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Research Article

MOLECULAR CHARACTERIZATION OF *BLA*TEM AND *BLA*CTX-M GENES IN ESBL-PRODUCING *PROTEUS MIRABILIS* ISOLATED FROM KLANG RIVER, MALAYSIA

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ABSTRACT

The emergence of multidrug-resistance (MDR) bacteria poses a health threat to the public. This is seen among the extended spectrum β-lactamase (ESBL) producing *Enterobacteriaceae* family often mediated by *bla*TEM and *bla*CTX-M genes. This study evaluates the molecular characteristics of ESBL-producing *P. mirabilis* isolated from Klang River, Malaysia. The physiochemical properties of the Klang River were determined. 150 water samples were collected, which 546 Gram-negative *bacilli* were detected. 39 (26.0%) *P. mirabilis* were isolated and tested against β-lactam antibiotics. 21 (53.8%) exhibited MDR, which 15 (71.4%) were ESBL-producing strains after screening with PCR and genetic sequencing approaches. Among the 15, 3 strains (20%) exhibited *bla*TEM gene and 12 (80%) exhibited *bla*CTX-M gene. Molecular docking was performed to understand the structure of the mutated β-lactamase. Findings suggest high prevalence of *bla*TEM and *bla*CTX-M genes among *P. mirabilis* strain isolated. There is a need of advanced molecular techniques to further elucidate MDR bacteria.

Keywords: Multidrug-resistance; *Proteus mirabilis*; *bla*TEM; *bla*CTX-M; β-lactamase

INTRODUCTION

Proteus mirabilis, a member of the *Enterobacteriaceae* family, is well known to be a pathogen of the urinary tract giving rise to symptomatic infections such as pyelonephritis and urinary cystitis especially in geriatric patients and those undergoing long-term catheterization[1]. While *P. mirabilis* associated infections are generally combatted through use of broad-spectrum penicillin and cephalosporin, it first emerged as ESBL-producing strains in the 1990s as nosocomial infections, which later caused outbreaks[2].

Continuous exposure towards β-lactam antibiotics has induced a dynamic production and mutation of β-lactamases in the bacteria that serves as a defense mechanism against newly developed β-lactam antibiotics via production of ESBLs[3]. ESBLs are enzymes that are capable of hydrolyzing third generation cephalosporins rendering these once useful antibiotics ineffective[4]. The production of ESBLs is generally associated with the *bla* genes with variants such as TEM, OXA, SHV and CTX-M[1]. These ESBL-producing strains are capable of causing severe conditions such as pneumonia and bacteremia[5].

The rise of multidrug-resistance (MDR) bacteria has raised concerns towards the integrity of global public health in the modern era citing numerous cases especially in Southeast Asian countries comprising of Malaysia, Singapore, Indonesia and Thailand with increasing mortality rates[6]. The presence of ESBL-producing MDR bacteria has far extended from the clinical environment as they are now found present in the natural environment, including water, soil, and especially food producing animals. This fact coincides with the use of antibiotics outside the clinical environment as well. In recent years, the presence of ESBL-producing strains has been confirmed virtually in all ecological niches. Aquatic ecosystems are often recognized as a common reservoir as these environments are enriched with antibiotics, specifically β-lactam antibiotics from agricultural runoffs and hospital waste waters[7]. This is seen in China's urban river as 39 ESBL-producing isolates were identified[8]. Similarly, in Iran, 38.5% isolates in water sources were found to be ESBL-producers[9].

Raising the possibility of ESBL-producing strains among us, the Klang River runs through Klang Valley, which is one of the most urban regions of Kuala Lumpur consisting of relatively high population densities of cities and towns with numerous aquatic environments such as rivers and lakes. These rivers and lakes are most likely to be exposed to chemical and biological wastes that might have been contaminated with ESBL-producing bacteria such as *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and others. Noting this fact, there is a lack of research concerning ESBL-producing bacteria in the rivers of Malaysia[10]. Thus, this study was done to investigate the existence of ESBL-producing *P. mirabilis* in the Klang River, and at the same time determine the physicochemical properties of the river. *P. mirabilis* was identified using biochemical characterization, and its phenotype was confirmed using the phenotypic study of β-lactamase activity against β-lactam antibiotics. Molecular

techniques such as PCR and DNA sequencing were performed to identify the genes encoding the ESBL-phenotype in *P. mirabilis*.

