MOLECULAR DETECTION OF MULTIPLE DRUG RESISTANT *KLEBSIELLA* SPECIES FROM URBAN RUNOFF*

Aye Chan Thu[1], Hla Htet Soe[2], Aye Aye Khine[3], Kay Lwin Tun [4]

Abstract

Wastewaters from community drainages may be reservoirs for various bacterial strains harboring drug-resistant genes associated among healthcare and community. Among pathogenic bacteria, *Klebsiella* is an important human pathogen causing opportunistic nosocomial and community acquired infections. In the present study, *Klebsiella pneumoniae* was recorded with the confirmation of phenotypic and molecular analyses. The 16s rRNA gene of the selected isolate (MHBD 10^{-2}(3)), collected from community drainage, Latha Township, was successfully amplified using PCR resulting in a sequence length of 996bp and comprehensive identification results using the phylogenetic analysis involved a comparison of the isolate's sequence with others obtained from GeneBank database. The studied isolate revealed a close clustering with *Klebsiella pneumoniae* species, supported by 100% nucleotide identities. This study may highlight a rapid and cost-effective diagnostic tool for detection of antibiotic-resistant pathogenic bacteria in the natural environment.

Keywords: phenotypic, molecular identification, phylogenetic, 16s rRNA, *Klebsiella pneumoniae*, community drainage

Introduction

Antibiotics are commonly used and easily available in pharmacies in Myanmar. The authority from pharmacists or doctors is not required to purchase the antibiotics in local markets. Inappropriate usage of antibiotics creates a selection pressure for bacteria to acquire antibiotic resistance. The increasing trends of antimicrobial agent (AMA) consumption are more likely to drive to the emergence and spread of antimicrobial resistant (AMR) microbes which cause the large burden of infectious diseases (Godinho, *et. al*., 2024 and Pwint, *et. al*., 2021). In 2019, the deaths of estimated 4.95 million people had associated with bacterial AMR. A global report estimated that without further action, by 2050, 10 million people per year will be dead and 100 trillion USD worth of global production will be lost due to AMR (Miyano, *et. al*., 2022). Thus, AMR has become a major threat to global health, food security and development today.

A research conducting national situational analyses from 11 countries of the South-East Asia, including Myanmar, reported high antibiotic use and poor implementation of policies to encourage appropriate use (Holloway., 2015). In Myanmar in 2019, there were 11,200 deaths attributable to AMR and 40,200 deaths associated with AMR. Myanmar has the 64th highest age-standardized mortality rate per 100,000 population associated with AMR across 204 countries (www.health-data.org). Hence, an ongoing challenge in Myanmar has been the high AMR-related mortality with a high burden of infectious diseases that effectively reach to about 55

*Third prize(2024)

[1] Department of Zoology, University of Yangon
[2] Department of Zoology (Fisheries and Aquaculture), University of Yangon
[3] Department of Zoology, University of Yangon
[4] Department of Zoology, University of Yangon

million population, most of whom battle tuberculosis, acute respiratory infections, diarrhea and malaria (national action plan, version 01, 2017 and www.health-data.org).

The widespread prevalence of antimicrobial resistant bacteria not only in humans and animals but also in the natural environment has resulted due to many common factors favoring AMR bacteria growth. In cases of developing countries like Myanmar, the drastically increasing rate of bacterial resistance to antibiotics has been linked with poor antibiotic quality, enormous amount of antibiotics use in healthcare, agriculture, aquaculture and livestock, easy purchase without prescription, misuse due to the negligence of careful consultation with professionals, lack of awareness and inadequate AMR surveillance (Figure 1). Besides, national policy on the scale or use of antimicrobials in humans and animals is not well established in Myanmar (Pwint, *et. al*., 2021, Mehanni, *et. al*., 2023 and www.health-data.org).

In addition, untreated or improperly treated discharged wastewater which contain hazardous pollutants such as radioactive, chemical and pharmaceutical wastes into the urban drainages and also free DNA encouraging pathogenic microorganisms to acquire multi-drug resistant genes by horizontal gene transfer is the main reservoir contributing to the inescapable consequence of environmental pollution by antimicrobials. Majority of runoff from hospitals, wastewater treatment plants, aquaculture run off and livestock breeding which are considered as hot spots for the dissemination antibiotic resistance, directly dispose to nearby water body via community drains (Thanda, *et. al*., 2021 and Mehanni, *et. al*., 2023).

Considerably, the current situation highlights that the suboptimal wastewater treatment infrastructures in the community, the hospital setting as well as aquaculture and livestock sectors in Myanmar may pose a significant biosafety risk, an emergence of multi-drug resistant bacteria and contamination of both surface and ground water. Moreover, according to the latest national AMR surveillance data and one study of Myanmar in 2017, the reported high levels AMR bacteria were *Stapholococcus aureus*, ESBL Enterobacteriaceae, *Pseudomonas* species and *Acinetobacter* species and *Enterococcus* species (Mehanni, *et. al*., 2023 and www.health-data.org).

On the other hand, there are very few and no evidence-based studies how much AMR bacteria cause contamination in the natural environment. Nowadays, factors influencing the abundance, distribution and potential transmission routes of antibiotic resistant bacteria remains poorly understood and are unclear as there is a limited data concerning resistant microbial profiles associated with wastewaters.

