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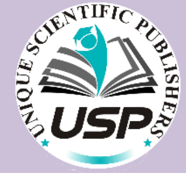

Research Article https://doi.org/10.47278/journal.ijvs/2020.016

Genetic Characteristics of Antibiotic Resistance Gene of *Vibrio cholerae* Isolated

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ABSTRACT

Vibrio cholerae is the factor of cholera disease in human and it has been becoming a serious problem in human health. This study was conducted to identify the genetic characteristics and antibiotic resistance of *V. cholerae* isolated in Tra Vinh Province. From the results, twenty-five (25) *Vibrio* spp. were isolated, including 6 strains of *Vibrio* (*V.*) *cholerae* (24%), 8 strains of *V. paraheamolyticus* (32%), 4 strains of *V. vulnificus* (16%), 5 strains of *V. fluvialis* (20%), and 2 strains of *V. alginolyticus* (8%). The serogroup results showed that all the 6 *V. cholerae* belonged to serogroup O1, with 50% positive to serotype Inaba, and 50% positive to Ogawa. No strain belonged to serogroup O139. The results also showed that 50% of *V. cholerae* were resistant to streptomycin and 33% were resistant to tetracycline and trimethoprim-sulfamethoxazole. Two of the 6 strains had an antibiotic resistance gene (gene *tetA)* encoding for a tetracycline-resistant factor. Gene *blaSHV*, gene *aac(3)-IV* and gene *dhfrI* encoding for β-lactam, aminoglycoside, trimethoprim resistant factors were not detected. Additionally, the results showed that adhesion indexes of *V. cholerae* strains T1 and T3 in the intestinal mucosa of rabbits shared the same antigens with the bacteria used to produce cholera vaccine (mORCVAX) available in Vietnam. *V. cholerae* was prevalent in the environment and clams in Tra Vinh province. Information from this research may be useful for further studies.

Key words: *Vibrio, V. cholerae,* Isolation and Identification, Genetic characteristics, Antibiotic resistance.

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INTRODUCTION

Vibrio cholerae is a Gram-negative bacteria (Nelson et al. 2009; Oladokun and Okoh 2016), the agent of cholera in humans, causing acute diarrhea and dehydration associated with other forms of local epidemic and pandemic. It is a facultative pathogen that has both human and environmental stages in its life cycle. Although many antibiotics are used to treat this disease, their effect is limited due to the resistance of *V. Cholerae* to many drugs. Among the seven global cholera pandemics between 1816 and 1923, six occurred in India and were caused by *V. cholerae* O1, the classical biotype. The 7th pandemic was different from the previous six; it was caused by *V. cholerae* El Tor and was obtained from the Indonesian island of Celebes in 1961. This was the longest pandemic and had a greater impact than the previous 6 pandemics. Until now, many countries have reported the cholera outbreak caused by this case (Hays 2005; Harris et al. 2012). WHO also declared that there is 3-5 million cases of this diseases per year (WHO 2017).

It was reported that patients with diarrhea caused by *V. Cholerae* were still available in Vietnam and it is increasing recently (Nguyen et al. 2017). The province of Tra Vinh is geographically exposed to numerous potential risks of cholera due to its 65 km shoreline and major river systems spanning 578 km, including large rivers such as the Hau river, Co Chien river, and Mang Thit river. In particular, *V. cholerae* can easily spread from Co Chien River adjacent to Ben Tre Province, where the disease occurred in 2010 (Nguyen et al. 2017).

Many of the antibiotics used to treat cholera are resisted by bacteria, including *V. cholerae*. This is a public health concern. A chromosome is proven to be one of the genetic factors of antibiotic resistance genes in bacteria. Other genetic factors include plasmids, integrons and transposons (Ghosh and Ramamurthy 2011; Sultan et al. 2018). One of the genetic factors is integrated into the chromosome and transmitted antibiotic resistance genes between species in the environment (Burrus and Waldor 2004; Bengtsson-Palme et al. 2018). From these reasons, the study was implemented to estimate the genetic characteristics and antibiotic resistance of *V. cholerae* isolated in Tra Vinh Province.