MATERIAL AND METHODS

Sample collection

150 samples were collected from the Klang River, which were taken at multiple sites downstream including Klang Gates Dam, city of Kuala Lumpur, Gombak River, Kuyoh River and Port Klang. The samples were collected in a 20 mL sterile container and a 300 mL dark amber bottle. Both the containers and the bottles were kept on ice to ensure viability of microorganisms present in it.

Determination of physicochemical properties of the river water

Properties such as pH level, temperature, presence of suspended debris, odor and turbidity were measured in-situ, while the biochemical oxygen demand (BOD) was analyzed in the laboratory. The pH level was measured with a pH meter (Eco Testr pH 2 Meter), which was calibrated with a pH 7.0 buffer solution. The temperature of the water samples was measured using a thermometer. The turbidity was observed by the unaided eye, and the odor was sniffed.

Determination of BOD value

The BOD value of the river water was determined by performing the Winkler method. 1.0 mL of manganese (II) sulphate solution was added onto the river water collected in the amber bottle followed by 1.0 mL of alkaline potassium iodide solution, which would cause the formation of a precipitate. As the precipitate settles, 1.0 mL of 50% v/v sulphuric acid solution was added to dissolve the precipitate. The mixture was transferred into a conical flask after being left to stand for 5 minutes. It was then titrated with 0.0018 M sodium thiosulphate. 1.0 mL of starch solution was added into the conical flask, and it was titrated again with 0.0018 M sodium thiosulphate until the solution turns colorless.

The volume of sodium thiosulphate in each titration was recorded and the average volume was calculated. The initial dissolved oxygen (DO_0) level was obtained and tabulated. The remaining water samples within the bottle was topped up with distilled water and was left to stand for 5 days before repeating the titration – this allows the calculation for the final dissolved oxygen (DO_1) level.

The BOD level was calculated using the formula[11]:

$$BOD\ (mg/L) = \frac{DO_1 - DO_0}{B} \times 300\ \text{(bottle volume)}$$

Where DO_0 = initial dissolved oxygen (immediately after preparation), DO_1= final dissolved oxygen (after 5 days of incubation), B = fraction of sample used.

Isolation of bacteria from water samples

The water samples were serially diluted to 10-folds from $10^{-1}$to 10^{-4} with distilled water (1 mL of water sample with 9 mL distilled water) prior to inoculating and streaking onto the culture media. 100 μL from 10^{-2} and 10^{-3} dilutions were inoculated onto MacConkey agar (MCA) plates using spread-plating technique, which were incubated at 37℃ for 24 hours.

In obtaining a pure culture, non-lactose fermenting (NLF) colonies were subcultured onto MCA again using streak-plating method. Colonies grown were further subcultured on Eosin Methylene Blue (EMB) agar and Blood agar, which were incubated at 37℃ for 24 hours.

The NLF colonies from MCA were also sub cultured into tubes containing 3 mL saline suspension and streaked onto a nutrient agar slant and were incubated at 37℃ for 24 hours[12]. The colonies from the nutrient slant were then inoculated into tubes containing 5 mL nutrient broth and were incubated at 37℃ for 24 hours. Positive growth was determined through a change in turbidity of the nutrient broth – these tubes were used to store antibiotic resistant strains at -80°C.

Identification of bacterial isolates

P. mirabilis is identified through several criteria; on its cultural characteristics, swarming colonies on blood agar and NLF on MCA and EMB agar. Microscopic examination of Gram stained bacterial isolates examined showed Gram-negative bacilli. Biochemical tests were also done to identify *P. mirabilis* isolates including Indole, Methyl red (MR), Voges-Proskauer (VP), citrate as IMViC test, Triple Sugar Iron (TSI) agar, mannitol fermentation, and motility tests[13].

Antibiotic Resistance Test (ART)

ART was performed according to Kirby-Bauer disc diffusion method using 9 β-lactam antibiotic discs: ampicillin (AMP), ampicillin/clavulanic acid (AMC), cefpodoxime (CPD), cefotaxime (CTX), cefuroxime (CXM), ceftazidime (CAZ), ceftriaxone (CRO), cefepime (FEP), and aztreonam (ATM). These antibiotic discs are categorized into 3 different classes: Penicillin (AMP and AMC), Cephalosporin (CPD, CTX, CXM, CAZ, CRO, and FEP), and Monobactam (ATM)[14].