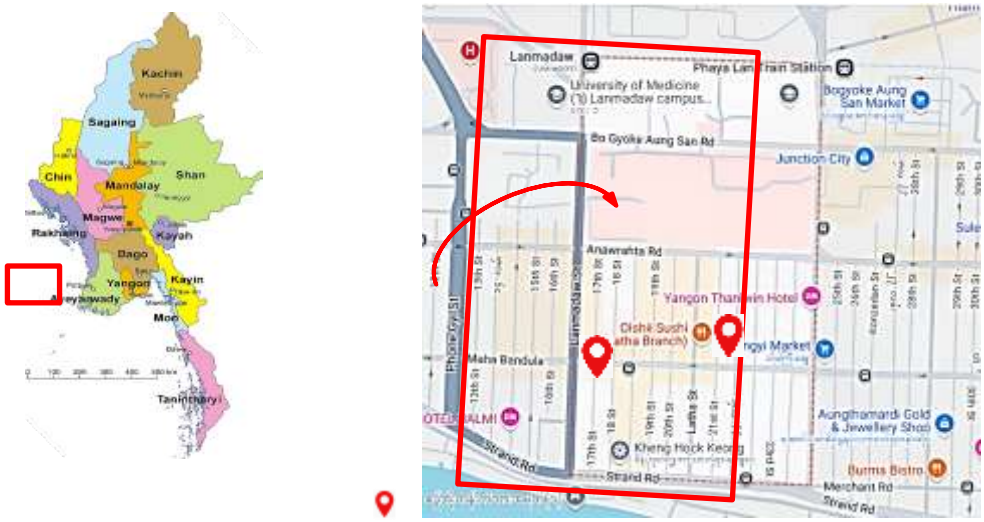
Therefore, this study aims to advocate insights on multi-drug resistant bacteria present in community drainage and improve awareness amongst general public and professionals by generating the evidence-based data to fulfill the research gaps for AMR organisms in Myanmar with One-Health perspective.

Materials and Methods

Sampling site and the study period

Three water samples were collected from three community drainages alongside of Maha Bandula Road, Shwedagon Pagoda Road and Anawrahta Road, Latha Township shown in the map (Figure - 1).

A volume of 500mL of water samples from community drainages were taken from each site with the use of sterile new plastic bottles (sterilized by shaking with 70% ethanol for three times then rinsing with sterile distilled water), preserved in cool box containing ice packs and transported to Microbiology Laboratory at Department of Zoology, University of Yangon. The research was conducted from May to September 2023.



Myanmar Latha Township, Yangon

Figure 1 Map showing location of Latha Township of Yangon, Myanmar (www.googlemap.com)

Sample preparation

One mL of each water sample was diluted into the 9mL of distilled water (1:10 concentration) then homogenized gently by pipetting. A 10 fold serial dilutions were made to the ninth level.

Isolation of bacterial isolates

0.1 mL of each prepared dilution mixture was aseptically inoculated on Nutrient agar medium by the spread plate method (Dubey and Mashwari, 2002). Then, all the plates were incubated at 37^{o}C for 24 hours. After incubation, separate colonies from each plate were selected and sub-cultured again on the same medium by streak plate method to obtain pure cultures. These plates were incubated at 37˚C for 48 hours. The pure collected bacterial isolates (8 isolates from MHBD, 8 isolates from ANYT and 4 isolates from SDG samples) were kept in nutrient agar slants (containing 20% glycerol) in sterile stock bottles with caps and stored at -20˚C for long term use (Bisen and Verma, 1998 and Dubey and Mashwari, 2002).

Determination of drug-resistant patterns by phenotypic confirmatory testing (Antibiotic Susceptibility Assay)

The Kirby-Bauer disk diffusion method according to the CLSI standards was performed to determine the antimicrobial susceptibility profiles of all 20 pure isolates. The test was carried out using Mueller-Hinton medium against five antibiotics; namely, Ampicillin AMP (10 µg), Erythromycin EM (15 µg), Tetracycline TE (30µg), Linezolid LZ (30µg) and Oxacillin (1µg). A total of five commercially available antibiotics was purposely chosen with the aim of targeting to

inhibit cell wall biosynthesis, protein synthesis and DNA replication (Pwint, *et. al*., 2021 and Godinho, *et. al*., 2024).

Bacterial inoculums were prepared by suspending the freshly grown bacteria in 4-5ml sterile nutrient broth. The suspensions were spread uniformly on Mueller-Hinton agar with a sterile swab. Antibiotic discs were then placed on the surface of the inoculated media, center to center at least 25mm apart. After that, the plates were incubated at 37^{o}C for 18-24 hours. After incubation, inhibition zone diameters were measured and interpreted as susceptible (S), intermediary (I) or resistant (R) with the standards of CLSI chart (Thanda, *et. al*., 2021, Mahmud, *et. al*., 2023, Gashaw, *et. al*., 2024, and Godinho, *et. al*., 2024).

Characterization of the selected bacteria

Among 20 bacterial isolates obtained, one strain ((MHBD 10^{-2}(3)) which demonstrated the highest level of antimicrobial resistance was selected as a suitable candidate for further morphological characterization and molecular analysis.

