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A Study on Multiple Antibiotic Resistance (MAR) and Biofilm Eradication of *Vibrio cholerae* in Shrimps

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Abstract

Antimicrobial resistance (AMR) poses a significant threat to global health security. One factor that exacerbates this issue is the formation of bacterial biofilms, which can increase the Multiple Antibiotic Resistance (MAR) of bacteria. Biofilms are particularly challenging to control due to their resistance to both chemical and physical stressors, including antibiotic therapy. This study focused on investigating the MAR in relation to the eradication of *Vibrio cholerae* biofilms. The bacteria were obtained from shrimp specimens collected during a recent outbreak in Limbang, Sarawak. Antibiotic Susceptibility Tests (ASTs) were conducted on sixteen *V. cholerae* isolates from environmental and clinical samples (shrimps). The results revealed six distinct antimicrobial resistance profiles, with MAR indices ranging from 0.10 to 0.38 against 21 antibiotics. Notably, the isolates VC006 and VC026 exhibited the highest MAR index of 0.38. An A MAR index greater than 0.2 indicates contamination from high-risk sources and a high probability of multidrug resistance. The antibiotic chloramphenicol was selected as the agent for determining the Minimal Biofilm Eradication Concentration (MBEC) due to its known efficacy in quantifying the zone of inhibition. The results showed that concentrations ranging from 3.125 mg/mL to 25 mg/mL eradicated about 50% of *V. cholerae* biofilm formation (MBEC50), while higher concentrations of 50 mg/mL and 100 mg/mL achieved 90% eradication (MBEC90). In conclusion, the findings suggest that chloramphenicol is a highly effective antimicrobial therapy against multidrug-resistant and biofilm-forming strains of *V. cholerae*. This study underscores the importance of understanding and addressing MAR in the fight against AMR.

Keywords antimicrobial therapy; AST; chloramphenicol; MBEC; MAR index; shrimps

1.0 INTRODUCTION

V. cholerae O1 has undergone tremendous alterations in its phenotypic and genetic traits over the last few years (1). Multidrug-resistant *V. cholerae* bacteria have since been identified, and strains with novel drug resistance characteristics have become abundant (2). As with other bacteria of medical significance, *V. cholerae* O1 is continuously gaining resistance to newer antibiotics and is found particularly in developing countries (3). Therefore, it is vital to closely monitor changes in the susceptibility of strains in order to adjust antimicrobial treatment accordingly (2).

Despite the emergence of multidrug resistance, antibiotics still remain the most effective treatment for food-borne diseases. The growing antibiotic resistance of food-borne microorganisms is concerning (4). Tetracyclines, chloramphenicol, ampicillin and trimethoprim-cotrimoxazole have been used effectively in the treatment of cholera. However, the cholera-causative bacteria have successfully adapted to produce resistance against these drugs (5).

In response to varying environmental conditions, bacteria have undergone adaptation and survival (6). Based on Čabarkapa et al. (6) and Nadell et al. (7), a biofilm is a highly structured colony of microorganisms adhered to a surface or in contact with a surface and immersed in a self-produced extracellular matrix (ECM) that binds cells together. Numerous fitness benefits accrue from the biofilm matrix, including resistance to environmental stressors and increased nutritional availability (7). When biofilms grow on these contact surfaces, they can serve as a constant source of pollution, posing major consequences in industrial, environmental, public health and medical settings (4).

The search for substances or ways to inhibit bacterial biofilm development or antibiotic resistance in biofilm-associated pathogens is crucial for the treatment of biofilm-associated illnesses (8). Antibiotics are often recommended to manage and control cholera epidemics, so the monitoring of *V. cholerae* antibiotic resistance by public health authorities is important (9). Therefore, this study was conducted to determine the antibiotic susceptibility of *V. cholerae* strains isolated from a variety of clinical and environmental sources (shrimps), and to investigate the lowest concentration of a specific antibiotic required to eliminate *V. cholerae* strains that have developed biofilm.

