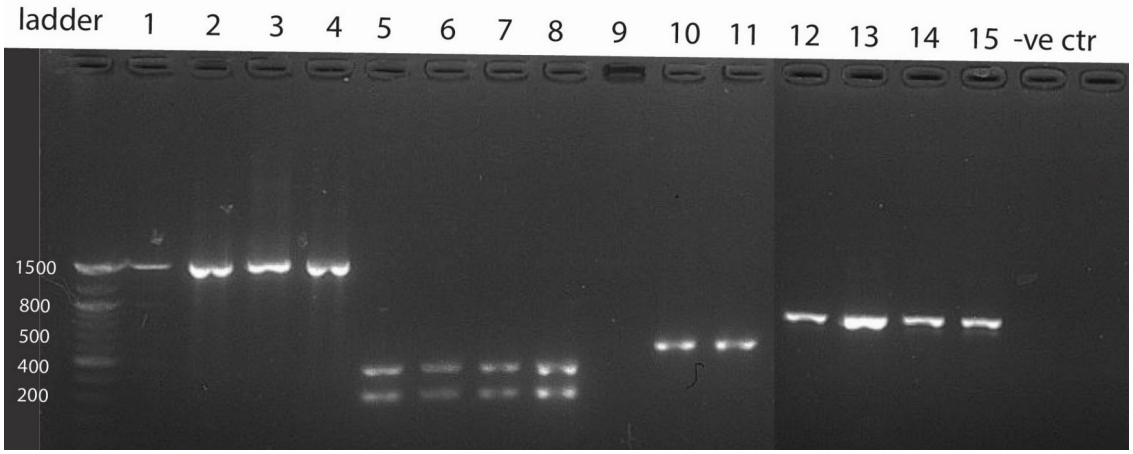

Figure 3.3: Gel picture of PCR amplification of 16s rRNA and detection of resistance genes in *P. mirabilis*.

Note; *Lane 1-4 represents that of 16s rRNA at an expected size of 1500bp. Lane 5-15 shows band sizes of resistant genes; lane 5-8 are for Sul1 and Sul2 of the expected size of 433 and 293 respectively; lane 10-11 contains TetA of the expected size of 577bp; lane 12-15 holds bla$_{TEM}$ of expected band size of 858bp and lastly the negative control.*

Discussion

Rampant crisis to antimicrobial response especially multidrug resistance (MDR) among many genera of the family *Enterobacteriaceae* to date indicates a major public health risk (D'Andrea *et al.*, 2011). Past studies reported that isolates of *P. mirabilis* were sensitive to most antimicrobial classes opposing recent findings from different countries across the globe, reporting increase resistance of *Proteus mirabilis* (Ramatla *et al*., 2019). The current study showed that all *P. mirabilis* isolates were 75% resistant to Tetracycline and Trimethoprim – Sulfamethaxzole. This disagrees with results from Ibadan Nigeria which reported 100% resistance to tetracycline for *P. mirabilis* isolates from rats (Ogunleye *et al*., 2016). Furthermore, this study reveals 100% resistance to Azithromycin and Ampicillin. Ampicillin resistance in our study concurs with reports from Tanzania, Iran, Nigeria, and Sudan which have reported similar patterns of resistance to Ampicillin from clinical patients, rats, and currency isolates (Doumith *et al*., 2009; Ogunleye *et al*., 2016; Mukhtar *et al*., 2018; Mirzaei *et al*., 2019). The 100% resistance found in Azithromycin in this study matches with results from India and Babylon (Demby *et al*., 2001; Al-Khafaji and Bunyan, 2013). The observed high resistance to the aforementioned antimicrobials may be contributed by several factors. This may include poor disposal of unused antimicrobials (Agbonlahor *et al*., 2017), misuse of antimicrobials, and high-frequency use in chemoprophylaxis, treatment, and enhancement of growth in livestock management. Also, this happens due to the readily available and cheap cost of antimicrobials, frequent self-prescribing of over-the-counter drugs, or several pre-exposures to various classes of antimicrobials before doctor's and veterinarian prescriptions (Katakweba *et al*., 2012; Agbonlahor *et al*., 2017). Moreover Sonola *et al.* (2021) suggests that sharing the same environment by human and rats can cause crossover of resistance genes from the two that can be deposited in the soil.