Single colonies that were subcultured onto the nutrient slant were transferred into a tube containing 3 mL saline solution to prepare bacteria suspension[12]. Lawn culture method was performed on Muller-Hinton Agar (MHA), and was incubated at 37℃ for 24 hours. Zone of inhibition around the discs were measured in diameters to the nearest millimeter (mm).

Phenotyping of β-lactamase activity

Phenotyping was done via two methods, Combination Disc Test (CDT) and Modified Double Disc Synergy Test (MDDST). CDT incorporates a cephalosporin disc, CAZ and another in combination with clavulanic acid (CAZ/CA) 20 mm apart to compare the zone of inhibition from both discs.

DDST method employs the use of CTX, CRO, CPD, and CAZ discs placed 20 mm apart, with AMC disc in the center. Any zone of inhibition augmented in the direction of AMC is considered as positive result.

Escherichia coli 25922 were used as negative control for ESBL production[15].

Molecular Characterization of MDR bacteria

Bacterial genomic DNA was extracted using innuPREP Bacteria DNA Kit (Analytik Jena, Germany). PCR was performed to identify genes responsible for ESBL-resistance using forward (F) and reverse (R) oligonucleotide primers (Rapfon Glamor, Malaysia) (Table 1).

Table 1: **Oligonucleotide primers used in PCR**

Primer name	Oligonucleotide sequence [5' to 3']	Expected product size [bp]	Target gene
TEM - F	ATGAGTATTCAACATTCCG	867	*bla*TEM
TEM - R	CTGACAGTTACCAATGCTTA		
CTX-M-F	ATGTGCAGYACCAGTAARGT	593	*bla*CTX-M
CTX-M-R	TGGGTRAARTARGTSACCAGA		

PCR was performed using a thermal cycler (Eppendorf Mastercycler EP S Thermal Cycler, US). However, different set of PCR conditions were subjected onto the DNA samples depending on the primers used for different genes, *bla*TEM and *bla*CTX-M[9,16] (Table 2).

Table 2: PCR conditions for *bla*TEM and *bla*CTX-M gene

Gene	PCR conditions
*bla*TEM	Initial denaturation: 1 cycle of 5 min at 96℃; Denaturation: 35 cycles of 1 min at 96℃; Annealing: 35 cycles 1 min at 43℃; Extension: 35 cycles min at 72 ℃; Final extension: 1 cycle of 10 min at 72 ℃
*bla*CTX-M	Initial denaturation: 1 cycle of 1 min at 94℃; Denaturation: 35 cycles of 50 secs at 94℃; Annealing: 35 cycles 40 secs at 50℃; Extension: 35 cycles of 1 min at 72 ℃; Final extension: 1 cycle of 5 min at 72 ℃

The products of PCR were electrophoresed at 70V on agarose gel along with a 10kb DNA ladder (Bio line Reagents Ltd, UK). The resulting DNA fragments were visualized at 302 nm using Alpha Imager.

Genomic sequencing of *bla*TEM and *bla*CTX-M genes

*bla*TEM and *bla*CTX-M genes were sequenced via Sanger sequencing, in which the acquired genomic sequences were compared with sequences available in Gen Bank (BLAST)[17]. The genomic sequences were also compared with available NCBI sequences for any presence of unique amino acid changes to indicate mutation[18].

Molecular docking of *bla*TEM and *bla*CTX-M gene

Forward and reverse sequences were assembled into a single gene with the Bio Edit tool, which was then transcribed into a protein sequence using Sequence Manipulation Suite (SMS). A 3D structure of the modelled β-lactamase protein using is SWISS-MODEL Server[14]. Potential binding sites of the protein (receptor) was predicted using CASTp server whereas 3D structures of antibiotics (ligands) were collected from PubChem database. Docking interaction was carried out with Auto Dock Vina and studied using PyMol[14].