2.0 Materials and Methods

2.1 Antibiotics Susceptibility Testing (AST)

The antibiotic susceptibility test (AST) of *V. cholerae* strains was performed using the Kirby–Bauer disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2018). A total of 21 antibiotics (OXOID, England) that were tested contained Chloramphenicol (C30, 30 mcg), Bacitracin (B10,10 mcg), Spectinomycin (SH100, 100 mcg), Norfloxacin (NOR10, 10 mcg), Kanamycin (K30, 30 mcg), Meropenem (MEM10, 10 mcg), Erythromycin (E15, 15 mcg), Ampicillin (AMP10, 10 mcg), Imipenem (IPM10, 10 mcg), Ceftazidime (CAZ30, 30 mcg), Nalidixic Acid (NA30, 30 mcg), Amoxycillin/Clavulanic Acid (AMC30), Amoxycillin (AML10), Penicillin (P10), Tetracycline (TE30), Amikacin (AK30, 30 mcg), Streptomycin (S25, 25 mcg), Rifampicin (RD2, 2 mcg), Cephalothin (KF30, 30 mcg), Gentamicin (CN10, 10 mcg) and Enrofloxacin (ENR5, 5 mcg) were used in this study. The classification breakpoints are shown in Table 1.

Table 1: Breakpoints for classification of Sensitive, Intermediate, or Resistant for (CLSI, 2018)

Antibiotics and Antimicrobial Agents	Zone Diameter Interpretive Standards (mm)		
	Susceptible	Intermediate	Resistant
Erythromycin	≥23	14-22	≤13
Ciprofloxacin	≥21	16-20	≤15
Nalidixic Acid	≥19	14-18	≤13
Penicillin G	≥15	-	≤14
Carbenicillin	≥23	20-22	≤19
Oxacilin	≥14	10-13	≤9
Tetracycline	≥15	12-14	≤11
Oxytetracycline	≥19	15-18	≤14
Gentamicin	≥15	13-14	≤12
Streptomycin	≥15	12-14	≤11
Kanamycin	≥18	14-17	≤13
Chloramphenicol	≥18	13-17	≤12
Ceftazidime	≥21	18-20	≤17
Ampicillin	≥17	14-16	≤13
Meropenem	≥23	20-22	≤19
Imipenem	≥23	20-22	≤19
Amikacin	≥17	15-16	≤14
Norfloxacin	≥17	13-16	≤12
Amoxycillin/Clavulanic Acid	≥18	14-17	≤13

The tube dilution method was used for antimicrobial susceptibility testing. The test organism suspension was standardized to a 0.5 McFarland standard or diluted to 10^6 CFU/mL. Using sterile cotton-wool swab sticks, each test suspension was streaked onto Mueller-Hinton Agar (MHA) plates. The 21 antibiotic discs were then aseptically placed on streaked MHA plates. The plates were incubated at 37°C for 24 hours.

The diameter of the zone of inhibition was used to determine the susceptibility or resistance of each isolate to various antibiotics. The zones were classified as resistant (R), intermediate-resistant (I), or susceptible (S) for each antimicrobial agent (CLSI, 2018). *E. coli* ATCC 25922 was used as a control for susceptibility testing. This assay has been repeated three times, and triplicate experiments have been performed to obtain the mean diameter of the zone.

2.2 Multiple Antibiotic Resistance (MAR) Index

To quantify the resistance of *V. cholerae* isolates against multiple antibiotics, the Multiple Antibiotic Resistance (MAR) index was applied using the method introduced by Igere et al. (10). This was calculated using the formula MARI = a/b, where "a" represents the number of antibiotics to which the isolates showed resistance and "b" represents the total number of antibiotics employed during the analysis. Antibiotics with a MAR index value of less than 0.2 have low antibiotic use against animals, so they are less likely to be multidrug resistant. MAR index values greater than 0.2 suggest a high exposure of antibiotics against animals; therefore, they are more likely to be multi-drug resistant (11).

2.3 Minimum Biofilm Eradication Concentration (MBEC)

Chloramphenicol powder (Acros Organics, Thermo Scientific, USA) was used as an antibiotic agent for the MBEC test in this study. Chloramphenicol was chosen for the MBEC test due to its broad-spectrum activity, chemical stability, and general usage as a control antibiotic during biofilm susceptibility testing (16). Additionally, decreased chloramphenicol susceptibility was revealed among aquaculture-related *Vibrio* spp., offering a stringent indicator of resistance associated with biofilms (33). According to Nillian et al. (12), to allow *V. cholerae* to form biofilm, approximately 100 µL of isolated *V. cholerae* has to be pipetted into each well of a 96-well, U-shaped microtiter plate. Column 1 was pipetted with a negative control containing just fresh broth, while column 2 was pipetted with a positive control (*V. cholerae* O1 El Tor ATCC 14033) containing only *V. cholerae* inoculum. *V. cholerae* biofilm formations were compared to these two controls. The plate was then sealed with parafilm and incubated at 37°C for 24 hours without agitation. After discarding the overnight medium, each well of a 96-well microtiter plate was loaded with approximately 100 µL of fresh MHB. Then, chloramphenicol was added into the wells in two-fold dilutions serially from 100 mg/mL to 0.78125 mg/mL. There are no 0 mg/ml tested as it is considered a negative control (fresh broth: free inoculum and free Chloramphenicol).