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MATERIALS AND METHODS

Chemical and biological for PCR

Identifying bacteria based on 16S rRNA gene segments

Primers for polymerase chain reaction (PCR)-based target gene were 27F-1492R as the most widely used primer (Frank et al. 2008; Hou et al. 2018), Vc.*sod*B (Tarr et al. 2007), O1*rfb,* O139*rf, ctx*A (Jeyasekaran et al. 2011; Bonnin-Jusserand et al. 2019)*,* and *tcp*A (Espineira et al. 2010; Bonnin-Jusserand et al. 2019).

Determining antibiotic resistance genes

Primers for determining antibiotic resistance genes using PCR were bla_{SHV}*, aac (3)-IV, tetA,* and *dhfrI* (Messele et al. 2017).

Materials

They included samples collected from different sources. Specifically, 160 clam samples were collected from Districts of Duyen Hai and Cau Ngang; 100 pig blood samples from the slaughterhouse in Tra Vinh City, Districts of Chau Thanh, Duyen Hai, Cau Ngang and Cang Long; 40 stool samples were collected from diarrhea patients in hospitals of Tra Vinh Province; 150 water samples were collected from rivers, the sea and the shrimp farms; and 50 shrimp samples were collected from Duyen Hai District. All samples were stored in cold storage tanks and transferred to the laboratory for isolation.

Other materials were oral cholera vaccine (mORCVAX) prepared from the strain of cholera bacteria including Classical biotype and El Tor biotype and the strain of *V. cholerae* O139 (Company Limited Vaccine and Biologicals No. 1, Hanoi); and 24 New Zealand white rabbits weighing between 2.0 and 2.5kg.

Methods

Different experimental methods were employed depending on the purposes of the experiments

For methods of isolation and identification of bacteria: a) biochemical reactions (ISO 2017) were used to determine *V. cholerae*; b) automated machine identifier (Vitex 2 compact- Biomerieux - Can Tho Hospital) was used to identify *V. cholerae* strain; c) serological method of *V. cholerae* was done by agglutination with 4 types of antiserum Inaba, Ogawa, and O139 and polyclonal antiserum Ogawa Inaba, O139 (ISO 2017); and d) *Vibrio* spp. was determined by PCR.

For analysis of 16S RNA gene sequence and establishment of the phylogenetic tree, the PCR product (DNA) including 6 sequences of 6 *Vibrio* strains: *V. cholerae*-Ng3, *V. cholerae*-O3.2, *V. cholerae*-O1.2, *V. cholerae*-81V1, *V. cholerae*-N8 and *V. cholerae*-85V1, was sequenced in Macrogen Inc (South Korea). The sequences were analyzed and read using Bio.Edit software, then compared with similar nucleotide sequences set on the GenBank, after which the genetic tree was established.

Besides, the disc diffusion (Kirby-Bauer) method and PCR method were used to determine the susceptibility pattern of the isolates to antimicrobial agents. Additionally, for sequence analysis of antibiotic resistance genes and establishment of the phylogenetic tree, the determined antibiotic resistance genes from DNA was sequenced in Macrogen Inc. The sequences were analyzed and read using a Bio. Edit software, then comparisons made with nucleotide sequences homologous on the GenBank, after which a genetic tree was established.