RESULTS

Physicochemical properties of the river water

150 water samples were categorized according to their physical appearance and odor. (6.0%) samples were slightly clear, odorless, with no debris; 9 (6.0%) samples were slightly clear, odorless with no debris; 36 (24.0%) samples were slightly clear, odorless, with debris; 9 (6.0%) samples were yellowish with foul smell, however with no debris; 81 (54.0%) samples were yellowish with foul smell and debris; 9 (6.0%) samples were brownish with foul smell and little debris.

The water quality parameters assessed were its pH, temperature, DO levels, and BOD levels (Table 3).

Table 3: Statistical normal distribution of Physico-chemical parameters of water samples (n = 150)

Physico-chemical parameters	Acidity (pH)	Temperature (℃)	Dissolved Oxygen (mg/L)	Biochemical oxygen demand (mg/L)
Mean	7.09	29.4	1.88	2.20
Min-Max	6.2-7.6	28-32	0.2-5.8	0.00-8.85
Standard deviation	7.09 ± 0.32	29.36 ± 1.00	1.88 ± 1.48	2.20 ± 2.17
Median	7.1	29	1.65	1.58
Mode	7.3	29	0.65	0.00

Isolation and Identification of *Proteus spp.*

546 NLF colonies were isolated from 150 samples, with 63 isolates identified as *Proteus spp.* based on their cultural characteristics: swarming growth in MCA, BA, and EMB agar. 21 isolates were confirmed at *P. mirabilis* via biochemical test: Indole negative, MR positive, VP negative, citrate utilization test variable, urease positive, glucose fermenter and H_2S producer in TSI agar[19].

ART Result

Multidrug resistance is determined when a bacterium shows resistance to three or more antimicrobial drug classes[20]. Accordingly, 21 (53.8%) of the isolates shown MDR. This can be seen from an example of MH plate no. 42 (P42), one of 21 isolates showing multidrug resistance (Figure 1-2).