The plate was then incubated at 37°C for 24 hours. The medium was discarded upon incubation. The biofilm was rinsed three times with sterile distilled water to eliminate non-adherent cells. The plate was inverted and left to air dry for 30 minutes inside a biosafety cabinet. The minimum biofilm eradication concentration (MBEC) was determined by scratching the biofilm formed at the bottom of wells with a metal loop and then streaking it on the MHA surface. MBEC values were established by observing the number of *V. cholerae* colonies formed on the streaked plate. This experiment was conducted in triplicate to calculate the mean. The MBEC50 value is defined as the eradication of 50% of biofilm formation, whilst the MBEC90 value is defined as the eradication of 90% of biofilm formation (12).

3.0 RESULTS AND DISCUSSION

Cholera causes diarrhea, and it can be characterized by rapid fluid loss, which could require intravenous rehydration therapy. Additional antimicrobial treatment is also used to shorten the duration of the sickness and lower stool volume (13). Some of the symptoms of *V. cholerae* infection include excessive vomiting and rice water stools, which can lead to dehydration and death if not handled appropriately. While rehydration therapy is the primary and easiest treatment for cholera, dehydrated patients are given oral antibiotics as soon as the vomiting ceases (14).

The ctx gene had been detected in our previous study (15) using primer sequences of CTXAB-F F: GCCGGGTTGTGGGAATGCTCCAAG R and CTXAB-R GCCATACTAATTGCGGCAATCGCATG at 536 bp. *V. cholerae* produces cholera toxin (ctx), which is encoded by the ctx gene located within its chromosome. This toxin has been recognized as one of the toxins responsible for cholera outbreaks. *V. cholerae* O1 type possesses the genetic ability to manufacture cholera toxin, the ctx gene. Epidemic cholera strains induce human illness, whereas *V. cholerae* strains adhere to the intestinal lumen and produce cholera toxin. The cholera toxin gene (ctx) plays a role in life-threatening cholera sickness (17).

A total of 21 antibiotics from various classes were utilized to test the susceptibility of 16 isolates of *V. cholerae*. The data obtained could help with recommending the medications that can be used for future therapy. Table 2 shows the distribution of the susceptibility activities of the 16 *V. cholerae* isolates tested against 21 antibiotics.

Table 2: Distribution of Antimicrobial Resistance (R), Intermediate (I), and Susceptible (S) of *V. cholera* isolates

Antibiotics	No. of *Vibrio cholerae* and Percentage (%) against selected antibiotics		
	Susceptible (S)	**Intermediate (I)**	**Resistance (R)**
Aminocyclitol			
SH100 - Spectinomycin	5 (31%)	9 (56%)	2 (13%)
Aminoglycosides			
AK30 (Amikacin)	12 (75%)	3 (19%)	1 (6%)
CN10 (Gentamicin)	11 (69%)	5 (31%)	0 (0)
K30 (Kanamycin)	5 (31%)	11 (69%)	0 (0)
S25 (Streptomycin)	9 (56%)	7 (44%)	0 (0)
Ansamycins			
RD2 (Rifampicin)	0 (0)	5 (31%)	11 (69%)
Carbapenems			
IPM10 (Imipenem)	6 (38%)	9 (56%)	1 (6%)
MEM10 – (Meropenem)	12 (75%)	3 (19%)	1 (6%%)
Cephems			
CAZ30 (Ceftazidime)	13 (81%)	0 (0)	3 (19%)
KF30 (Cephalothin)	15 (94%)	0 (0)	1 (6%)
Fluoroquinolones			
ENR5 (Enrofloxacin)	13 (81%)	2 (13%)	1 (6%)
NOR10 (Norfloxacin)	16 (100%)	0 (0)	0 (0)
Quinolone			
NA30 (Nalidixic Acid)	14 (88)	1 (6%)	1 (6)
Macrolides			
E15 (Erythromycin)	0 (0)	15 (94%)	1 (6%)
Penicillins and β-lactam			
AMP10 (Ampicillin)	14 (88%)	2 (13) %	0 (0)
AML10 (Amoxycillin)	9 (56%)	6 (38%)	1 (6%)
AMC30 (Amoxycillin/Clavulanic Acid)	10 (63%)	5 (31%)	1 (6%)
P10 (Penicillin)	1 (6%)	0 (0)	15 (94%)
Phenicols			
C30 (Chloramphenicol)	15 (94%)	1 (6%)	0 (0)
Tetracyclines			
TE30 (Tetracycline)	15(94%)	0(0)	1(6%)
Others			
B10 (Bacitracin)	0 (0)	0 (0)	16 (100%)