For experiment of *V. cholerae* strains isolated and the immune response in rabbits against cholera vaccine, a) Fluid accumulation (FA) in the intestine segments was determined by the amount of fluid (ml)/length of the rabbits' intestines (cm), genetic characteristics of antibiotic resistance gene of *V. cholerae* isolated in Tra Vinh; b) The adhesion of bacteria to the intestinal surface was investigated by cutting intestinal segments, shaving the intestinal mucosa or fluid in the intestine, then diluting liquid into decimal (log_{10}). Results were calculated using the formula: Mi (CFU/ml) = Ai × D1/v (Mi: the number of bacteria in the initial solution; Ai: the average number of colonies/plate; D1: dilution; v: suspension volume/disk), from which the dilution series were prepared; and c) Rate of bacterial adhesion to intestinal mucosa of rabbit was calculated as follows:

The formula for the adhesion (%) is given as 100 × intestine surface bacteria/intestine surface bacteria + CFU fluid (Richardson 1991). The average value of *V. cholerae* prevalence was calculated using Microsoft Excel.

RESULTS

Result of identifying *Vibrio* spp. by PCR

The electrophoresis result of Fig. 1 shows a 1500bp length PCR product including high conservation areas and present in most branched bacteria with close relationships.

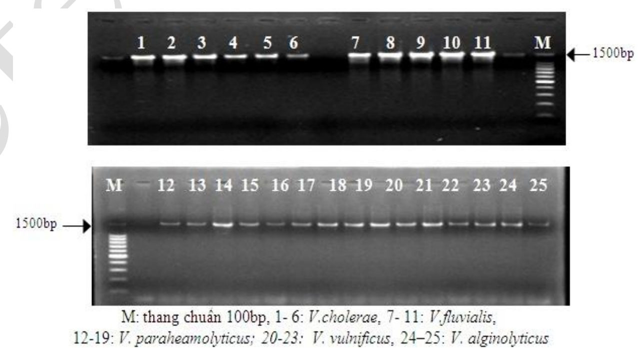

Fig. 1: Genetic results of 16S-27F and 1492Rbp.

Detection rate of *Vibrio* spp. isolated

There were 25 isolated strains including 6 strains of *V. cholerae* (24%); 8 strains of *V. paraheamolyticus* (32%); 4 strains of *V. vulnificus* (16%); 5 strains of *V. fluvialis* (20%) and 2 strains of *V. alginolyticus* (8%). Results of the homology between species of Vibrio on GenBank using BLAST have been shown in Fig. 2.

In this study, the isolated strains were *Vibrio* spp., including Ng3, O3.2, O1.2, N8, and 85V1 and 81V1 had similar rates of nucleotide sequences of gene segments 16S-27F and 1492R of the analysed strains with other strains of *V. cholerae* which was very high.

V. cholerae antibiotic resistance by Kirby Bauer method and by PCR

Table 1 showed the results of *V. cholerae* antibiotic resistance which were sensitive to many antibiotics such as norfloxacin, ampicillin, and amoxicillin-clavulanic acid, vancomycin, streptomycin, and tetracycline.

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Table 1: The sensitive and antibiotic resistance of *V. cholerae*

Antibiotic	Code	Number of samples	Sensitive		Antibiotic resistance	
			Samples	%	Samples	%
Streptomycin	Sm	6	3	50	3	50
Norfloxacin	Nr	6	6	100	0	0
Ampicillin	Am	6	5	83	1	17
Tetracycline	Te	6	4	67	2	33
Azithromycin	Az	6	4	67	2	33
Amoxicillin-clavulanic acid	Ac	6	5	83	1	17
Trimethoprim-sulfamethoxazole	SXT/Bt	6	4	67	2	33
Vancomycin	Van	6	2	33	4	67

Table 2: Comparing the amino acid position of the wild-type *V. cholerae* strains N16961 with *V. cholerae* strains T1

