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ORIGINAL ARTICLE

Phenotypical analysis of vibrio cholerae 01 (Eltor and classic) biotypes by scanning electron microscope and transmission electron microscope

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ABSTRACT

Background: Due to repeated cholera outbreaks in Iran and neighboring countries, the present study was performed to determine the prevalence of phenotypes of Ogawa and Inaba serotypes of Vibrio cholera 01 and classic Vibrio cholera 01 biotypes.

Materials and methods: Scanning and transmission electron microscopy (SEM and TEM) were applied on 4 species, of Ogawa and Inaba serotypes and two classic serotypes of Vibrio cholera 01.

Results: Membrane diameter of Eltor was wider as compared to classic biotype. Number of ribosomes, protein synthesis, length and number of flagella were quite more in comparison with classic biotype.

Conclusion: According to our findings, genome of classic biotype is more compact.

Keywords: *Vibrio cholera, Ogawa serotype, Inaba serotype, Transmission Electron Microscopy, Scanning Electron Microscopy.*

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INTRODUCTION

Recent Cholera outbreaks in Iran and other neighboring countries and its high mortality rate seek further attention towards its possible route of transmission through contaminated water. Cholera outbreak was started from Bengal in 1817 and spread to other countries. In 1993, seventy–eight countries had reported outbreaks of this disease to WHO, which was the highest rate so far. However, the most infected regions were Africa, Latin America, and Asia with a total 376,845 infected subjects, of whom 6,781 were died. Related mortality rates were 3.3, 1.2, and 2% for Africa, Latin America and Asia, respectively. Vibrio cholera 01 classic biotype was reported as the seventh pandemic which is now substituted by Eltore biotype. It should be mentioned that Inaba serotype of Eltor Vibrio cholera 01 biotype was known as the cause of epidemic in 1999 in Iran.

During the recent epidemic in Madras, Vibrio cholera 0139 Bengal type was the causative agent that seems to be the same etiologic agent of the eighth pandemic (1).

Pathogenicity of Vibrio cholera is related to its toxins. First, the bacteria attach to their receptors

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on the cells, then colonize and produce the toxins. Therefore, in order to inhibit the attachment of vibrio to GM_1, specific gangeliozide are required. Needless to say, recognition of surface molecules is basically important for which studying the bacteria morphology is of paramount importance. In our study, scanning electron microscopy and transmission electron microscopy were used to determine Vibrio cholera 01 Ogawa and Inaba serotypes, as well as Eltor and classic biotypes.

PATIENTS and METHODS

Four species of Ogawa and Inaba serotypes of Vibrio cholera 01 and two classic serotypes of Vibrio cholera 01, 2000-2001-2005 PTCC were provided by the Microbial Bank of Razi Institute. The lyophilized species were transferred to the laboratory in special vials, with the vials being already sterilized by ethylic alcohol 70%, recapped and 1cc BHI (Brain Heart Infusion) media was added and then covered by parafilm. The vials were incubated in 37^{o}C for about 24 hours; afterwards 5cc of suspension was poured in labeled tubes. The specimens were centrifuged and fixed with glutaraldehyde and osmium tetraoxide. Then, the specimens were washed out with buffer. Following the fixation, the specimens were placed in 40^{o}C melted agar for dehydration and clotting, after which we cut down them to small pieces. Finally, the specimens were dehydrated with ethanol. In order to evaluate the samples with SEM, they were placed on the stub with specific glue and incubated for 24 hours. Finally, the stubs were placed in vacuum, being prepared for coating.

During the coating process, fixed specimens were coated by 40-70nm gold, being ready for inspection with SEM. Subsequently, the photos were taken by SEM. The microscope had 50×2000×5000 magnifications for determining the dimensions of the bacteria.

For TEM evaluation of the specimens, they were placed in ethanol and acetone complex for 30 minutes then in pure acetone for another 30 minutes, while being shaken at each stage. During the next phase, they were infiltrated in rotator for 4 steps including 3:1 resin-propylene complex over night, 1:1 complex for 10 hours, 1:3 complex over night and pure resin for 12 hours.

In the last phase, the specimens were put in special dishes for blocking and pure resin was added. These specimens were put in the laboratory temperature and then in 60^{o}C oven for polymerization which were prepared for trimming process after 72 hours. During the trimming phase, models were initially cut and prepared for ultra microtome cut. Sections (40nm) were obtained from each specimen with ultra microtome. Finally, each specimen was prepared for TEM study.

RESULTS

Ten sections were prepared and studied from each species. Of 10 blocks of classic biotype, 2 had 2×12mm diameter and 8 were measured 0.9×0.63mm. Flagella length and dimensions of Inaba and Ogawa serotypes are presented in tables 1 and 2.

Table 1. *Inaba and Ogawa serotypes, Vibrio cholerae Eltor biotype dimensions with electron microscope with ×5000 magnification*

Dimension	Serotype		
	Ogawa	**Inaba**	**Total**
1.2×1.8mm	5(50)	3(30)	8(40)
1.3×4mm	5(50)	7(70)	12(60)
Total	10(100)	10(100)	20

Ninety percentages of classic biotypes had 1.5–2 mm flagella length, whereas the remaining were measured 2-2.5 mm. Terminal flagella were presented in 40 and 80% of Ogawa and Inaba serotypes, respectively, however, 30% of the classic biotype had terminal flagella while 70% had peripheral flagella. Monoflagellated species were found in 40 and 80% of Ogawa and Inaba

serotypes, respectively, as compared to 30% of classic biotypes.

Table 2. *Inaba and Ogawa serotypes, Vibrio cholerae Eltor biotype flagella length with SEM with ×2000 magnification*

Flagella length	Serotype		
	Ogawa	Inaba	Total
1.2×2mm	2(20)	4(40)	6(30)
2.2×5mm	8(80)	6(60)	14(70)
Total	10(100)	10(100)	20

DISCUSSION

We have studied 4 different species of Vibrio cholera 01. Vibrio cholera 01 classic biotype was known as the causative agent of the sixth pandemics and Eltor biotype as the causative agent of the seventh pandemic (1).

Using bacterioribotyping for diagnosis, we suggest a method for Vibrio cholera detection. Photos showed that this technique can be used as a reliable diagnostic modality for specific species of Vibrio cholera (2).

Toxins are of utmost importance in Vibrio cholera pathogenicity. Different vaccines have been developed so far to prevent the disease; however, each has induced partial immunity. Therefore, different regions' biotypes should be concerned due to their genotype variations and, of course, recent antibiotic resistance. In addition, phenotype and antigenic structure of the bacteria must be observed by vaccine developers. Recent theories have proposed that preventing the bacterial attachment to intestinal tissues and colonization could inhibit toxin production.

Preparation samples for electron microscopy varied greatly and at least 5 different approaches have been introduced. For instance, specimen fixation may be achieved via physical, chemical or thermal techniques. However, our unique approach revealed to be the best.

Prior investigators have emphasized the necessity of further studies on different bacterial antigens (3), especially on specific binding protein receptors. Kiellberg and colleagues explained BC-NMR technique, through which O antigenic polysaccharide of Vibrio cholera 01 is repeated tetra saccharid monomers (3). Therefore, if the coding gene for the specific proteins are found and cloned, it would be possible to develop a new cholera vaccine.

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