Table 3: Overview of Minimum Biofilm Eradication Concentration (MBEC) (mg/mL) of 16 Strains of *V. cholerae* Treated with Chloramphenicol.

V. cholerae strains	*V. cholerae* ctx gene	Chloramphenicol (mg/ml)		
		10 mg/mL	50 mg/mL	100 mg/mL
VC001	+	Incomplete eradication	Eradicated	Eradicated
VC002	+	Incomplete eradication	Eradicated	Eradicated
VC003	+	Incomplete eradication	Eradicated	Eradicated
VC004	-	Incomplete eradication	Eradicated	Eradicated
VC005	+	Incomplete eradication	Eradicated	Eradicated
VC006	+	Incomplete eradication	Eradicated	Eradicated
VC007	-	Incomplete eradication	Eradicated	Eradicated
VC008	-	Incomplete eradication	Eradicated	Eradicated
VC009	-	Incomplete eradication	Eradicated	Eradicated
VC010	-	Incomplete eradication	Eradicated	Eradicated
VC011	-	Incomplete eradication	Eradicated	Eradicated
VC012	+	Incomplete eradication	Eradicated	Eradicated
VC013	-	Incomplete eradication	Eradicated	Eradicated
VC014	-	Incomplete eradication	Eradicated	Eradicated
VC015	+	Incomplete eradication	Eradicated	Eradicated
VC016	+	Incomplete eradication	Eradicated	Eradicated
VC017	+	Incomplete eradication	Eradicated	Eradicated
VC018	+	Incomplete eradication	Eradicated	Eradicated
VC019	+	Incomplete eradication	Eradicated	Eradicated
VC020	+	Incomplete eradication	Eradicated	Eradicated
VC021	+	Incomplete eradication	Eradicated	Eradicated
VC022	-	Incomplete eradication	Eradicated	Eradicated
VC023	+	Incomplete eradication	Eradicated	Eradicated
VC024	-	Incomplete eradication	Eradicated	Eradicated
VC025	-	Incomplete eradication	Eradicated	Eradicated
VC026	+	Incomplete eradication	Eradicated	Eradicated
VC027	-	Incomplete eradication	Eradicated	Eradicated
VC028	+	Incomplete eradication	Eradicated	Eradicated

(+): positive result for the detection of *V. cholerae* through PCR

(-): negative result for the detection of *V. cholerae* through PCR.