Codon	Nucleotide changes	Amino acid changes	Nucleotide T1 loss	Mutant type
14	AGT→CTG	Ser→Leu	Loos 1	Replication error
51	CCT→TGA	Pro →End	-	Recombination error
52	TGG→TCA	Trp→Ser	1	Replication error
69	CGT→TAG	Arg→End	-	Recombination error
71	GTT→TAA	Val→End	-	Recombination error
74	A-T→TCA	→ Ser	Adding	Error
105	CA - →CG -	→	1	Error
106	- TG→-TG -	→	2	Error
121	GCT→TAG	Ala→End	-	Recombination error
142	GGT→TGA	Gly →End	-	Recombination error
160	AAA→TGA	Gly →End	-	Recombination error
161	GTA→TGA	Val →End	-	Recombination error
164	CTT→TGA	Leu →End	-	Recombination error
165	GAA→TGA	Glu→End	-	Recombination error
166	TCA→TGA	Ser→End	-	Recombination error

Table 3: Comparing the nucleotide position of wild *V. cholerae* strain N16961 with nucleotide position isolated *V. cholerae* strains T3

Codon	Nucleotide changes	Amini acid change	Nucleotide T3 loss	Mutant type
6	TAA→TGA	End →End	Adding	Recombination error
14	AGT→CTG	Ser→Leu	Loss 1	Replication error
17	TGA→ATC	End→Ile	Loss 1	Error
19	ACA→CCC	Thr→Pro	Loss 1	Replication error
23	GAT→CTC	Asp→Leu	Loss 3	Replication error
24	TCA→TAC	Ser→Tyr	Loss 3	Replication error
32	AAC→ATC	Asn→Met	Loss 1	Replication error
48	ACA→CGC	Thr→Ala	Loss 1	Replication error
52	TGG→CTA	Trp→Leu	Loss 1	Replication error
53	GAT→GCG	Asp→Gly	Loss 3	Replication error
54	CTA→GCG	Leu→Gly	Loss 3	Replication error
55	AAA→TAT	Lys→Tyr	Loss 2	Replication error
63	GGA→GAA	Gly→Glu	Loss 1	Replication error
71	GTT→GAG	Val→Glu	Loss 2	Replication error
74	A-T→G- -	→	2	Error
95	AAA →TGA	Lys →End	-	Recombination error
100	TT→TGA	Ile→End	-	Recombination error
106	GAA → TAA	Glu→End	-	Recombination error
124	CA→TAA	Ala→End	-	Recombination error
161	TA→TAA	Val→End	-	Recombination error

Genetic relationship of *V. cholerae* strains based on antibiotic resistance genes *tetA*

Evaluation results of *V. cholerae* mutant strains for rabbits without cholera vaccine

After putting the inoculum in the small intestine of rabbits at doses of 10^5 to 10^7, the amount of secreted fluid was withdrawn according to Fig. 5.

The number of bacteria counted at 3, 6, 9, and 16 hours were synthesized according to Fig. 6. From the figure, the adhesion of bacteria in the intestinal mucosa was highest at 6 hours and then decreased at 9 and 16 hours in all bacteria strains. Particularly, for 2 strains T1 and T3, the number of bacteria adhered only temporarily at 6 hours, from 5×10^5 to 65×10^4, and then decreased significantly at 9 hours, for only 4×10^5 and 35×10^4 and at 16 hours there was no adhesion of *V. cholerae*. In summary, the experiment on rabbits show that mutant-bacteria were temporarily adhesive at 6 hours, then decreased and finally got lost at 16 hours.

Evaluations of the immune response in rabbits with cholera vaccine

Fig. 7 shows the amount of secreted fluid withdrawn after putting the inoculums in the small intestine of rabbits at doses of 10^5 to 10^7. Fig. 8 shows the number of bacteria counted at 3, 6, 9 and 16 hours, respectively. As can be seen from the figure, the adhesion of bacteria in the intestinal mucosa was highest at 6 hours and then decreased at 9 and 16 hours in all bacteria strains. Particularly in 2 strains T1

