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The changes in antigenic components of *Vibrio cholerae* strains isolated in Vietnam

Sự biến đổi thành phần kháng nguyên của các chủng Vibrio cholerae phân lập ở Việt Nam

Research article

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Whole cells of *Vibrio cholerare* serotype Inaba and serotype Ogawa (strains I389 and O395) were injected into rabbits to obtain antiserum. The antiserums were used for immune reaction with antigenic components of 25 strains of *V.cholerae* isolated from five provinces of Vietnam and the two standard strains I389 and O395 by Western-blot technique. Analysis of immune hybrid results showed that there were 11 antigenic components with molecular weights approximately 79kDa, 62kDa, 52kDa, 45kDa, 42kDa, 38kDa, 35kDa, 31kDa, 26kDa, 23kDa and 20kDa. In which the antigens of 45kDa, 42kDa, 31kDa and 20kDa were similar to OmpT, OmpS, Omp-31kDa and TcpA that have been considered as vaccine-candidate antigens. Among 25 *V.cholerae* strains, there were 6 antigenic components in common including 79kDa, 62kDa, 45kDa, 35kDa, 31kDa and 20kDa. 23/25 strains contained 42kDa antigen; 5/25 strains contained 38kDa and 23kDa antigens; 11/25 had 26kDa antigen. In addition, 7/25 strains contained antigens identical to *V.cholerae* I389 serotype Inaba; 6/25 strains contained antigens of I389 and O395; 12/25 strains had changes of antigenic components. These changes were actually the lack of antigens, not appearing new antigens. These results are considered as basis for researches about immune response and prevention of cholera disease.

Toàn bộ tế bào của các chủng Vibrio cholerare typ huyết thanh Inaba và typ huyết thanh Ogawa (chủng I389 và O395) được sử dụng để gây miễn dịch trên thỏ để thu kháng huyết thanh. Các kháng huyết thanh được dùng để thực hiện phản ứng miễn dịch với các thành phần kháng nguyên của 25 chủng V.cholerae phân lập từ 5 tỉnh thành của Việt Nam và hai chủng chuẩn I389 và O395 bằng kỹ thuật Western-blot. Phân tích kết quả lai miễn dịch cho thấy, có tổng số 11 thành phần kháng nguyên có kích thước khoảng 79kDa, 62kDa, 52kDa, 45kDa, 42kDa, 38kDa, 35kDa, 31kDa, 26kDa, 23kDa và 20kDa. Các kháng nguyên này chủ yếu là các protein màng ngoài (Omp) và kháng nguyên lông (TcpA). Trong đó các kháng nguyên 45kDa, 42kDa, 31kDa và 20kDa trùng với các kháng nguyên OmpS, OmpT, Omp-31kDa và TcpA được xem là những kháng nguyên dự tuyển vacxin tả. Có 6 kháng nguyên chung giữa 25 chủng với kích thước 79kDa, 62kDa, 45kDa, 35kDa, 31kDa và 20kDa. 7/25 chủng có các kháng nguyên giống với kháng nguyên của chủng V. cholerae I389 typ huyết thanh Inaba; 6/25 chủng có các kháng nguyên giống với kháng nguyên của cả hai chủng V.cholerae I389 và O395; 12/25 chủng có sự biến đổi thành phần kháng nguyên. Tuy nhiên, sự biến đổi này thực chất là sự thiếu hụt chứ không phải là sự xuất hiện các thành phần kháng nguyên mới. Các kết quả nghiên cứu này có thể được xem là nền tảng ban đầu cho các nghiên cứu về miễn dịch và dự phòng bệnh tả.