Proteus mirabilis isolates were more susceptible to Cephalosporin, Quinolones, and Aminoglycosides (Cefotaxime, Ciprofloxacin, and Gentamicin) respectively with 100%

activity. These findings concur with several studies from rats and poultry isolates (Odhiambo and Oguge, 2003; Ogunleye *et al*., 2016; Mirzaei *et al*., 2019). This report indicates that the presence of *P. mirabilis* among rats trapped in houses and around peri-domestic areas may be due to environmental interactions and commonalities in the food chain which further contributes to the spread of antimicrobial resistance traits among various species sharing the ecosystem with humans (Ahmed *et al.*, 2007; Hamilton *et al*., 2018). Tetracyclines and Sulfamethaxole - Triomethoprim antimicrobials have been frequently used in animals (Katakweba *et al*., 2012; Kissinga *et al*., 2018; Kimera *et al*., 2020). This affects small mammals that share the environment with humans and animals as the Arusha region is the potential in keeping livestock like cattle, poultry, sheep, goats, and pigs (Gaspary *et al*., 2016).

Emanation and expansion role of multidrug-resistant *P. mirabilis* particularly those producing ESBLs is a setback in the medical and veterinary fields (Owoseni *et al*., 2021). In our understanding, this is the first report on *P. mirabilis* isolates from rats in Tanzania. In this study, isolates from rats showed intrinsic acquisition of resistance genes; *TetA, Sul1, Sul2,* and bla_{TEM}. Detection of bla_{TEM} a narrow spectrum B- lactamase gene that accounts for resistance against narrow-spectrum cephalosporins and penicillins explains why all isolates showed phenotypic resistance to ampicillin. The results are similar to a study in Japan (Begon, 2003) in zoo animals isolates. Occurrence of *TetA, Sul1, Sul2* in all isolates resistant and susceptible to Tetracycline's and Sulfamethaxole - Triomethoprim suggests that some antimicrobial agents in bacterial are "silent" and agreeing to a report in India (Deekshit *et al*., 2012). This indicates a threat as silent genes can be expressed *in vivo* under antimicrobial utilization pressure and transferred to other intestinal and environmental microflora (Wang *et al*., 2011). In Tanzania, these findings can be supported through a study by Katakweba *et al.* (2018), Kissinga *et al*. (2018) and Sonola

et al. (2021) who revealed the *Tet* and *sul* genes from faecal samples collected from livestock.

This study has some limitations, rodents trapping was conducted on 5 consecutive nights; therefore, lacking seasonality; as a result, we urge that the study be extended to include the dry and wet seasons. Accounting for seasonality enables for more in-depth study to understand how much if any season contributes to bacterial prevalence in rodents. Despite this limitation, however, the study gives useful insight on how rodents act as a reservoir of bacterial resistant strains and their risk to its transmission to other organisms.

Conclusion

Presence of multi-drug-resistant *P. mirabilis* in isolates from house rats implicates the possibility of widespread transmission of resistance genes and bacteria in the studied area, with the possibility of causing infections that are difficult to treat. The antimicrobials used in this study are the ones that are commonly used in the area for treating both human and animal infections, implying that they have limited success in their intended use. Also, dissemination of MDR *P. mirabilis* from rat's faecal and urine droppings could result in environmental contamination that can result in the acquisition of the trait by other pathogenic bacteria like *Salmonella* and other unintended bacteria and hence serious public health concern. Therefore, comprehensive interventions, using a one-health approach, would be required to control the situation. Such measures should include improving (1) awareness of the community on the proper use of antimicrobial drugs in humans and animals, (2) good house conditions and hygienic waste management (3) integrated rodent control measures (4) improving treatment regimens for humans and other veterinary animals by doing microbial susceptibility testing before drug prescription.

Ethical statement: Ethical approval was obtained from the directorate of research, technology transfer, and consultancy review board of the Sokoine University of Agriculture with reference and publication committee reference number SUA/DPRTC/R/12/2021.

Author's contribution: FPN, MEB, AM, AASK conceptualized the study, FPN, MEB, AM, AASK designed the experiment, FPN and AASK captured and identified rats, FPN and AM performed microbiological, biochemical and molecular biology works, FPN, AM, AASK analysed and interpreted data FPN and MEB drafted the manuscript and AM and AASK edited the manuscript.