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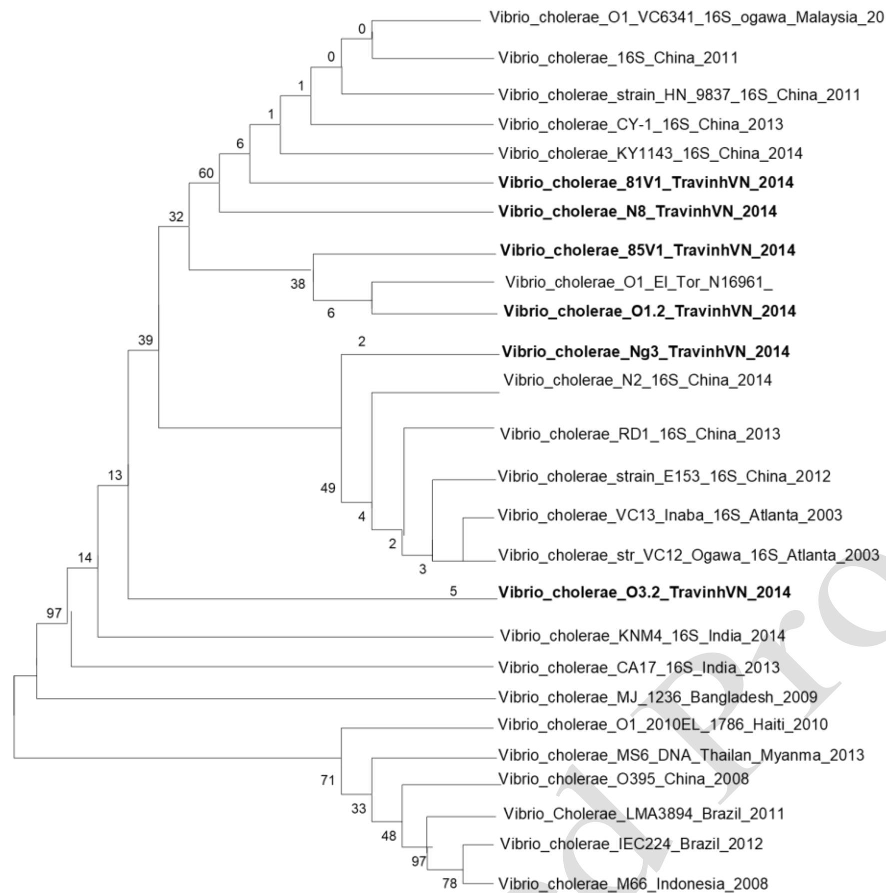

Fig. 2: Tree performances of evolutionary relationship based on 16srDNA of isolated *Vibrio* spp. and some reference strains.

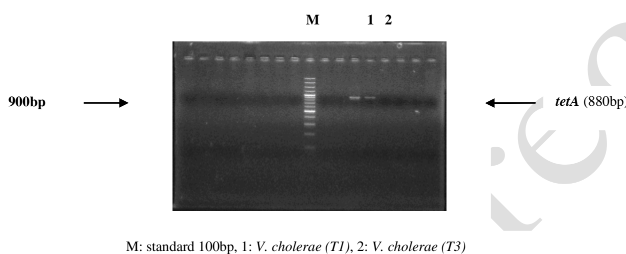

Fig. 3: Results of gene *tet*A.

and T3, the number of bacteria adhered only temporarily at 6 hours, from 1×10^5 to 8×10^4, and then also decreased significantly at 9 hours, for 12×10^3 and 20×10^3, and at 16 hours there was no adhesion of *V. cholerae*.

DISCUSSION

To compare the test results with the biochemical characteristics of PCR products, the study recorded the various species of *Vibrio,* respectively: *V. cholerae* from line 1-6; *V. fluvialis* from line 7-11; *V. paraheamolyticus* from line 12-19; *V. vulnific* from line 20-23; and *V. alginolyticus* from line 24-25. Also it was unable to detect any strains of *Vibrio* carrying the gene O139rfb and strains carrying the cholera toxins gene CTXA in water and seafood samples. Thus, PCR detected strains did not carry the gene of *Vibrio* O139rfb and cholera toxin genes CTX in water and seafood samples. This indicates that *Vibrio* O139 is not present in Tra Vinh province. The result was equivalent to the 16S rRNA gene in all strains of *Vibrio* spp (9). The result also showed that the isolated strains had similar characteristics as the strain that originated from the environment, implying that the strains of *Vibrio* spp. always pose potential risks and will easily cause the disease by receiving the gene from the virulent strain of CTX.