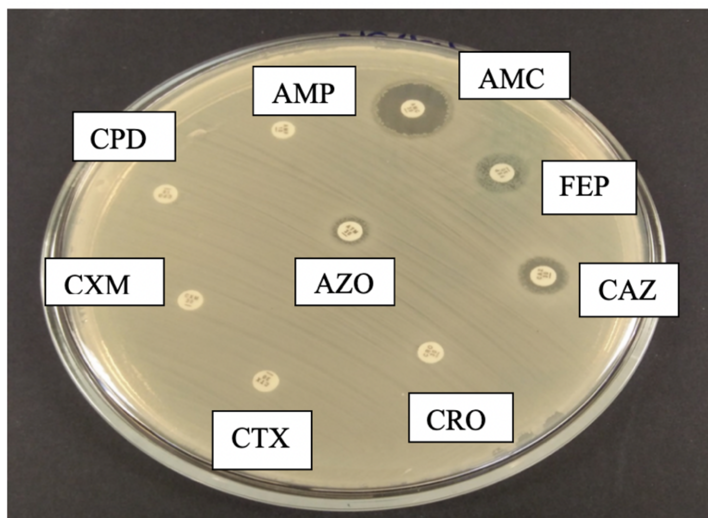

Figure 1: Zone of inhibition seen in MDR *P. mirabilis*, P42

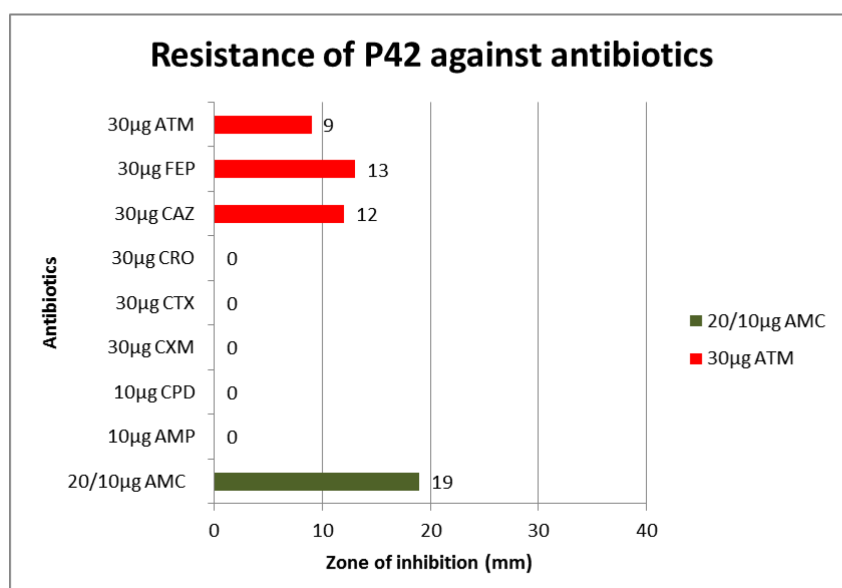

Figure 2: Antibiogram of P42

Reflecting the source of water sample collection, there were no MDR isolates of *P. mirabilis* from the Klang Gates Dam, Kuala Lumpur, and Gombak River. However, 6 (15.4%) MDR isolates of *P. mirabilis* were obtained from Kuyoh River whereas 15 (38.5%) MDR isolates obtained from Port Klang making up a total of 21 (53.8%) MDR isolates out of 39 non-MDR isolates of *P. mirabilis*.

High resistance rates were seen in these MDR isolates towards cefpodoxime (100%), ampicillin (85.7%), cefotaxime (71.4%), aztreonam (71.4%), cefuroxime (57.2%), cefepime (57.2%), and ceftriaxone (57.1%). Antibiotic resistance patterns of MDR *P. mirabilis* were tabulated (Table 4).

Table 4: MDR pattern of 21 MDR *P. mirabilis* isolates according to antibiotic type

Antibiotic (g/disk)	*P. mirabilis* (n=21)		
	Sensitive (%)	Intermediate (%)	Resistant (%)
Amoxicillin/clavulanic acid (20/10 g)	12(57.1)	3 (14.3)	6 (28.6)
Ampicillin (10 g)	3 (14.3)	0 (0.0)	18 (85.7)
Cefpodoxime (10 g)	0 (0.0)	0 (0.0)	21 (100.0)
Cefuroxime (30 g)	9 (42.9)	3 (14.3)	9 (42.9)
Cefotaxime (30 g)	6 (28.6)	0 (0.0)	15 (71.4)
Ceftriaxone (30 g)	9 (42.9)	0 (0.0)	12 (57.1)
Ceftazidime (30 g)	12 (57.1)	3 (14.3)	6 (28.6)
Cefepime (30 g)	9 (42.9)	3 (14.3)	9 (42.9)
Aztreonam (30 g)	6 (28.6)	3 (14.3)	12 (57.1)

Extended spectrum β-lactamase Phenotyping

Qualitative MDDST and quantitative CDT were performed on all 21 MDR *P. mirabilis* isolates, noting a high frequency of ESBL-producing *P. mirabilis*. 15 (71.5%) isolates found to be ESBL producers. In MDDST, it is observed that the edge of inhibition around CAZ and CTX discs had extended towards AMC disc showing a synergistic key-hole shaped zone of inhibition. CDT shows a comparison of zone of inhibition between CAZ and CAZ/CA whereby the zone of inhibition in CAZ/CA is larger than that of CAZ.

Detection and genomic sequencing of *blaTEM* and *bla*CTX-M gene

A total of 15 isolates showing resistance to 4 or more groups of β-lactam antibiotics were chosen for the detection of ESBL resistant genes, *bla*TEM and *bla*CTX-M is using PCR. All ESBL positive *P. mirabilis* had one or more ESBL genes. The PCR assay results indicated that 12 (80%) isolates had *bla*CTX-M gene that corresponds to the expected product size of 593bp, while 3 (20%) had *bla*TEM genes that corresponds to the expected product size of 867bp. Upon detection, Sanger sequencing was performed on the amplified DNA of the *P. mirabilis* strains. However, only *bla*CTX-M genes were able to be sequenced, while *bla*TEM genes could not be sequenced due to unsatisfactory sample condition of isolates possessing the *bla*TEM genes.