Morphological examination was carried out by determining on the characteristics features such as size, shape, color, edges of the colonies, texture and elevation and observing Gram-staining nature and shapes of the bacterial cells (Bisen and Verma, 1998).

The biochemical reactions of the isolate were examined by utilizing the tests such as (i) Citrate Utilization, (ii) Triple Sugar Iron, (iii) Methyl-Red, (iv) Voges-Proskauer, (v) Indole formation, (vi) H_2S production, (vii) Motility, (viii) Oxidase, (ix) Catalase and (x) Urease (Buchanan, *et al*., 1974).

Molecular Analysis

Molecular Identification

The selected bacterial isolate ((MHBD 10^{-2}(3)), shown resistant to five tested antibiotics, was identified at the species level based on the analysis of their 16S ribosomal RNA gene sequences.

DNA Extraction and PCR amplification of 16S rRNA gene

The total genomic DNA from the selected bacteria was extracted by using Total Nucleic Acid Extraction Kit (MAX DETECT BEYOND BOUNDARIES) according to the manufacturer's instructions. The quantity, purity and concentration of extracted DNA from the isolate was measured by NanoDrop spectrophotometer (Thermo Scientific, USA). Amplification of PCR assay was performed with a set of the primers; the forward primer U1 (5'- CCA GCA GCC GCG GTA ATA CG - 3') and the reverse primer U2 (5'- ATC GGY TAC CTT GTT ACG ACT - 3') by using GoTaq DNA polymerase (Promega, Madison, WI, USA) (Table 1). 20µL of PCR mixture was prepared containing 2µL of template DNA, 1µL of forward and revere primer, 7µL of distilled water and 10µL of Go PCR Master Mix (Promega, Madison, WI, USA). The reaction mixture was run through PCR conditions: 94^{o}C for 10 min, 35 cycles of 94 ^{o}C for 1min, 55 ^{o}C for 1min and 72 ^{o}C for 2min, with a final extension cycle of 72 ^{o}C for 10min (Lu. *et al*., 2000).

The PCR product (996 bp) was visualized and separated in agarose gel electrophoresis (1% agarose gel in 1X Tris-Acetate EDTA (TAE) buffer stained with Ethidium bromide and photographed by UV transilluminator,

Table 1 Primers utilized in PCR amplification

Primers	Sequence (5′-3′)	Target gene	PCR product (bp)	Reference
U1 Forward	CCA GCA GCC GCG GTA ATA CG	16s rRNA	996	Lu. *et al*., 2000
U2 Reverse	ATC GGY TAC CTT GTT ACG ACT	16s rRNA		

DNA sequencing of isolated bacteria

PCR amplication

50µL of PCR amplification was performed to proceed DNA sequence analysis. each 50µL PCR mixture contained 5µL of template DNA, 2.5µL of forward and revere primer, 17.5µL of distilled water and 25µL of Go PCR Master Mix (Promega, Madison, WI, USA). The conditions used for PCR amplification will be as follows: 94^{o}C for 10 min, 35 cycles of 94 ^{o}C for 1min, 55 ^{o}C for 1min and 72 ^{o}C for 2min, with a final extension cycle of 72 ^{o}C for 10min (Lu. *et al*., 2000). Subsequently, amplified PCR product (50µL) was separated 1% agarose gel containing SYBR Safe DNA gel stain (WizPure, Seongnam, South Korea) and visualized under LED transilluminator to observe approximate base pair from the resulted band.

Gel purification for sequencing

For DNA sequencing, DNA was then extracted and purified from distinct bands using the FastGene® Gel/PCR Extraction Kit (Nippon Genetics Europe GmbH, Bunkyo, Tokyo). To isolate DNA, first excise the fragment from an agarose gel using a clean scalpel. Transfer up to 300 mg of the gel slice into a microcentrifuge tube, add 500 µl of binding buffer GP1, vortex and incubate at 55°C for 10-15 minutes, inverting the tube every 2-3 minutes. Next, apply up to 800 µl of the sample mixture from previous step into the GP Column and centrifuge at 13,000 rpm for 30 seconds. Add 600 µl Wash Buffer GP2, and centrifuge at 13,000 rpm for 30 seconds. Discard the flow-through, return the column to the tube, and centrifuge again for 2 minutes to dry the column. Finally, add 20-50 µl elution buffer GP3 to the column, let it stand for 2 minutes and centrifuge at 13,000 rpm for 2 minutes to elute the purified DNA.

DNA sequencing

The DNA sequencing of the isolated amplicons was transmitted to Macrogen, Inc., Korea. Following the manufacturer's instructions, sequencing operations were carried out using the ABI Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD). Every template underwent single pass sequencing with the U1 (Forward) and U2 (Reverse) primers. One was sequenced to compared 16s rRNA genes.