Conflicts of interest: we declare no conflict of interest

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CHAPTER FOUR

4.0 General Discussion, Conclusions and Recommendations

4.1 General Discussion

Generally, the study accomplished to report on rodent species that are routinely found in houses and peri-domestic interfaces in Arusha municipality, Tanzania. Information retrieved from this objective is of crucial advantage in economic, disease and rodent control in the city. Our study was able to show three species of rodents commonly found in the study area. *Rattus rattus* (Black rats) was a dominant species in houses than in peri domestic areas. These results are concurrent with most studies conducted worldwide. For example, studies conducted in Nigeria, South Africa, United kingdom, Canada, Trinidad and Tobago revealed that *Rattus rattus* was a dominating species in houses (Hilton *et al.,* 2002; Meerburg and Kijlstra, 2007; Nkogwe *et al.,* 2011; Wakawa *et al*., 2015; Ramatla *et al*., 2019).

Black rats are commonly associated with shedding of pathogens and parasites through their fecal, urine, and fur droppings to the surrounding, foodstuffs, grain stores and other consumable materials (Singleton *et al*., 2003). These pathogens and parasites mostly are etiological agents for diseases in domestic animals and humans (Ramatla *et al.,* 2019). Several studies have reported an occurrence of pathogenic bacteria in black rats that are listed on the WHO list of preferential pathogens such as of the family *Enterobacteriaceae* like *Escherichia coli, Salmonella* spp, *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp and *Proteus* spp (Ayyal *et al*., 2019). Generally, species of the genus *Rattus* have conceivably accounted to more agony to humans since far back in the centuries through their role played in infrastructural damage, disease transmission and destructive impression on agricultural crops and stored grains.

The second abundant species from the study area was the multimammate mouse; *Mastomys natalensis*. The abundance of these species was relatively higher compared with

other studies reported elsewhere. For example, a study conducted by Agbonlahor *et al*. (2017), presented low abundance of 2% and Katakweba *et al*. (2012b), reported 14.3%. This could be attributed by features like high reproductive rates in the study area due to enough food availability and appropriate geographical location (Demby *et al*., 2001; Odhiambo and Oguge, 2003). *M. natalensis* are spatially distributed species and are known to appear in cultivated lands, fallow lands, woodlands, forest clearances, savannahs and houses (Agbonlahor *et al*., 2017). These findings coincide with the current study where majority of *M. natalensis* were trapped on peri-domestic fields especially in the agricultural fields interfaces, this could be attributed to variations due to season of the year particularly after rainy seasons where *M. natalensis* populations are pronounced to grow, plenty availability of feeds in the field crops and fallow lands and the distance from the house to the farms, crops and fallows (Agbonlahor *et al*., 2017). In this study, the lowest abundant species was *M. musculus* 22 (15.8%).The occurrence of this species in Arusha was relatively low contrary to Agbonlahor *et al*. (2017) who found the abundance of 39.4% southern part of Nigeria.

Rats have been reported to be carriers of pathogenic microbes for centuries (Singleton *et al*., 2003) include *P. mirabilis*. Despite the low prevalence of 2% for *P. mirabilis* from the current study, these findings are consistent with results from Iraq and Nigeria where *P. mirabilis* isolates from rats had a prevalence of 2% (Ayyal *et al*., 2019, Ogunleye and Carlson, 2016, Wakawa *et al*., 2015) and almost similar to a study in Nigeria which reported a prevalence of 3% (Jemilehin *et al*., 2016). However, our findings were lower compared with results from Sudan 22.2% (Mukhtar *et al*., 2018). This variation could be due to differences in sanitary conditions and different climatic conditions in relation to temperature and humidity that affects bacterial growth and multiplication also due to environmental contamination by human and animal fecal materials. Molecular detection

methods for microbial identification and characterization outruns conventional methods like biochemical tests which rely on expression of phenotypic traits (Macrae, 2000). 16s rRNA is a powerful molecular detection tool as it is accurate and sensitive in detection of bacterial isolates (Schmidt *et al*., 1991). In this study, all isolates were sequenced after DNA extraction and with help of BLAST and multiple sequence alignment of sequenced isolates showed all isolates were 100% similar to *P. mirabilis* in the gene bank. Using 16s rRNA partial sequences, evolutionary tree was constructed and showed that all isolates from the study area were almost similar as they clustered together. This could be due to presence of conserved and variable regions in their genomes (Mukhtar *et al*., 2018). These isolates were closely related to isolates from Tunisia and Venezuela.