V. cholerae strains in this study were highly sensitive to many antibiotics such as norfloxacin (100%), ampicillin (83%), and amoxicillin-clavulanic acid (83%). Also, *V. cholerae* was resistant to vancomycin (67%), streptomycin (50%), and tetracycline (33%). This result is consistent with those in studies of Tran et al. (2012), Dörr et al. (2016) and Das et al. (2020).

Strains carrying antibiotic resistance genes *blaSHV*, *aac (3) -IV* and *dhfrI* were discovered. *V. cholerae* strains (T1) and (T3) isolated from the water environment all carried genes resistant to tetracycline, Aminoglycoside group. Thus some strains in the study did not contain tetracycline resistance gene (*tetA)*, but were resistant to tetracycline. This was probably due to the presence of other genes encoding resistance to tetracycline such as *blaSHV*, *aac (3)-IV* and *dhfrI* (Dang et al. 2006; Obayiuwana and Ibekwe 2020). When comparing the nucleotide position of wild *V. cholerae* strain N16961 with that of isolated *V. cholerae* strains T1, *V. cholerae* strain T1 had the mutation of adding or losing 1- 3 nucleotides in multiple codons, changing its position and altering the amino acid protein structure. *V. cholerae* strains T1 had codon ending at 10 positions, corresponding to 10 amino acid positions; this was a frame shift mutation by adding or losing 1 or 2 nucleotides to stop the codons. This in turn will stop the polypeptide synthesis chain, consequently stopping the

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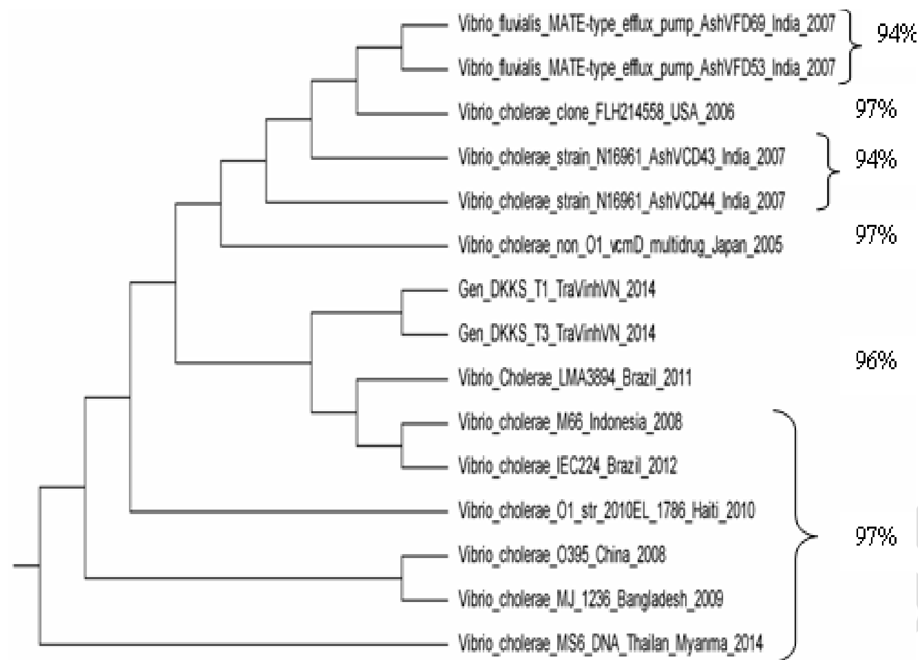

Fig. 4: Genetic relationship of *V. cholerae* strains based on antibiotic resistance genes *tetA*: The results of the genetic tree in Figure 4 also show that two strains of *V. cholerae* isolated from T1 and T3 in Tra Vinh river water carried antibiotic resistance genes.