Phylogenetic analysis

The obtained data of 16S rRNA gene sequences was edited, aligned and compared with Bioedit and NCBI Genbank database using NCBI's Standard Nucleotide BLAST search. MEGA 11 Program was utilized for constructing phylogenetic trees to determine the taxonomic position of the selected bacteria by applying a neighbor-joining method of 1,000 replicates used for bootstrapping (Kobayashi, *et. al.*, 2016, Kobayashi, *et. al.*, 2017, Mahmud, *et. al*., 2023, Mehanni, *et. al.*, 2023 and Godinho, *et. al*., 2024). The analysis confirmed the species belonging to *Klebsiella pneumoniae* and *Salmonella bongori* and *Salmonella enterica* subsup.*arizonae* were

included as outer groups to form a separate clade that confirms its divergence from *Klebsiella pneumoniae*.

Results

Antibiotic resistance pattern and identification of the bacterial isolate using phenotypic characters

Among 20 tested isolates, MHBD 10^{-2}(3) exhibited resistance to all antibiotics, showing no clear zones, whereas the other isolates were susceptible to at least one antibiotic (Table 2 and Figure 2).

The selected bacterial isolate (MHBD 10^{-2}(3)) showing the highest multi-drug resistance was tentatively identified by colony morphology, cell shape and biochemical reactions. The colony was creamy white, circular, raised, fluidal with 2 to 3mm diameter (Figure 3). Cell shapes of the isolate under light microscope appeared rod-shaped with gram negative reaction(Figure 3).

Biochemical characters of the isolate are depicted in the table 3. The isolate was able to ferment glucose, lactose, and sucrose, as indicated by the yellow coloration observed in the Triple Sugar Iron (TSI) test. Positive reactions were recorded in the Citrate Utilization, Urease, and Catalase tests, evidenced by a color change to blue, pink, and the formation of gas bubbles, respectively. The positive result in the Citrate Utilization test indicates that the bacterium can utilize citrate as its sole carbon and energy source. In the Catalase test, the production of oxygen gas bubbles confirmed the presence of the catalase enzyme, which breaks down hydrogen peroxide into water and oxygen. Similarly, the pink coloration in the Urease test demonstrated that the isolate produces urease, an enzyme responsible for the hydrolysis of urea into ammonia and carbon dioxide. Conversely, the isolate exhibited negative results for the Oxidase, Methyl Red (MR), Voges-Proskauer (VP), hydrogen sulfide (H_2 S) production, Indole, and Motility tests (Table 3 and Figure 3).

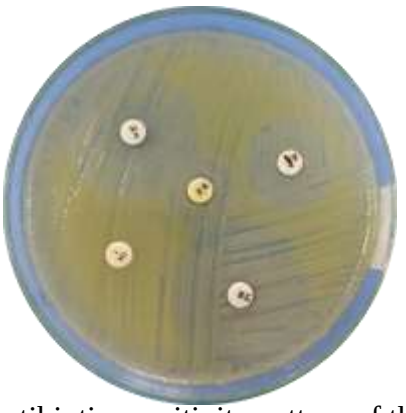


Figure 2 Antibiotic sensitivity pattern of the isolate

Colony on nutrient agar

Cell shape (1000x)

Biochemical reaction.
(1) TSI (A/A), (2) Citrate (+),
(3) MR (-), (4) VP (-), (5) H_2S (-),
Indole (-), Motility (-), (6) Urease (+)

Figure 3 Phenotypic Characteristics of the selected isolate

Table 2 Zone of inhibition (mm) of different antibiotics against the tested isolate

Isolate	Antibiotics	Zone of Inhibition (mm)	Inhibitory zone diameter to nearest millimeter (mm) (CLSI standards)		
			Sensitive (S) ≥	Intermediate (I)	Resistant (R) ≤
MHBD 10^{-2}(3)	EM (15 µg)	0 (R)	23	14-22	13
	TE (30µg)	0 (R)	15	12-14	11
	AMP (10 µg)	0 (R)	17	14-16	13
	LZ (30µg)	0 (R)	23	21-22	20
	OX (1µg)	0 (R)	20	-	-

Table 3 Biochemical characters of the selected isolate

Isolate	Biochemical tests												Tentative Genus
	TSI			Cit	MR	VP	SIM			Oxidase	Catalase	Urease	
	Slant	Butt	Gas				H_2S	Indole	Motility				
MHBD 10^{-2} (3)	A	A	+	+	-	-	-	-	-	-	+	+	*Klebsiella* spp.

DNA sequence analysis and phylogenetic tree construction

From the measurement of nanodrop spectrophotometry, DNA concentration was high with the range of 169.9ng/µL accompanying with the acceptable DNA purity with the range of 1.94 even though there was possible contamination with the absorbance ratio value of 1.06. Continuously, PCR analysis of MHBD 10^{-2} (3) isolate successfully amplified 16S rRNA gene using U1 (Forward) and U2 (Reverse) primers, showing the expected single band at specific size: approximately 996 base pairs (bp) in length (Figure 4). DNA sequences of a 1506 bp fragment obtained from the isolated were illustrated in figure 5.

NCBI BLAST analysis resulted the 14 sequences which are shown in Table 4. Query sequence of the isolate was evaluated and compared with the database sequences based on query coverage, e-value and percent identity. BLAST results revealed that the isolate is 99.64% similar to *Klebsiella pneumoniae* with the accession number AP018750 with sequence submissions originated in Thailand (Table 4). The phylogenetic tree was constructed with the examined isolate (MHBD 10^{-2} (3)) and the 14 similar sequences obtained from the GenBank database using the neighbor-joining method; the accession numbers are shown in Figure 6. The constructed phylogenetic tree clearly resolved the relationships among these sequences.