Rampant crisis to antimicrobial response especially multi drug resistance (MDR) among many genera of the family *Enterobactericeae* to date indicates a major public health concern. Previous studies reported that isolates of *P. mirabilis* were sensitive to most antimicrobial classes opposing recent findings from different countries across the globe, reporting increase resistance of *Proteus mirabilis* (Wilfred *et al*., 2021). The current study showed that all *P. mirabilis* isolates were 75% resistant to Tetracycline and Triomethoprim – Sulfamethaxole. This disagrees with results from Ibadan Nigeria which reported 100% resistance to tetracycline for *P. mirabilis* isolates from rats (Ogunleye and Carlson, 2016). Furthermore, this study revealed 100% resistant to Azithromycin and Ampicillin. Ampicillin resistance in our study is in accordance with reports from Tanzania, Iran, Nigeria and Sudan that have reported similar patterns of resistance to Ampicillin from clinical, rats, and currency isolates (Ogunleye and Carlson, 2016, Mukhtar *et al*., 2018, Mirzaei *et al*., 2019; Wilfred *et al*., 2021).

The 100% resistance found in Azithromycin in this study matches with results from India and Babylon (Mishra *et al*., 2001; Al-khafaji and Bunyan, 2013). The observed high

resistance to the aforementioned antimicrobials may be contributed by several factors. This may include poor disposal of un used antimicrobials (Kissinga *et al*., 2018), mis-use of antimicrobials, high frequent use in chemoprophylaxis, treatment and enhancement of growth in livestock management also due to ready availability and cheap cost of antimicrobials, frequent self-prescribing of over-the-counter drugs or several pre-exposures to various classes of antimicrobials before doctor's and veterinarian prescriptions (Katakweba *et al*., 2012a).

Proteus mirabilis isolates were more susceptible to Cephalosporin, Quinolones and Aminoglycosides, Cefotaxime, Ciprofloxacin and Gentamicin respectively with 100% activity. These findings are in harmony to several studies from rats isolates (Ogunleye and Carlson, 2016; Mirzaei *et al*., 2019; Owoseni *et al*., 2021). This report indicates that the presence of *Proteus mirabilis* among rats trapped in houses and around peri-domestic areas may be due to environmental interactions and commonalities in food chain which further contributes in the spread of antimicrobial resistance traits among various species sharing the same ecosystem with humans (Hamiliton *et al*., 2018). Tetracyclines and Sulfamethaxole - Triomethoprim antimicrobials have been reported frequently used in animals therefore accounting to development of resistance (Katakweba *et al*., 2012a; Sonola *et al*., 2021), This has effect to small mammals that share the same environment with humans and animals as Arusha region is potential in keeping livestock like cattle, poultry, sheep, goats and pigs (Katakweba *et al*., 2018).

Emanation and expansion role of multi drug *P. mirabilis* particularly those producing ESBLs is a setback in medical and veterinary fields. In our understanding, this is the first report on *P. mirabilis* isolates from rats in Tanzania that may require detailed investigation

on the impact to public health. In this study, isolates from rats showed intrinsic acquisition of resistance genes; *TetA*, *Sul1, Sul2* and bla_{TEM}. Detection of bla_{TEM} a narrow spectrum B-lactamase gene which accounts to resistance against narrow spectrum cephalosporins and penicillins explains why all isolates showed phenotypicresistance to ampicillin. The results are similar to Ahmed *et al.* (2007) in Japan isolates from zoo animals. Occurrence of *TetA,Sul1, Sul2* in all isolates resistant and susceptible to Tetracyclines and Sulfamethaxzole - Triomethoprim suggests that some antimicrobial agents in bacteria are "silent" and agreeing to a report in India (Deekshit *et al*., 2012). This indicates a threat as silent genes can be expressed *in vivo* under antimicrobial utilization pressure and transferred to other intestinal and environmental microflora (Adesiji *et al*., 2014). In Tanzania these finding can be supported through a study by Katakweba *et al*. (2018), who revealed the *Tet* and *sul* genes from faecal samples collected from livestock and animals and humans.

4.2 Conclusion

In conclusion, this was the first study of *Proteus mirabilis* in rats conducted in Tanzania and it was able to show various domestic rat species that are distributed throughout Arusha municipality.