Keywords: *Vibrio cholerae* serotype Inaba, *Vibrio cholerae* serotype Ogawa, antiserum, antigens of *Vibrio cholerae*, outer membrane protein, TcpA

1. Introduction

Vibrio cholerae is the etiologic agent for the diarrheal disease (cholera disease) in many areas of Asia, Africa and Latin America (Kenneth Todar, 2002). *V.cholerae* settled on mucosa membrane of small intestine, but did not invade into the intestinal epithelial cells. However, the vaccine trials showed that antibodies producing in cholera

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immune responses are present in both of the serum and intestine (Forrest B.D., 1992).

According to previous studies, *V.cholerae* has a variety of antigens causing immune responses such as O antigen, B subunit of cholera toxin, toxin co-regulated pili (Tcp), mannose sensitive haemagglutinin factor (MSHA) and outer membrane proteins (Omps) (Richardson K. et al., 1989; Tacket C.O. et al., 1998). Particularly, the O antigen is capable to cause immune responses against bacteria, while sub-section B subunit is capable to cause immune responses against cholera-toxin (Taylor D.N. et al., 2000). Both of them are co-effects causing immune responses against cholera. The remaining antigens such as Tcp, MSHA and Omps play an important role for immune response against settlement of *V.cholerae* in the small intestine.

Beside researches on detection of antigens for development of vaccines, researches on antigenic components of *V.cholerae* strains isolated in different areas are necessary to control cholera disease. Thus, the purpose of this paper is research on changes in the antigenic components of *V.cholerae* strains isolated in different geographical areas of Vietnam to provide information for molecular epidemiological surveillance and cholera prevention.

2. Materials and methods

2.1. Materials

V.cholerae strains were isolated in the different areas of Vietnam. After that they were stored and managed by Institute of Hygiene and Epidemiology, Ministry of Health, Vietnam.

Table 1. *Vibrio cholerae* strains and isolated places in Vietnam

Strains	Isolated places	Strains	Isolated places	Strains	Isolated places	Strains	Isolated places
1AG	An Giang	8HN	Hanoi	15HN	Hanoi	22HP	Hai Phong
2AG	An Giang	9HN	Hanoi	16QN	Quang Ninh	23HP	Hai Phong
3AG	An Giang	10HN	Hanoi	17HN	Hanoi	24HP	Hai Phong
4CM	Ca Mau	11HP	Hai Phong	18HN	Hanoi	25HP	Hai Phong
5HN	Hanoi	12AG	An Giang	19HN	Hanoi	I389	Standard strain
6HN	Hanoi	13HP	Hai Phong	20HN	Hanoi	O395	Standard strain
7HN	Hanoi	14HP	Hai Phong	21HN	Hanoi		

2.2. Methods

2.2.1. Bacterial culturing

V.cholerae were cultured in APW media (peptone 10g/l, NaCl 10g/l). Time for culturing was from 16 to 24 hours at 37ºC.

2.2.2. Antiserums producing

Steps for producing antibodies in rabbits: (1) 1ml of *V.cholerae* cells serotypes Inaba and Ogawa (OD_{600nm} = 1) was inactivated by heat, and injected directly to rabbits; (2) injection again after 5, 14 and 30 days to exploit primary immune responses (production of primary antibodies) and secondary immune responses (production of antibodies quickly and much); (3) After 10 days since last injection, blood of rabbits was obtained, incubated for 2 hours at 37ºC, held for 2 hours at 40ºC; (4) centrifuging blood of rabbits for 10 min, collecting serum, preserving for immune hybrid.

2.2.3. Checking antigenic components of *V. cholerae* strains by Western-blot

Main steps of Western-blot: (1) denaturing cultured bacterial liquids at 100ºC for 10 minutes; (2) analyzing proteins of bacteria by SDS-PAGE electrophoresis; (3) transferring proteins to PVDF membrane (Polyvinylidene fluoride); (4) dyeing PVDF membrane with 0.1% ponceau solution; (5) coating PVDF membrane with 5% skim milk at 40ºC overnight to cover positions of PVDF membrane without proteins; (6) adding Inaba antiserum and Ogawa antiserum (diluted with 5% skim milk) to coat PVDF membrane for 2 hours. (7) coating PVDF membrane with anti-rabbit antibody attached horse-radish peroxidase (diluted with 5% skim milk) for 2 hours; (8) washing PVDF membrane with TTBS and TBS each 5 minutes, repeating two times; (9) adding solution containing 5ml methanol + 15mg 4-chloronaphthol + 25ml TBS + 15µl H_2O_2 30% for detecting colour.