Table 5: Forward and reverse nucleotide sequence of *bla*CTX-M gene acquired from MH plate 33

Nucleotide sequence of *bla*CTX-M (F) (602bp)	GGGGATGTGGGGAGCTGAGACGTGAGCGACCGAATCTGTTAATCAGCGAGTTGAGATCAAAAAATCTG ACCTTGTTAACTATAATCCGATTGCGGAAAAGCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCG CGGCCGCGCTACAGTACAGCGATAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCGGCTA GCGTCACCGCGTTCGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAA ACACCGCCATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCGGAATC TGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACATGGATGAAAGGCAATACC ACCGGTGCAGCGAGCATTCAGGCTGGACTGCCTGCTTCCTGGGTTGTGGGGGATAAAACCGGCAGCGGT GGCTATGGCACCACCAACGATATCGCGGTGATCTGGCCAAAAGATCGTGCGCCGCTGATTCTGGTCAAT TTTTTTCCCCAAAATGGGTGGGGGTTTTTTTTTTTCCCTCTTCCGGAACAC
Nucleotide sequence of *bla*CTX-M (R) (594bp)	CAAAAGATGATAAATGTGGCTAGATCACCGCGATATCGTTGGTGGTGCCATAGCCACCGCTGCCGGTTT TATCCCCCACAACCCAGGAAGCAGGCAGTCCAGCCTGAATGCTCGCTGCACCGGTGGTATTGCCTTTCA TCCATGTCACCAGCTGCGCCCGTTGGCTGTCGCCCAATGCTTTACCCAGCGTCAGATTCCGCAGAGTTTG CGCCATTGCCCGAGGTGAAGTGGTATCACGCGGATCGCCCGGAATGGCGGTGTTTAACGTCGGCTCGGT ACGGTCGAGACGGAACGTTTCGTCTCCCAGCTGTCGGGCGAACGCGGTGACGCTAGCCGGGCCGCCAA CGTGAGCAATCAGCTTATTCATCGCCACGTTATCGCTGTACTGTAGCGCGGCCGCGCTAAGCTCAGCCA GTGACATCGTCCCATTGACGTGCTTTTCCGCAATCGGATTATAGTTAACAAGGTCAGATTTTTTGATCTC AACTCGCTGATTTAACAGATTCGGTTCGCTTTCACTTTTCTTCAGCACCGCGGCCACGGCCATCACCTTA CGTGCTGGGGCCCCTTAAAATTTTTTTTTTTCCCCCCGG

Molecular docking of *bla*CTX-M gene

Forward and reverse genomic sequences of *bla*CTX-M gene were assembled into a single gene and were transcribed into a protein sequence to be modelled into a protein structure. Docking interactions between the modified β-lactamase and β-lactam antibiotics, AMP, CPD, CTX, CXM, CAZ, CRO, FEP and ATM were modelled to visualize the different bonds involved as well as their corresponding binding energy. It was discovered that the amino acid residues ASN 107, ASN 135, ASN 137, TYR 108, SER 133, and ASP 242 in the active site of the target has led to β-lactamase mutation (Table 6). Findings also suggest that the ligand-receptor complex between the modified β-lactamase and ATM had the highest binding energy score of -6.9 Kcal/mol (Figure 4).

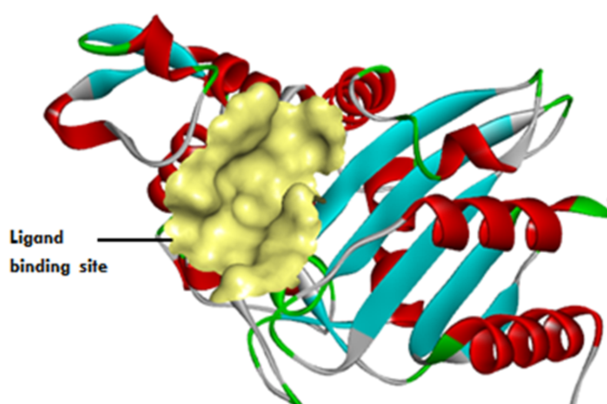

Figure 3: 3D structure of β-lactamase in ESBL-producing *P. mirabilis* and its ligand binding site