The 16S rRNA gene sequence of the isolate formed a well-defined cluster, aligning in a distinct clade with *Klebsiella pneumoniae* (AP018750). This clustering was strongly supported with 100% bootstrap value in the analysis. *Salmonella bongori* and *Salmonella enterica* subsp. *arizonae* constituted a distinct clade, affirming their separation from the *Klebsiella pneumoniae* species cluster.

Table 4 16s rRNA gene partial sequence identities of the studied bacteria to other species in the Gene Bank

No.	Description	Query Cover	E value	Percentage Identification	Accession	Origin	Country
1.	*Klebsiella pneumoniae* KP64	100%	0.0	99.64%	AP018750.1	Wound	Bangkok, Thailand
2.	*Klebsiella* sp. VITAJ23	100%	0.0	99.64%	KX770742.1	Contaminated soil	India
3.	*Klebsiella pneumoniae* F17KP0054	100%	0.0	99.64%	CP052136.1	Blood	South Korea
4.	*Klebsiella pneumoniae* 8695	100%	0.0	99.64%	CP085889.1	Patient's ascites	Wenzhou, China
5.	*Klebsiella pneumoniae* JX-CR-hvKP-10	100%	0.0	99.64%	CP064258.1	Blood	Jiangxi, South China
6.	*Klebsiella pneumoniae* KpWEA2 DNA	100%	0.0	99.64%	AP024570.1	Fecal sample	Fukuoka, Japan
7.	*Klebsiella pneumoniae* K56	100%	0.0	99.64%	MW843607.1	Ward	Saudi Arabia
8.	*Klebsiella pneumoniae* NK_H4_026	100%	0.0	99.64%	CP152609.1	Blood	Norway
9.	*Klebsiella pneumoniae* KSB1_7G	100%	0.0	99.64%	CP110723.1	Rectal swab	Australia
10.	*Klebsiella pneumoniae* UHD-50_PH	100%	0.0	99.64%	CP121131.1	Rectal swab	United Kingdom
11.	*Klebsiella pneumoniae* 15017	100%	0.0	99.64%	CP132688.1	Urine	Virginia, USA
12.	*Klebsiella pneumoniae* K014	100%	0.0	99.64%	CP128719.1	Blood	Chicago, USA
13.	*Klebsiella pneumoniae* KPN528	100%	0.0	99.64%	CP020853.1	Respiratory	Houston, USA

No.	Description	Query Cover	E value	Percentage Identification	Accession	Origin	Country
14.	*Klebsiella pneumoniae* 2017-45-134-04A	100%	0.0	99.64%	CP109720.1	Dispensary sink drain	Florida, USA

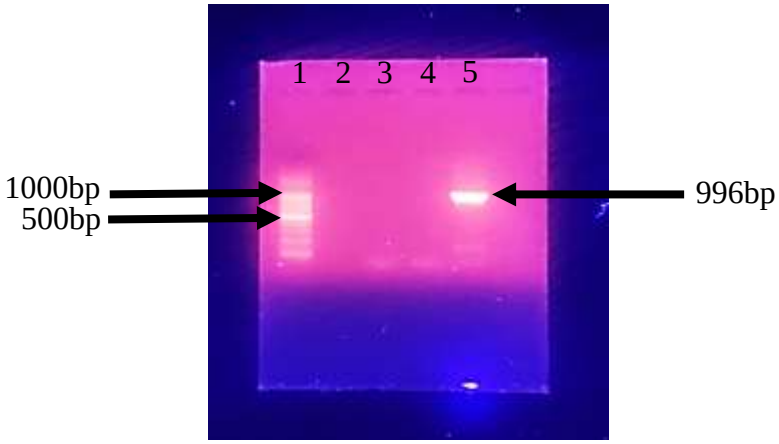


Figure 4 Agarose gel image of amplified fragment of the selected isolate 16s rRNA gene (996bp) using PCR. Lane 1: 1000bp DNA ladder, Lane 5: amplified PCR products from the bacterial isolate MHBD 10^{-2} (3)