3. Results and discussions

3.1. The antigenic components of *V.cholerae* I389 serotype Inaba and O395 serotype Ogawa

Inaba antiserum and Ogawa antiserum were used to check antigenic components of *V.cholerae* serotype Inaba and serotype Ogawa by immune hybrid (Figure 1).

The antigenic components of *V.cholerae* I389 contained 10 bands with molecular weights approximately 79 kDa, 62kDa, 45kDa, 42kDa, 38kDa, 35kDa, 31kDa, 26kDa, 23kDa and 20kDa. And the antigenic components of *V.cholerae* O395 contained 8 bands with molecular weights approximately 79kDa, 62kDa, 52kDa, 42kDa, 35kDa, 26kDa, 23kDa and 20kDa. So there were 7 antigens in common between *V.cholerae* serotype Inaba and serotype Ogawa. The difference between two serotypes mainly based on lacking of three bands (sizes of 31kDa,

38kDa and 45kDa) and appearing one more band (size 52kDa) of serotype Ogawa.

The antigen with size 20kDa was similar to TcpA antigen. TcpA is usually used as a component in vaccines against cholera (Asaduzzman M. et al., 2004; Du Yan et al., 2004).

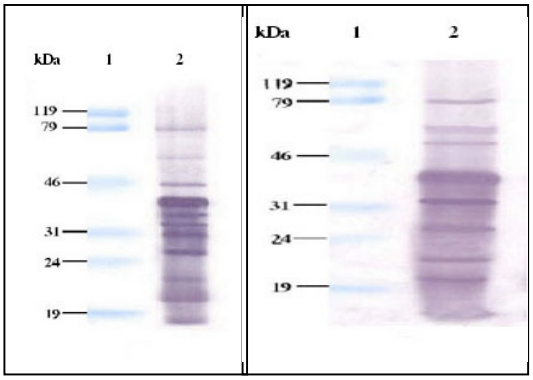


Figure 1. (A) Antigenic components of *V.cholerae* serotype Inaba (I389 strain). (B) Antigeinc components of *V.cholerae* serotype Ogawba (O395 strain); A1, B1: protein marker

The 23kDa antigen was same to a heat shock protein -Hsp and the antigen with size 26kDa was similar to OmpV- an outer membrane antigen. It is also a heat induced-protein. Both of these antigens were described by Sahu G.K. et al. (1994).

The antigen with 31kDa size was an outer membrane protein (Omp-31kDa) that was researched by Kuma S. et al. (2001). The 35kDa antigen was said to be OmpA that was easy to be changed by heat (Simonet V.C. et al., 2003). The 38kDa antigen was similar to OmpU, an outer membrane protein with pore-forming ability mentioned by Sperandio V. et al. (1995) and Simonet V.C. et al. (2003). The antigens with 42kDa and 45kDa sizes were described like to OmpT with pore-forming ability (Simonet V.C. et al., 2003; Stewart-Tull D.E. et al., 2004) and OmpS (Stewart-Tull D.E. et al, 2004). Both of these antigens have proteinases activity.

Kumar S. et al. (2001) mentioned and described the 52kDa antigen as a proteinase, named Omp-52kDa. The antigen with 62kDa size was similar to a Hsp of the outer membrane. It is also a protein with proteinase activity (Sahu G.K. et al., 1994). The antigens of 26kDa, 31kDa, 42kDa and 45kDa have been considered as vaccine-candidate antigens for development of vaccines against cholera (Stewart-Tull D.E. et al, 2004).