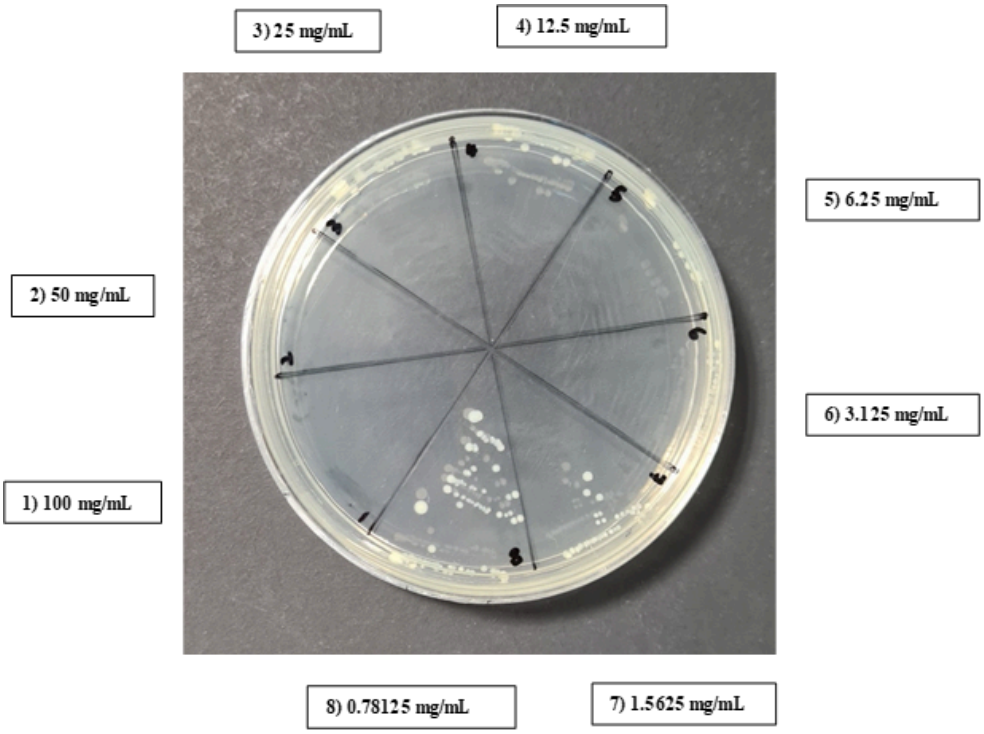

Figure 1 : Representative of triplicates for Minimum Biofilm Eradication Concentration (MBEC) of *V. cholerae* strains after treatment with 100 mg/mL of chloramphenicol with a negative control (free medium).

According to the Clinical and Laboratory Standards Institute guidelines, each isolate is classified as Resistant (R), Intermediately resistant (I), or Susceptible (S) based on the size of the zone of inhibition revealed in the disc diffusion of antimicrobial activities. A bacterial strain is considered to be resistant (R) against a certain antibiotic if it is suppressed in vitro by a concentration of the medication associated with a high risk of therapeutic failure. The sensitivity of a bacterial strain to a certain antibiotic is referred to as intermediate (I) if it is inhibited in vitro by a concentration of this medication associated with an unknown therapeutic effect. Meanwhile, Susceptible (S) bacteria are those that are suppressed in vitro by a concentration of an antibiotic that is linked with a high chance of therapeutic success. This set of assessment criteria is based on the current understanding of antibiotic pharmacokinetics and pharmacodynamics.

As shown in Table 2, *V. cholerae* isolates were shown to be 100% susceptible to Norfloxacin, an antibiotic tested. This is followed by Chloramphenicol and Tetracycline, whose susceptibilities are at 94% respectively. Meanwhile, *V. cholerae* isolates showed 100% resistance against Bacitracin, and 94% of *V. cholerae* isolates were resistant to penicillin, followed by 69% of isolates were resistant to rifampicin. The highest rate of penicillin resistance was similarly reported by Al-Dulaimi et al., 2019 (11). Penicillin resistance, which is nearly universal in clinical and environmental *V. cholerae* isolates worldwide, is caused by changes in the penicillin-binding proteins 1 and/or 2, as reported for multiple sequenced *V. cholerae* strains (18).

As shown in Table 2, *V. cholerae* isolates showed 6% of resistance (R) and 94% of Intermediate Resistant (IR) against the erythromycin antibiotic. This indicates that erythromycin has a significant level of resistance among the isolates. Resistance to erythromycin might develop over time or as a result of the widespread use of antibiotics for prophylaxis in asymptomatic people (19). Interestingly, the majority of *V. cholerae* isolates in this study were susceptible to third-generation cephalosporins, ceftazidime at 81% and first-generation cephalosporins, cephalothin at 94%, with a resistance profile of 19% and 6%, respectively. Numerous antibiotics are currently being used in clinical practice to treat *Vibrio* spp. infections. These include cephalothin, cefuroxime, cefotaxime, ceftazidime, tetracycline, doxycycline, or fluoroquinolone (20). Additionally, this study discovered that the isolates have the highest susceptibility at 88% to ampicillin in the β-lactam antibiotic family, compared to amoxycillin at 56% and amoxycillin with clavulanic acid at 63%. None of the isolates were ampicillin-resistant. However, 6% were resistant to both amoxycillin and amoxycillin plus clavulanic acid.

The antibiotic spectinomycin demonstrated a low sensitivity, being detected in only 31% of isolates and being resistant in 13% of isolates. The same goes for imipenem, which has a 38% sensitivity rate among isolates. Meropenem was shown to be sensitive to 75% of the isolates. Only 6% of isolates were resistant to both carbapenems. This investigation discovered that *V. cholerae* isolates were highly susceptible to antibiotics such as ampicillin at 88%, ceftazidime at 81%, cephalothin at 94%, chloramphenicol at 94%, enrofloxacin at 81%, nalidixic acid at 88% and tetracycline at 94%.