It was also able to report the prevalence of *P. mirabilis* 2% (4/139) from rats through bacterial culture methods and 16SrRNA molecular techniques and also demonstrated antimicrobial patterns and clinically important resistance genes (*TetA*, bla_{TEM}, *Sul1* and *Sul2*).

Detection of MDR *P. mirabilis* in this study is a public health risk as several consequences are to be expected as these isolates are mainly responsible for urinary tract infections and merely as food poisoning agents. Such account may result into difficulties in treatment of

such infections and other deadly infections in opportunistic individuals. Also dissemination of MDR *P. mirabilis* from rats fecal and urine droppings results to environmental contamination that can result to acquisition of the trait by other pathogenic bacteria like *Salmonella* and other unintended bacteria and hence a serious public health concern. Therefore, appearance of rats in human homes and peri-domestic areas is a risk to humans, animals, microbes and environmental health.

4.3 Recommendations

i. Education on rat control and prevention methods should be provided to people in the study area not basing only on their destructive behavior but as well as the role they play on zoonotic disease transmission
ii. Further surveillance on zoonotic diseases and their pathogens that can be transmitted between rodents, humans and animals should be conducted
iii. Good sanitation practices in houses and peri domestic areas to eliminate rodents habitats from humans surrounding should be encouraged
iv. Further studies are recommended on the detection of resistance genes found in bacterial isolates from environment samples, rats, humans and animals to understand the interconnectedness of transmission patterns
v. The government should strengthen national control programs to customary conduct antimicrobial susceptibility testing and surveillance through National antimicrobial resistance multi-secterial coordination committee (MCC) and its sub technical working groups (STWG) of humans and agricultural sector

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APPENDICES

Appendix 1: Extraction of bacterial DNA (Quick-DNA™ Universal kit Zymo research)

Procedure

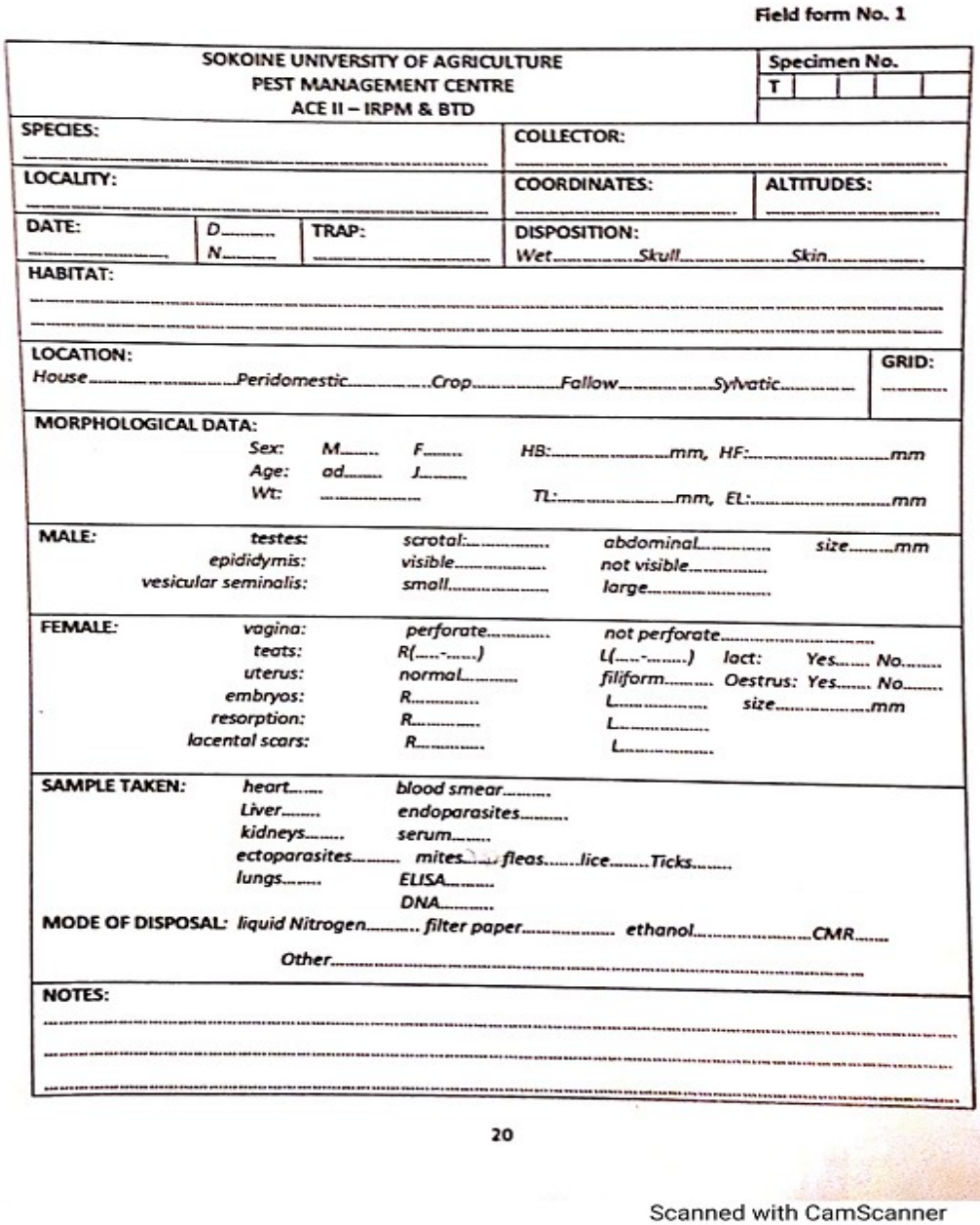
1. Add up to approximately 5×10^6 bacterial cells to a microcentrifuge tube and add; 200µl biofluid and cell buffer and 20µl proteinase K.
2. Mix thoroughly and then incubate the tube at 55^0C for 10 minutes.
3. Add of 450µl Genomic binding buffer to the digested sample and mix thoroughly
4. Transfer the mixture to a Zymo-Spin ™IIC-XL Column in a collection tube. Centrifuge (13,000×g) for 1 minute. Discard the collection tube with the flow through
5. Add 400µl g-DNA pre wash buffer to the column in a new collection tube and centrifuge at the same rpm for 1 minute. Then empty the collection tube
6. Then add 700 µl g-DNA wash buffer and centrifuge for 1 minute. Empty the collection tube
7. Then add 250 µl g-DNA wash buffer and centrifuge for 1 minute. Discard the collection tube with the flow through
8. To elute the DNA, transfer to a clean micro-centrifuge tube. Add 100 µl DNA Elution buffer, incubate for 5 minutes and then centrifuge for 1 minute.
9. Store the eluted DNA in -20 or can be used immediately