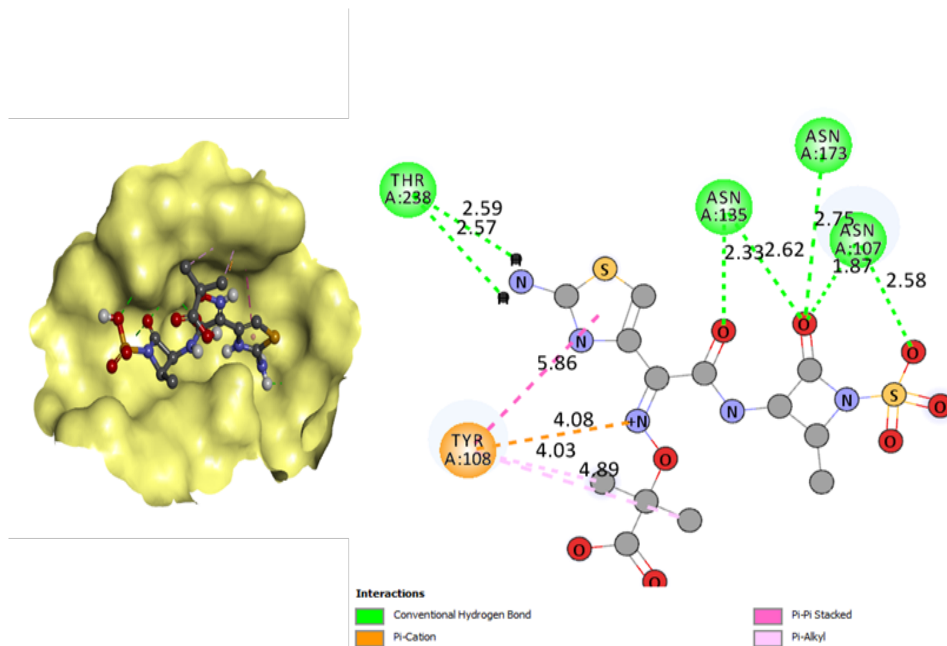

Figure 4: Docking interaction of Aztreonam (ATM) with modeled ligand binding site in 3D and 2D

Table 6: Binding energy and amino acid residues involved in the binding of β-lactamase with different β-lactam antibiotics

Antibiotic	AMP	CPD	CTX	CXM	CAZ	CRO	FEP	ATM	Amino acid residues involved (%)
Binding energy	-7.2	-7.4	-7.3	-7.7	-7.5	-9.0	-7.6	-6.9	-
Amino Acid Sequence	SER 73	SER 73	SER 73				SER 73		50.0
						LYS 76			12.5
	ASN 107		ASN 107	ASN 107		ASN 107	ASN 107	ASN 107	75.0
	TYR 108	TYR 108	TYR 108	TYR 108	TYR 108	TYR 108	TYR 108	TYR 108	100.0
	SER 133	SER 133	SER 133		SER 133	SER 133	SER 133		75.0
		ASN 135	ASN 135	ASN 135			ASN 135	ASN 135	62.5
					PRO 170	PRO 170			25.0
	ASN 173	ASN 173			ASN 173	ASN 173		ASN 173	62.5
							THR 219		12.5
					LYS 237		LYS 237		25.0
			THR 238	THR 238		THR 238		THR 238	50.0
		SER 240	SER 240	SER 240	SER 240	SER 240	SER 240		75.0
		GLY 241				GLY 241			25.0
		ASP 242	ASP 242		ASP 242	ASP 242	ASP 242		62.5
		GLN 271							12.5
		ASN 272							12.5
			ARG 277	ARG 277		ARG 277	ARG 277		50.0

DISCUSSION

MDR towards antibacterial agents such as antibiotics has becoming a prevalent phenomenon worldwide. Focusing on MDR bacteria in the midst of an urban settlement, the Klang River serves a potential reservoir for these antibiotic resistant pathogens. Prior to discussing *P. mirabilis* as an ESBL-producing isolate, evaluation of the aquatic environment in necessary.

Being essential to life, the aquatic environment supports human life by providing not only water but the resources that are utilized in many fields including agriculture, energy generation and industry. Having such dependency towards this environment calls for the need to assess river water and its pollution level, reflecting onto the Klang River, which runs through densely populated urban settlements. The pH, a main indicator to assess water pollution, satisfies the standards set by the Interim National Water Quality Standards for Malaysia[21] showing no possible threats to biological life.