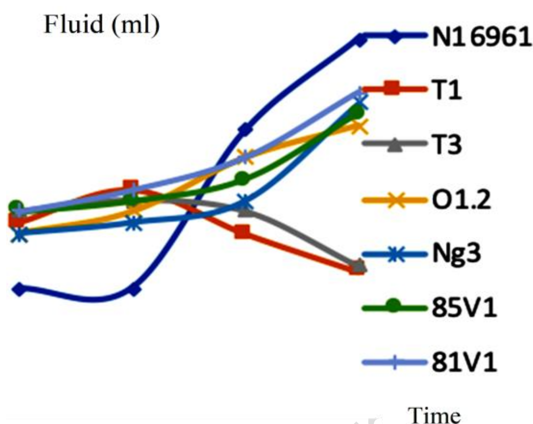

Fig. 5: Chart of liquid rate after injection of bacteria in the intestine of rabbits.

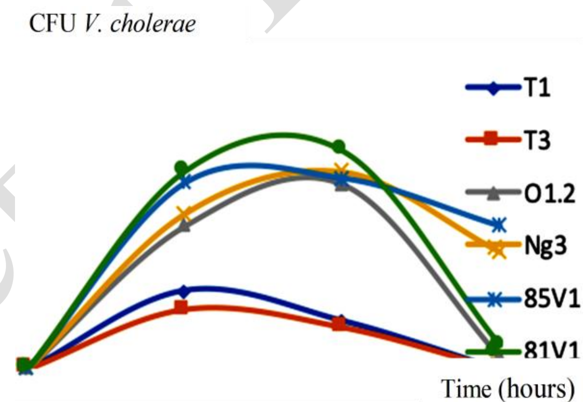

Fig. 6: Chart of *V. cholerae* adhesion in rabbit intestinal mucosa.

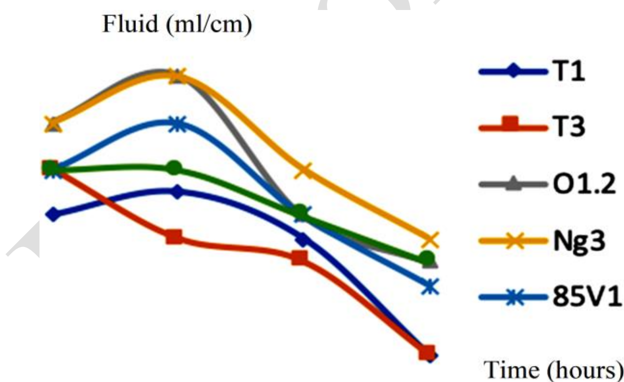

Fig. 7: Fluid chart after injection of bacteria in the small intestine.

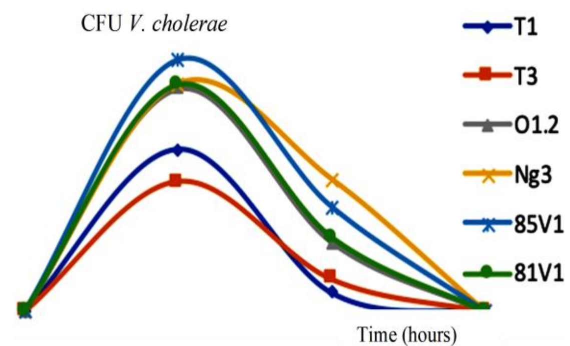

Fig. 8: Chart of *V. cholerae* adhesion in rabbit intestinal mucosa.

enzyme activities (Nguyen et al. 2007). When comparing the nucleotide position of wild *V. cholerae* strain N16961 with the nucleotide position of isolated *V. cholerae* strains T3, the results are similar. However, *V. cholerae* strains T3 had codon ending at 6 positions, corresponding to 6 amino acid positions. The frame shift mutation is the same and has the same effect on polypeptide synthesis chain and enzyme activities (Nguyen et al. 2007).