As shown in Table 2, Norfloxacin was found to be the most sensitive antibiotic in this study, as it was completely susceptible to all isolates. This is consistent with the findings of Dutta et al. (21), who found that multidose norfloxacin therapy considerably decreased stool production, length of diarrhea, and hydration demand when compared to other regimens.

Shrestha et al. (19) reported that the fluoroquinolones group is generally effective against cholera. Further study on biofilm formation could be beneficial to gain more understanding of the treatment of severe infection of cholera using the fluoroquinolone group of antibiotics. Nowadays, cholera treatment failures have become common due to the recurrence of resistant strains. Consequently, there is a growing concern regarding the emergence of antibiotic resistance in *V. cholerae* strains, particularly in low-income nations (22). Research done in African countries indicated that *V. cholerae* outbreaks happen due to its developing resistance against effective antimicrobials such as tetracycline and fluoroquinolones (23). However, (3) discovered that *V. cholera* isolates were susceptible to chloramphenicol.

Table 4: Patterns of antibiotic resistance profiles and MAR index of *Vibrio cholerae*

Patterns	Strain no.	Antibiotic Resistant Profiles	MAR Index	No. of Isolates/Total of isolates (% of occurrence)
A	VC001 VC002 VC015 VC016 VC018 VC020 VC021 VC023	B10P10RD2	0.14	8/16 (50.0%)
B	VC003	B10SH100CAZ30P10ENR5	0.24	1/16 (6.25%)
C	VC005 VC012 VC017 VC028	B10P10	0.10	4/16 (25.0%)
D	VC006	B10SH100MEM10E15CAZ30NA30TE30RD2	0.38	1/16 (6.25%)
E	VC019	B10IPM10P10RD2	0.19	1/16 (6.25%)
F	VC026	B10CAZ30AMC30AML10P10AK30RD2KF30	0.38	1/16 (6.25%)

According to Igere et al. (24), the emergence of potential MAR pathogens such as *V. cholerae* from environmental estuaries poses a concern because the water is used for daily domestic activities. As indicated in this study, the samples were from villages in rural areas in Limbang, Sarawak, where their primary water resources are nearby rivers. Table 3 summarises the antibiotic resistance profiles of *V. cholerae* as measured by the MAR index. This study established six patterns of antimicrobial resistance profiles from the Antibiotics Susceptibility Test (AST). The MAR index ranges from 0.10 to 0.38, with the Pattern of Antibiotic Resistance Profiles D and E isolates VC006 and VC026 having the highest values, which is 0.38. *V. cholerae* strain VC006 was obtained from a Moore swab, and VC026 from human stool samples. As shown in Table 3, Patterns of Antibiotic Resistance Profiles B, D, and E had a MAR index greater than 0.2. An MAR index greater than 0.2 may be the result of contamination from high-risk sources, posing a risk to human health (25). Meanwhile, contamination of the MAR index less than 0.2 is determined to come from low-risk sources (very rarely or never exposed to antibiotics). The widespread use of antibiotics in human medicine leads to the rise of MAR, harmful bacteria in human excrement, contaminating aquatic systems and habitats (26). The MAR index of VC005, VC012, VC017 and VC028 was 0.10, indicating that they were resistant to at least one class of antibiotic. According to Table 3, Pattern of Antibiotic Resistance Profiles C (B10SH100CAZ30P10ENR5) contains four out of sixteen samples originating from Moore swab, rectal swab and human stool.

Approximately 50% (8/16) of the isolates were resistant to three distinct antibiotics and possessed the antibiotic-resistant profile Pattern of Antibiotic Resistance Profiles A (B10P10RD2). This pattern profile has a MAR index of 0.14, which consists of isolates from water samples and rectal swabs. Thus, 50% of the isolates from this source would express resistance if they were tested with the antibiotic profile. It demonstrated that even though the clinical and environmental samples were obtained from separate sources, they shared similar features and were related.

The Pattern of Antibiotic Resistance Profiles E (B10IPM10P10RD2), which is V. cholerae VC019 from the rectal swab, has a MAR index of 0.19, which is lower than 0.2, thus showing a lowered rate of contamination from high-risk sources, thereby potentially posing a risk to human health. Rectal swab examination is an examination that is generally required in food or beverage premises where staff or employees come into contact with food or drinks. The transmission of these bacteria from the rectum happens due to inadequate hand washing and poor hygiene, where bacteria get under the nails and spread to food or drinks that are produced or served. Thus, a MAR index lower than 0.2 shows that the workers practice a high standard of hygiene (27).