Appendix 2: PCR amplification using AccuPower® PCR premix

Procedure;

1. Add template DNA into AccuPower® PCR premix tubes
2. Add distilled water into AccuPower® PCR premix tubes to a total volume of 20µl. Do not calculate the dried pellets.
3. Dissolve the lyophilized blue pellet by flick with your finger or pipetting, and briefly spin down
4. Perform reaction under optimal PCR conditions
5. Maintain the reaction at 4^0C to 8^0C after cycling. The samples can be stored at -20^0C until use.
6. Load samples on agarose gel without adding a loading-dye mixture, and perform electrophoresis.

Appendix 3: Field collection form



Field form No. 1

SOKOINE UNIVERSITY OF AGRICULTURE
PEST MANAGEMENT CENTRE
ACE II – IRPM & BTD

Specimen No. T

SPECIES:

COLLECTOR:

LOCALITY:

COORDINATES:

ALTITUDES:

DATE: D..... N.....

TRAP:

DISPOSITION: Wet..... Skull..... Skin.....

HABITAT:

LOCATION: House..... Peridomestic..... Crop..... Fallow..... Sylvatic.....

GRID:

MORPHOLOGICAL DATA:
Sex: M..... F..... HB:.....mm, HF:.....mm
Age: ad..... J.....
Wt: ..... TL:.....mm, EL:.....mm

MALE: testes: scrotal:..... abdominal..... size.....mm
epididymis: visible..... not visible.....
vesicular seminalis: small..... large.....

FEMALE: vagina: perforate..... not perforate.....
teats: R(.....-.....) L(.....-.....) lact: Yes..... No.....
uterus: normal..... filiform..... Oestrus: Yes..... No.....
embryos: R..... L..... size.....mm
resorption: R..... L.....
lacental scars: R..... L.....