Growing conditions are major factors affecting expression of antigens of *V.cholerae* (such as Omp, Tcp and cholera toxin). The expression of the antigens is controlled by a regulatory protein - ToxR (same to regulatory mechanism for expression of cholera toxin). Activity of ToxR is controlled by environmental signals. According to Richardson et al. (1989), the antigens with higher molecular weights (> 84kDa) of *V.cholerae* only appear when *V. cholerae* grows in in-vivo condition. In our experiments, *V.cholerae* strains were cultured in in-vitro condition so that may be why antigens with molecular weights less than or equal to 79kDa were expressed.

3.2. Changes of the antigenic components of *V.cholerae* strains

Inaba and Ogawa antiserums were used for immune hybrid with antigens of 25 *V.cholerae* strains isolated in some provinces of Vietnam and two standard strains (I389 and O395). Western-blot results are showed in Figure 2 and Figure 3. The statistics of antigenic components of *V.cholerae* strains are indicated in Table 2 and Table 3.

In the Figure 2 and Table 2, the antigenic components of 25 *V.cholerae* strains were divided into five groups. The group of 13 strains (5-6HN, 8HN, 17-21HN, 22-25HP, and 1AG) had 10 antigens similar to I389; the group of 3AG and 7HN strains lacked 4 antigens (42kDa, 38kDa, 26kDa and 23kDa); the group of 2AG and 4CM strains lacked 3 antigens (38kDa, 26kDa and 23kDa); the group of 11HP strain lacked 2 antigens (38kDa and 23kDa); and the group of 9HN, 10HN, 12AG, 13HP, 14HP, 15HN and 16QN strains lacked 26kDa antigen.

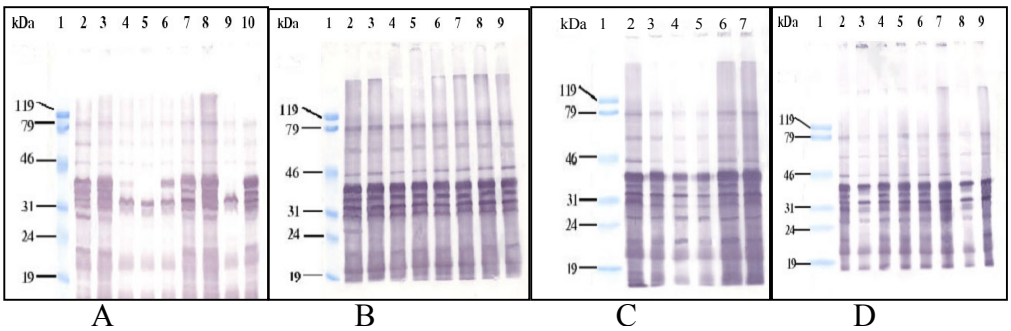


Figure 2. Western-blot of Inaba antiserum and *V.cholerae* strains (see tab. 1) (A) strains 1-8; (B) strains 9-10; 12-16; (C) strains 17-21; (D) strains 11, 22-25 and O395. (Protein marker: A1, B1, C1, D1; I389: A2, B2, C2, D2; O395: D9; 11HP:D3, D8)

In the Figure 3 and Table 3, the antigenic components of 25 *V. cholerae* strains were divided into six groups. The group of 1AG, 5HN, 6HN, 8HN, 20HN and 21HN strains contained antigens similar to O395; the group of 17HN, 18HN, 19HN, 22HP, 23HP, 24HP and 25HP strains lacked 52kDa antigen; the group of 9HN, 10HN, 12AG, 13HP, 14HP, 15HN and 16QN lacked 26kDa antigen; the group of 11HP strain lacked 23kDa antigen; the group of 2AG and 4CM strains lacked 26kDa and 23kDa antigens; and the group of 3AG and 7HN strains lacked 42kDa and 26kDa antigens.