Another isolated strain, VC003, developed antibiotic resistance characteristics at a higher MAR index of 0.24, which led to Pattern of antibiotic resistance profiles B at B10SH100CAZ30P10ENR5. The other two *V. cholerae* isolates are VC006 with Pattern of Antibiotic Resistance Profiles D at B10SH100MEM10E15CAZ30NA30TE30RD2 and VC0026 at Pattern of Antibiotic Resistance Profiles F at B10CAZ30AMC30AML10P10AK30RD2KF30, which have generated the highest MAR index in this investigation, which is 0.38. By implying a common clonal origin, it is possible to anticipate a similar resistance trend for each year (28).

The minimum biofilm eradication concentration (MBEC) is defined as the smallest concentration of a substance capable of preventing bacteria from re-growing in treated biofilms (29). At the MBEC, biofilm elimination should be complete, which means that no viable bacteria should grow and recolonize (30). As previously reported by (31), chloramphenicol prevents the growth of bacteria; however, at high doses, it will eventually kill the bacteria, potentially treating microbial biofilm. It is an antibiotic that may be used against Gram-positive, Gram-negative and anaerobic bacteria. Chloramphenicol prevents the synthesis of protein by attaching to the 50S ribosomal subunit and directly blocking bacterial protein production (31).

As shown in Table 2, Chloramphenicol has 94% susceptibility to antibiotics toward V. cholerae isolates; hence, chloramphenicol was chosen as the antibiotic agent for the minimal biofilm eradication concentration (MBEC) test in this study. The minimum biofilm eradication concentration (MBEC) of sixteen (n=16) different strains of *V. cholerae* was determined using the antibiotic chloramphenicol. According to the results in Table 4, no full eradication of the *V. cholerae* biofilm was achieved when treated with chloramphenicol at a dosage of 10 mg/mL. When the *V. cholerae* biofilms were treated with 1.5625 mg/mL, observable growth was slightly inhibited. However, treatment with concentrations ranging from 3.125 mg/mL to 100 mg/mL has significantly reduced the visible growth of eradication of the *V. cholerae* biofilm. Thus, the eradication of the V. cholerae biofilm was fully achieved with chloramphenicol at 50 mg/mL and 100 mg/mL concentrations. MBEC50 was determined as the lowest concentration of the antibacterial agent, at which 50% of resistant *V. cholerae* biofilms at OD650 were eradicated, while MBEC90 was reported as the lowest concentration of antibacterial agent, at which 90% of resistant *V. cholerae* biofilms at OD650 were eradicated (26).

Therefore, it is possible to remove *V. cholerae* biofilms at low concentrations. About 50% of biofilm formation (MBEC50) of *V. cholerae* was eradicated from 3.125 mg/mL to a 25 mg/mL concentration of chloramphenicol, as shown in Figure 1. Meanwhile, eradication of 90% of biofilm formation (MBEC90) was achieved by using 50 mg/mL and 100 mg/mL concentrations of chloramphenicol. Bacterial proliferation in a particular well is indicative of planktonic bacteria re-growing from a surviving biofilm. MBEC is defined as an antibiotic concentration at which no bacterial growth is seen on the plate (32).

The resistant patterns for *V. cholerae* strains vary significantly according to geographical location, antibiotic use patterns in the examined population and time period of the study. Despite not completely killing *V. cholerae*, chloramphenicol

antibiotics can eradicate it at low concentrations and are appealing drugs for cholera treatment due to their excellent therapeutic ratio and relatively extended half-life. Because of these qualities, chloramphenicol antibiotics could potentially be used to treat *V. cholera* biofilm.

4.0 CONCLUSION

In conclusion, the formation of *V. cholerae* biofilms with high resistance against antibiotics in this study would be mitigated using chloramphenicol at 3.125 mg/mL to 25 mg/mL in about 50% of biofilm formation (MBEC50) and in 90% of biofilm formation (MBEC90) at 50 mg/mL and 100 mg/mL concentrations. Chloramphenicol was shown to be capable of eradicating *V. cholerae* biofilms, which could potentially be used in treating severe outbreaks of cholera related to biofilm in the near future.

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