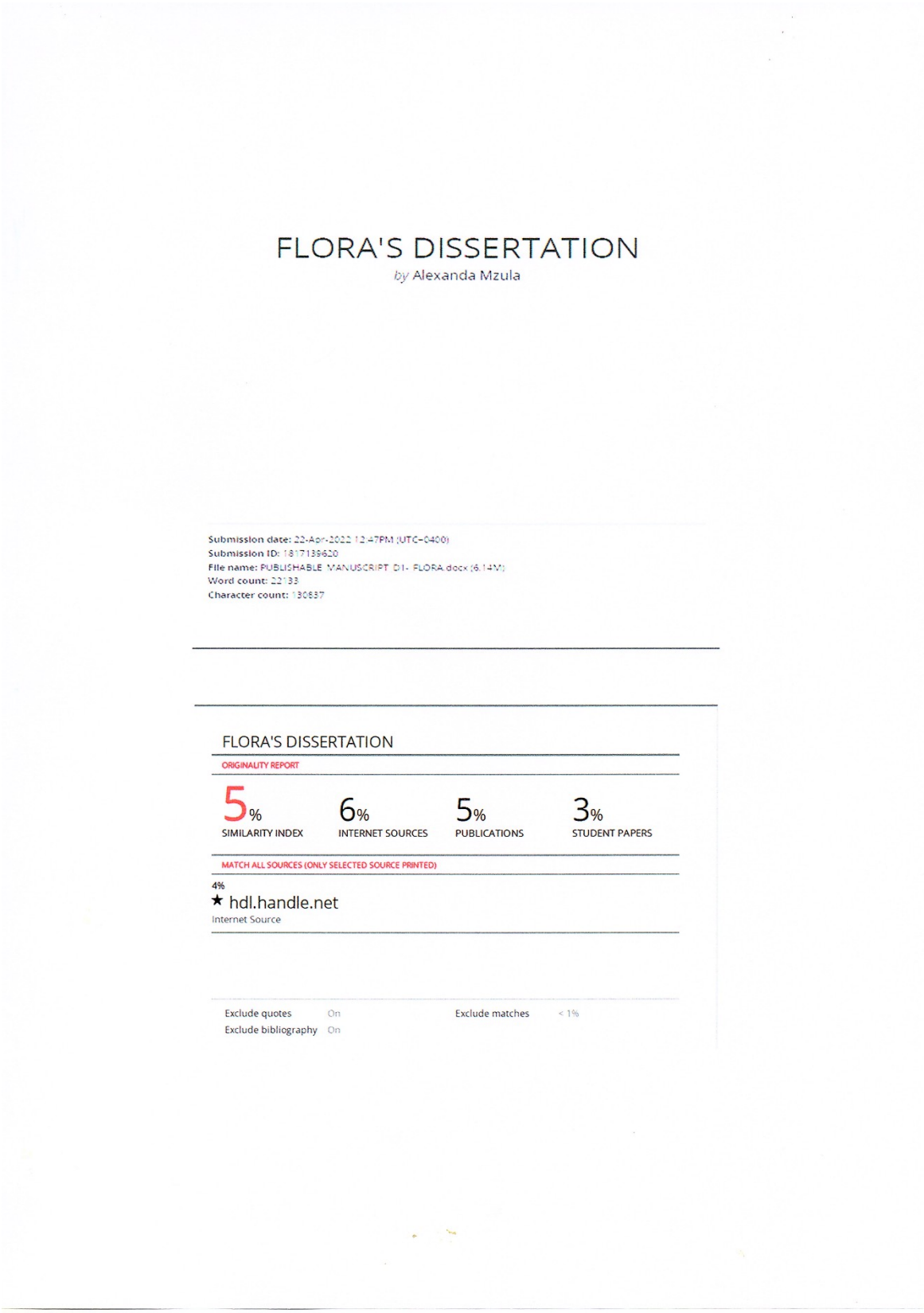
SAMPLE TAKEN: heart..... blood smear.....
Liver..... endoparasites.....
kidneys..... serum.....
ectoparasites..... mites..... fleas.....lice.....Ticks.....
lungs..... ELISA.....
DNA.....

MODE OF DISPOSAL: liquid Nitrogen..... filter paper..... ethanol.....CMR.....
Other.....

NOTES:

20

Appendix 4: Plagiarism checkup report



FLORA'S DISSERTATION

by Alexanda Mzula

Submission date: 22-Apr-2022 12:47PM (UTC-0400)
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FLORA'S DISSERTATION

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