GTCGAACGTTATCGGATTACTGGGCGTAAGCGCACGCAGGCGGTCTGTCA --- --- --- --- --- --- --- 50
AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTG --- --- --- --- --- --- --- 100
GCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAA --- --- --- --- --- --- --- 150
ATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACA --- --- --- --- --- --- --- 200
AAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC --- --- --- --- --- --- --- 250
CCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGA --- --- --- --- --- --- --- 300
GGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGG --- --- --- --- --- --- --- 350
CCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG --- --- --- --- --- --- --- 400
AGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGAC --- --- --- --- --- --- --- 450
ATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAGAC --- --- --- --- --- --- --- 500
AGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGT --- --- --- --- --- --- --- 550
CCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGA --- --- --- --- --- --- --- 600
ACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTC --- --- --- --- --- --- --- 650
AAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCAT --- --- --- --- --- --- --- 700
ATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATATAGTATGT --- --- --- --- --- --- --- 750
CGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCT --- --- --- --- --- --- --- 800
AGTAATCGTAGATCAGAATGCTACGGTGAATACATTCCCGGGCCTTGTAC --- --- --- --- --- --- --- 850
ACACCGCCCGTCACACCATGAGAGTGGGTTGCATACAAATTAAGTAGCTT --- --- --- --- --- --- --- 900
AACCTTCGGGAGGGCGCTTACCACTTTGTGATTCTGACTGCGGTGAGCCC --- --- --- --- --- --- --- 950
TACATTTACGGGGGGGGGAGGAAAAAAAAGCAGCAGCCTGGAATGCAGTG --- --- --- --- --- --- --- 1000
TATTGGGAGATTATGTAGAGCTGAAGTTCTGGAAAAGCATCGATGCTTGT --- --- --- --- --- --- --- 1050
GATTAGAACGAAAGGCAACGTTAGAATAGTTGGATTTGAGTTGGTCACAT --- --- --- --- --- --- --- 1100
GCATTGCTAATTTAGTGGGCAATGGTAATGAAGTAAGCGCAGCTGGTGGT --- --- --- --- --- --- --- 1150
TATGTTGAAAGAGTAGCATCAGTGGGCGATGAAAGGAGGTGACGTTGTGA --- --- --- --- --- --- --- 1200
TAAGATGCTTTTGACGCCTTTGGTGAGCGTTCTTTGGATGGATGGAATCA --- --- --- --- --- --- --- 1250
TACAATGTAGTGCCCAAATGATTTAGCGATAAGTCGAAGGATGTCGCTAT --- --- --- --- --- --- --- 1300
AATAAACAAGTGAGATGTTGTGCCGATGAACATCTCTGTATAGTAATAGG --- --- --- --- --- --- --- 1350
CGATGGCTAACGACATGAGTATGGCTGTGAACTAGAGCAATGATGAATGG --- --- --- --- --- --- --- 1400
TAGAACATAAAACATTGTTTTTCCGTCTATCACTATGACTTGGTATAGCG --- --- --- --- --- --- --- 1450
ATGAGATGGGGCTGCATCGAGGACGCGTATACGCGGGTTGGTTACACTCC --- --- --- --- --- --- --- 1500
ATTGGA --- --- --- --- --- --- --- 1506

Figure 5 Partial sequences of 16s rRNA gene of the studied bacteria

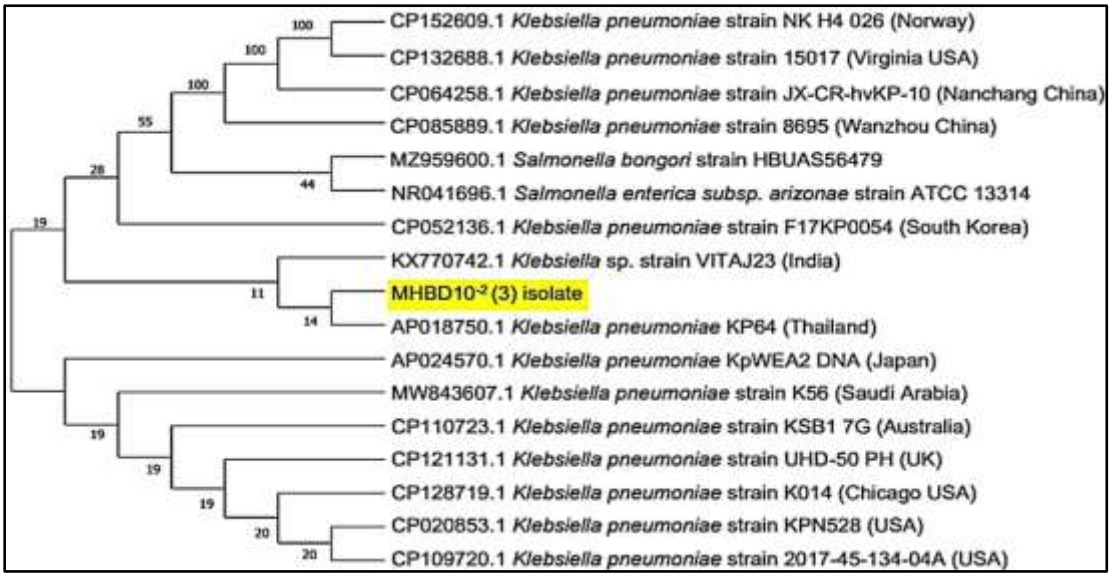


Figure 6 Phylogenetic tree of 16s rRNA gene of the studied bacterial isolate using the neighbor-joining method. *Salmonella bongori* and *Salmonella enterica* subsp. *Arizonae are used as out groups on phylogenetic analysis.*

Discussion

The present study aimed to isolate multi-drug resistant pathogenic bacteria from community drainage systems in urban areas. Biochemical and phenotypic characterization, along with molecular analysis through 16S rRNA gene sequencing, were performed to identify the selected bacterial isolate (MHBD 10^{-2} (3)) exhibiting the highest level of antibiotic resistance at the species level. Additionally, antimicrobial susceptibility test was carried out by the Kirby Bauer disc diffusion method to detect the pathogenic bacteria resistant to different classes of antibiotics. Based on phenotypic and genotypic analyses, the selected isolate, resistant to all tested antibiotics, was identified as *Klebsiella pneumoniae*.