Table 2. The difference of the antigenic components of *V.cholerae* strains (using Inaba antiserum in Western-blot)

Strains	Antigens Size (kDa)									
	79	62	45	42	38	35	31	26	23	20
I389	+	+	+	+	+	+	+	+	+	+
1AG	+	+	+	+	+	+	+	+	+	+
2AG	+	+	+	+	-	+	+	-	-	+
3AG	+	+	+	-	-	+	+	-	-	+
4CM	+	+	+	+	-	+	+	-	-	+
5HN	+	+	+	+	+	+	+	+	+	+
6HN	+	+	+	+	+	+	+	+	+	+
7HN	+	+	+	-	-	+	+	-	-	+
8HN	+	+	+	+	+	+	+	+	+	+
9HN	+	+	+	+	+	+	+	-	+	+
10HN	+	+	+	+	+	+	+	-	+	+
11HP	+	+	+	+	-	+	+	+	-	+
12AG	+	+	+	+	+	+	+	-	+	+
13HP	+	+	+	+	+	+	+	-	+	+
14HP	+	+	+	+	+	+	+	-	+	+
15HN	+	+	+	+	+	+	+	-	+	+
16QN	+	+	+	+	+	+	+	-	+	+
17HN	+	+	+	+	+	+	+	+	+	+
18HN	+	+	+	+	+	+	+	+	+	+
19HN	+	+	+	+	+	+	+	+	+	+
20HN	+	+	+	+	+	+	+	+	+	+
21HN	+	+	+	+	+	+	+	+	+	+
22HP	+	+	+	+	+	+	+	+	+	+
23HP	+	+	+	+	+	+	+	+	+	+
24HP	+	+	+	+	+	+	+	+	+	+
25HP	+	+	+	+	+	+	+	+	+	+
O395	+	+	-	+	-	+	-	+	+	+

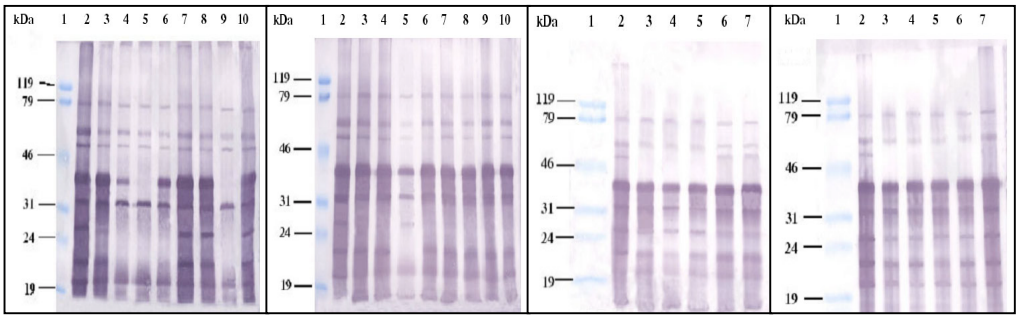
The immune hybrid results of Inaba antiserum with O395 and Ogawa antiserum with I389 did not appear 52kDa, 45kDa, 38kDa, and 31kDa antigens. It can be explained that I389 did not have 52kDa antigen and O395 did not have 45kDa, 38kDa and 31kDa antigens. Thus, Inaba and Ogawa antiserums did not have corresponding antibodies to react to these antigens.