In reference to Rivers and Drainages of Peninsular Malaysia (RDWPM)[22]; the mean for both water temperature and DO fall within the normal range of 27.8 – 35.5°C and 0.39-7.26 mg/L. It was noted that according to the Department of Environment (DoE), DO above 3 mg/L are considered safe for livestock drinking though water treatment is required. However, when not taking into account of the mean DO, water samples from the Gombak River, Kuyoh River, and the city of Kuala Lumpur had DO recorded less than 3 mg/L, which indicates the need for irrigation. Unsatisfactory DO registering at less than 1 mg/L was recorded in Port Klang – an integral center for fisheries, tourism, and transportation. This was in line with the yellowish and foul-smelling water sample present with debris collected in Port Klang.

As for BOD, it is associated to an increasing growth of microorganism resulting from the abundance of organic effluents and wastewater as bacterial respiration and degradation of organic matter consumes DO in the water[16,23]. Low BOD was recorded in Klang Gates Dam whereas the highest BOD is seen in the Gombak River. From this it could be deduced that the Gombak River is polluted following construction effluents on the rise.

The isolation of ESBL-producing *P. mirabilis* in an urban water source is alarming as this study had managed to isolate 21 MDR *P. mirabilis*. The trend was also evident as none of any MDR *P. mirabilis* were isolated from the Klang Gate Dam (cleanest source of Klang River according to the physicochemical assessment), whereas *P. mirabilis* isolated downstream in Kuala Lumpur and the Gombak River expressed resistance towards β-lactam antibiotics, but not considered MDR yet. Isolates showing resistance to 4 or more β-lactam antibiotics were detected in more polluted stretches of the Klang River including the Kuyoh River and Port Klang. The recovery rate of MDR *P. mirabilis* in this study was 53.9%, which was relatively high. Another trend observed was the prevalence of MDR *P. mirabilis* was higher downstream compared to the river source upstream. Similar findings were reports upstream *P. mirabilis* were mainly wild strains susceptible to penicillin and cephalosporin[12].

This was especially true as *P. mirabilis* are known to be intrinsically susceptible to cephalosporins and β-lactamase inhibitors. However, the dawn of resistance emerged in the 1990s as *P. mirabilis* had acquired resistance towards β-lactams through β-lactamases. This study reports MDR *P. mirabilis* against second, third and fourth generation cephalosporin due to production of ESBLs. Moreover, it was antibiotic resistance against cefepime, a fourth-generation cephalosporin, was relatively high at 42.9%. These findings were consistent with previous reports on the emergence of resistance to fourth generation cephalosporins[18] whereby *bla* CTX-Mgene coded β-lactamase is responsible for hydrolytic activities towards cefepime. Although resistance is seen towards cephalosporin classes, a high rate of susceptibility towards AMC was witnessed. Therefore, it could be deduced that β-lactamase inhibitors such as clavulanic acid may be opt for a treatment of ESBL-associated infections.

The employment of MDDST and CDT were successful in uncovering traits of MDR *P. mirabilis* competent for ESBL production. High ESBL production rate (71.5%) in the isolated *P. mirabilis* was seen, which when based on the sampling site it can be said that the Kuyoh River and Port Klang are potential reservoirs for ESBL-producing *P. mirabilis*. Molecular detection via PCR had narrowed down the ESBL subtypes of TEM, SHV, OXA, CTX and AmpC into just CTX-M and TEM subtypes.

Furthermore, the forward and reverse genomic sequence of the isolates expressing the *bla*CTX-M was modeled into a 3D protein structure to study the molecular docking between the β-lactam antibiotics and the binding site of the modified β-lactamase. Here, the binding energy in the conformation of the ligand-receptor complex between different antibiotics is measured in which the most stable complex is characterized by the lowest binding energy. In this case, CTX-β-lactamase complex exhibited the lowest binding energy of -9.0 Kcal/mol indicating that it is easily hydrolyzed by the mutated β-lactamase. This method of molecular docking opens an avenue to further understand effective binding scores of drugs towards the β-lactamase binding site, thus enabling the potential of designing novel drugs against ESBL-producing *P. mirabilis*.

CONCLUSION

To date, there has been no published data on ESBL bacteria specifically on *P. mirabilis* in the river waters of Malaysia. The present study shows prominence in *bla*CTX-M genes having resistance towards cefepime in ESBL strains isolated in urban Kuyoh River and Port Klang. Stressing on MDR, knowledge on its patterns, resistance, as well as prevalence in respect to our geographical area is integral for control and surveillance.

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