On the nutrient agar medium, *Klebsiella pneumoniae* isolate were as large colonies with creamy white, circular, raised, fluidal appearance. The microscopic examination showed cells of gram-negative bacilli. Positive results were recorded in Citrate Utilization, Triple Sugar Iron, Urease and Catalase tests followed by gas production. Negative responses were observed in oxidase, Methyl-red, Voges Proskauer, H_2S production, Indole and Motility tests. These results were agreed with AI Jadar and Ibrahem, 2022. The present studied isolate was entirely resistant to Ampicillin AMP (10 µg), Erythromycin EM (15 µg), tetracycline TE (30µg), linezolid LZ (30µg) and oxacillin (1µg). Other researches has also reported these antibiotics to be among the least effective against *K.pneumoniae* isolates. (Badger-Emeka, *et. al*., 2021). Considerably, these results further highlight the global public health threat resulting MDR bacterial isolates.

In the present study, the PCR method was performed with the use of U1 (forward) and U2 (reverse) primers that amplify conserved regions (16S rRNA) in various bacteria for DNA sequencing (Lu, *et. al.,* 2000). DNA sequence analysis was carried out by NCBI BLAST search database. The isolate exhibited 100% query coverage and 99.64% sequence identity to the standard strain recorded in gene bank, *Klebsiella pneumoniae* KP64 (AP018750). A phylogenetic tree was constructed to determine the kinship relationship of MHBD 10^{-2}(3) isolate resistant to

multiple antibiotics. From the result, the close relationship is evidenced by their shared clade, indicating a high level of genetic similarity between the two strains. Moreover, the clustering pattern indicates that MHBD10-2(3) shares a more recent common ancestor with the Thailand strain than with other *Klebsiella pneumoniae* strains from different geographical locations, suggesting possible regional or environmental influences and diverse sources contributing to the observed genetic similarities. This finding may be relevant for understanding the spread of multi-drug resistant *K. pneumoniae*, highlighting the potential for cross-border transmission or similar selective pressures in these environments (AI Jadar and Ibrahem, 2022).

Klebsiella is the second most common cause of nosocomial infections among Enterobacteriaceae. *Klebsiella* spp. are opportunistic pathogens that cause severe diseases in hospital setting and are difficult to identify and are often misclassified in clinical microbiology laboratories. Majority of *Klebsiella* infections are caused by *K. pneumoniae*. This organism cause pneumonia, urinary tract infection, soft tissue infection and septicemia, which often leads to septic shock. Isolates from various sources are just as virulent as clinical isolates from hospitals and can produce important virulence factors. In this study, referenced isolates from various sources from different countries are carbapenem-resistant and ESBL (extended-spectrum-β-lactamase producing *K.pneumoniae*. From sequence alignment and phylogenetic analysis, the studied isolate had the closet genetic similarity with KP 64 (Thailand strain), resistant to carbapenems and causing disease outbreak in Thailand (Sakamoto, *et al*., 2018). So, the isolate may be considered as potentially harboring hypervirulent, multidrug- resistant and transferable genes because of its specific resistance-associated genes against different antibiotics. Therefore, precise confirmation of *Klebsiella* identification is very important for taxonomic and molecular characterization. Also, PCR analysis has been reported to be more accurate in the identification of *Klebsiella* species when compared with other preliminary identification tests (Rawy, *et. al*., 2020).

Conclusion

The identification of the studied isolate (MHBD 10^{-2}(3)), isolated from community drainage of Latha township was carried out through both morphological and molecular methods. The isolate showed highest resistance to multiple antibiotics. The PCR of 16S rRNA gene sequence gave an approximately 996bp amplicon for the isolate. Phylogenetic analysis showed that the isolate was *Klebsiella pneumoniae*, with 100% similarity to *Klebsiella pneumoniae* KP64 (AP018750). Further studies are needed to elucidate specific antibiotic resistance genes using genome sequencing.

Acknowledgements

I would like to express sincere gratitude to all of my professors and colleagues, Department of Zoology, University of Yangon.

References

Al Jader, Z. W. and Ibrahem, S. N. 2022. Molecular detection of some pathogenic bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa and Escherichia coli*) from human saliva. *Microbial Biosystems* 7(1).2022.1058. 10.21608/MB.2022.138972.1058.

Badger-Emeka, L.I., Al-Sultan, A.A., Bohol, M.F.F., Al-Anazi, M.R., Al-Qahtani, A.A. Genetic Analysis, Population Structure and Characterisation of Multidrug-Resistant *Klebsiella pneumoniae* from the Al-Hofuf Region of Saudi Arabia. *Pathogens*, 10, 1097. https://doi.org/10.3390/ pathogens10091097.

Bisen, P.S. and Verma, K. 1998. *Handbook of Microbiology*. CBS Publishers and Distributors, New Delhi, India.

Buchanan, R.E. and Gibbons, N.E., eds. 1974. *Bergey's Manual of Determinative Bacteriology*, Eight edition. Williams and Wilkins Company, United States of America.