Table 3. The difference of the antigenic components of *V.cholerae* strains (using Ogawa antiserum in Western-blot)

Strains	Antigens Size (kDa)							
	79	62	52	42	35	26	23	20
O395	+	+	+	+	+	+	+	+
1AG	+	+	+	+	+	+	+	+
2AG	+	+	+	+	+	-	-	+
3AG	+	+	+	-	+	-	-	+
4CM	+	+	+	+	+	-	-	+
5HN	+	+	+	+	+	+	+	+
6HN	+	+	+	+	+	+	+	+
7HN	+	+	+	-	+	-	-	+
8HN	+	+	+	+	+	+	+	+
9HN	+	+	+	+	+	-	+	+
10HN	+	+	+	+	+	-	+	+
11HP	+	+	+	+	+	+	-	+
12AG	+	+	+	+	+	-	+	+
13HP	+	+	+	+	+	-	+	+
14HP	+	+	+	+	+	-	+	+
15HN	+	+	+	+	+	-	+	+
16QN	+	+	+	+	+	-	+	+
17HN	+	+	-	+	+	+	+	+
18HN	+	+	-	+	+	+	+	+
19HN	+	+	-	+	+	+	+	+
20HN	+	+	+	+	+	+	+	+
21HN	+	+	+	+	+	+	+	+
22HP	+	+	-	+	+	+	+	+
23HP	+	+	-	+	+	+	+	+
24HP	+	+	-	+	+	+	+	+
25HP	+	+	-	+	+	+	+	+
I389	+	+	-	+	+	+	+	+

Note: + yes; - no

As can be seen, the strains of 17HN, 18HN, 19HN 22HP, 23HP, 24HP and 25HP (7/25 strains) contained antigens of I389 and O395 but 52kDa antigen did not appeared. So it could be confirmed that their antigenic components were identical to antigens of *V.cholerae* I389 serotype Inaba.



A B

Figure 3. Western-blot of Ogawa antiserum and *V.cholerae* strains (see Table 1) (A) strains 1-8; (B) strains 9-16; (C) strains 17-21; (D) strains 22-25 and I389 (Protein markers: A1, B1, C1, D1; O395: A2, B2, C2, D2; I389: D7)

The strains of 1AG, 5HN, 6HN, 8HN, 20HN and 21HN (6/25 strains) contained antigens of I389 and O395 including 52kDa antigen. This could happen due to serotype Inaba and serotype Ogawa can be transformed into each other easily, especially transformation from Ogawa to Inaba (Villeneuve S. et al., 2000; Kenneth Todar, 2002).

The remaining strains of 2AG, 3AG, 4CM, 7HN, 9HN, 10HN, 11HP, 12AG, 13HP, 14HP, 15HN and 16QN (12/25 strains) had antigens that were not identical to standard strains; appearing 52kDa antigen of O395 but lacking some antigens of I389. So they were classified in a new group with changed antigenic components. However, the changes are just deficiency of antigens, not appearing new antigens.

Among 25 *V.cholerae* strains, there were 6 antigens in common including 79kDa, 62kDa, 45kDa, 35kDa, 31kDa and 20kDa antigens. 23/25 strains had 42kDa antigen; 5/25 strains contained 38kDa and 23kDa antigens and 11/25 contained 26kDa antigen.

4. Conclusion

There were 11 different antigens including sizes of 79kDa, 62kDa, 52kDa, 45kDa, 42kDa, 38kDa, 35kDa, 31kDa, 26kDa, 23kDa and 20kDa. In which, antigens of 45kDa, 42kDa, 31kDa and 20kDa are similar to OmpS, OmpT, Omp-31kDa and TcpA, and are considered as cholera vaccine-candidate antigens. Based on reported researches, we identified the antigenic components of *V.cholerae* strains in this research are the Out Membrane Proteins (Omp) and Toxin Coregulated-Pili A (TcpA).

There were 6 antigenic components in common among 25 strains including 79kDa, 62kDa, 45kDa, 35kDa, 31kDa and 20kDa. 23/25 strains contained 42kDa antigen; 5/25 strains contained 38kDa and 23kDa antigens; 11/25 strains contained 26kDa antigen.

Among 25 *V.cholerae* strains; 7/25 strains had antigens identical to *V.cholerae* I389 serotype Inaba; 6/25 strains contained antigens of I389 and O395; 12/25 strains had changes of antigenic components. The changes are lacking of antigens, not appearing new antigens.

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