In this study, two strains of *V. cholerae* isolated in Tra Vinh carried tetracycline antibiotic resistance. Isolated from water and clams with similar nucleotide sequence compared with other strains in Thailand, Japan, China,

Indonesia, Brazil and India, *V. cholerae* strain was 97% similar to 10 strains, 96% similar to 1 strain and 94% similar to 4 strains. Moreover, two strains of *V. cholerae* isolated from T1 and T3 in Tra Vinh river water carried antibiotic resistance genes which were closely related by nucleotide sequences (97%) with other isolated strains of *V. cholerae* in Indonesia, China, Bangladesh, Haiti, Brazil, and Thailand in 2008, 2008, 2009, 2010, 2012, and 2014, respectively and *V. cholerae* non-O1 isolated from vim in Japan in 2005. Thus, 2 strains in this study had antibiotic resistance mechanisms similar to comparative strains, and the risk of genetic antibiotic resistance genes was very high in the *V. cholerae* strains.

It was found that wild *V. cholerae* strain N16961 started accumulating fluid at 9 hours and 16 hours after injecting with 1.45-2.3ml/cm bacteria; this was higher than *V. cholerae* strains T1 and *V. cholerae* strains T3 in this study that carried the tetracycline resistance gene. When injected into the small intestine of rabbits, it did not stimulate the rabbit intestinal mucosa to secrete fluid, it only secreted from 0.8-0.9ml/cm at 6 hours, and then decreased to 0.15 and 0.2ml/cm at 16 hours. This was because the wild *V. cholerae* strain N16961 contained flagella responsible for strong mobility, easy adhesion to the intestinal, and stimulates the intestinal mucosa to produce toxic CT, thus causing diarrhea (Dixit et al. 2012).

This result showed that all the *V. cholerae* strains T1, T3, O1.2 Ng3, 85V1, and 81V1 were high when injected into the intestine of rabbits with vaccine (mORCVAX), the fluid accumulation commenced at 3 hours, but then decreased at 6, 9 and 16 hours, the amount of liquid was less because the intestinal mucosa had more antibodies attached to the receptors on the surface of bacteria, thus hindering the bacteria from sticking on the host's intestinal mucosa. Intestinal mucosa thus could not secrete toxins and the small intestine epithelial cells could not secrete fluids (Allaire et al. 2018). Therefore, for all rabbits with cholera vaccine, the presence of antibodies prevents the secretion of fluid in the intestine.

The result also proved that *V. cholerae* is inhibited by antibodies secreted by the intestinal mucosa. Therefore, for all rabbits with cholera vaccine containing antibody, the bacteria were only temporarily adhesive at 6 hours, and then there was no adhesion to the intestinal mucosa at 16 hours. Particularly, T1 and T3 strains had the least bacteria content.

Conclusion

Among the 25 strains isolated from seafood, river water, shrimp pond water, seawater and pig's blood in Tra Vinh Province there were 6 strains of *V. cholerae* (24%), 8 strains of *V. paraheamolyticus* (32%), 4 strains of *V. vultificus* (16%), 5 strains of *V. fluvialis* (20%), and 2 strains of *V. alginolyticus* (8%). Besides, there was no detection of antisera O139, gene *ctx*A and *tcp*. 100% of the strains of *V. cholerae* were sensitive to norfloxacin and 83% were sensitive to ampicillin and amoxicillin-clavulanic acid. A total of 50% were resistant to streptomycin and 33% were resistant to tetracycline and trimethoprim-sulfamethoxazole. Two strains of *V. cholerae* out of the 6 tested strains contained antibiotic resistance genes *tetA*, an encoding gene for tetracycline.

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