Dubey, R.C. and Maheshwari, D.K. 2002. Practical Microbiology. S.Chand and Company Ltd., Ram Nagar, New Delhi.

Gashaw, M., Gudina, E.K., Tadesse, W., Froeschl, G., Ali, S., Seeholzer, T., Kroidl, A., Wieser, A. 2024. Hospital Wastes as Potential Sources for Multi-Drug-Resistant ESBL- Producing Bacteria at a Tertiary Hospital in Ethiopia. *Antibiotics* (13), 374. https://doi.org/10.3390/ antibiotics13040374.

Godinho, O., Lage, O.M., and Quinteira, S. 2024. Antibiotic-Resistant Bacteria across a Wastewater Treatment Plant. *Appl. Microbiol*. (4), 364–375.https://doi.org/10.3390/ applmicrobiol4010025.

Holloway, K., 2017. Antibiotic use in South East Asia and policies to promote appropriate use: reports from country situational analyses. *Antimicrobial Resistance in South East Asia*, *BMJ* ;358 :j2291.
https://www.healthdata.org/antimicrobial-resistance

Kobayashi, T., Taguchi, C., Kida, K., Matsuda, H., Terahara, T., Imada, C., Moe, N, K, T. and Thwe, S, M. 2016. Diversity of the bacterial community in Myanmar traditional salted fish yegyo ngapi. *World J Microbiol Biotechnol* (32), 166. DOI 10.1007/s11274-016-2127-z.

Kobayashi, T., Hitomi Taya, H., Pongtep Wilaipun, P., Werawan Chinaksorn, W., Kenta Yonezuka, K., Tomoko Harada, T., Wakana Ishida, W., Hirona Yano, H., Takeshi Terahara, T., Chiaki Imada, C. and Michiya Kamio, M. 2017. Malachite‐green‐removing properties of a bacterial strain isolated from fish ponds in Thailand. *Fish Sci* (83):827–835. DOI 10.1007/s12562-017-1102-4.

Lu, J. J., Perng, C. L., Lee, S. Y., and Wan, C. C. 2000. Use of PCR with universal primers and restriction endonuclease digestions for detection and identification of common bacterial pathogens in cerebrospinal fluid. *JOURNAL OF CLINICAL MICROBIOLOGY*, p. 2076–2080.

Mahmud, MS., Hosen, MA., Hossion, MI., Sabuj, MS, S., Rumi, NA., Hossain, MK., Dauelbait, M., Nafidi, H-A., Dawoud, TM., Ibrahim, M. and Bourhia, M. 2023. Isolation, identification, and characterization of resistant bacteria to antibiotics from pharmaceutical effluent and study of their antibiotic resistance. *Front. Microbiol.* 14:1307291. doi: 10.3389/fmicb.2023.1307291.

Mehanni, MM., Gadow, SI., Alshammari, FA., Modafer, Y., Ghanem, KZ., El-Tahtawi, NF., El-Homosy, RF. and Hesham, AE-L. 2023. Antibiotic-resistant bacteria in hospital wastewater treatment plant effluent and the possible consequences of its reuse in agricultural irrigation. *Front. Microbiol*. 14:1141383. doi: 10.3389/fmicb.2023.1141383.

Miyano, S., Htoon, TT., Nozaki, I., Pe, EH., Tin, HH. 2022. Public knowledge, practices, and awareness of antibiotics and antibiotic resistance in Myanmar: The first national mobile phone panel survey. *PLoS ONE* 17(8): e0273380.

National Action Plan for Containment of Antimicrobial Resistance: Myanmar, 2017-2022. (Draft) July, 2017(Version 01).

Pwint, K. H., Min, K. S., Tao, W., Shewade, H. D., Wai, K. T., Kyi, H. A., Shakya, S., Thapa, B., Zachariah, R., Htun, Z. T. 2021. Decreasing Trends in Antibiotic Consumption in Public Hospitals from 2014 to 2017 Following the Decentralization of Drug Procurement in Myanmar. *Trop. Med. Infect. Dis*. (6), 57. https:// doi.org/10.3390/tropicalmed6020057.

Rawy, D. K., El-Mokhtar. A. M., Hemida, S. K., Askora, A., Yousef, N., 2020. Isolation, characterization and identification of *Klebsiella pneumoniae* from ASSIUT university Hospital and sewage water in Assiut governorate, Egypt. Assiut Univ. J. of Botany and Microbiology 49(2), pp.60-76. ISSN: 1687-4927.

Sakamoto, N., Akeda, Y., Sugawara, Y., Takeuchi, D., Motooka, D., Yamamoto, N., Laolerd, W., Santanirand, P., Hamada, S. 2018. Genomic characterization of carbapenemase-producing Klebsiella pneumoniae with chromosomally carried blaNDM-1. *Antimicrob Agents Chemother*. 62:e01520-18. https://doi.org/10.1128/AAC.01520-18.

Thandar, M., Win, H, H., Oo, K, M., Kyi, M, M. and Khine, S. 2021. Antimicrobial resistance in wastewater of Yangon Region, Myanmar from one health perspective. *Int J Community Med Public Health*. 8(12):5714-5721. http://www.ijcmph.com. pISSN 2394-6032 | eISSN 2394-6040.