Virulence factors of non-O1 non-O139

*Vibrio cholerae*

Project in biotechnology with measurement systems, 12hp

N. Mähler, F. Högdahl, L. Rautio, R. Lindquist, F. Torell, S. Öhman

Course Leader
Mark Dopson
Abstract

Cholera is a medical condition caused by *Vibrio cholerae*. The most common symptoms of the disease are diarrhea, vomiting, oedema and dehydration. *V. cholerae* is also known to possess putative virulence factors such as hemagglutinin protease, hemolysin/cytolysin which may assist in bacterial detachment and/or host cell death. On the basis of presence of surface O-antigen, *V. cholerae* can be divided into different serogroups. Some belong to serogroup O1 or O139 while strains of serogroups other than these are collectively known as non-O1 and non-O139 strains. Members of O1 and O139 serogroups are responsible for all known cholera endemics while those belonging to non-O1, non-O139 serogroups can occasionally cause gastro-intestinal or extra-intestinal illness and septicemia. The mechanisms for how O1, O139 *V. cholerae* cause cholera are known, while further studies on non-O1, non-O139 *V. cholerae* are needed. Study described in this report was made to detect virulence factors of six strains; C6706, L1, L4, USS, KI, and V5 belonging to different serogroups. The strains were tested for hemagglutination, proteolysis and hemolysis. We also tried to detect the presence of various virulence factor genes in these strains using polymerase chain reaction (PCR). PCR analysis showed the presence of *hlyA* (hemolysin), *prtV* and *hapA* (metalloproteases) genes in all the tested strains. Furthermore the PCR assays showed the absence of *ctx* (cholera toxin) gene in all the tested strains except *V. cholerae* strains C6706 and USS, both belonging to serogroup O1. All six strains showed hemolytic activity, hemagglutination and at least L1, L4, KI, USS and V5 exhibit proteolytic activity. The strains were also tested for serum resistance. The two O1 strains, namely C6706 and USS, did not survive in active blood serum while the non-O1, non-O139 strains had a survival rate of 51-97% in the active blood serum. Since the non-O1, non-O139 *V. cholerae* can survive in the blood serum, the blood might be a possible invasive pathway for these bacteria.
Contents

Introduction 2
Materials and methods 3
Results 6
Discussion 6
References 8
Introduction

Cholera is a medical condition caused by *Vibrio cholerae*, which is a Gram-negative, waterborne bacterium. It is commonly transmitted to humans orally [1]. In its most serious form, a patient suffering from cholera excretes large amounts of watery stool which in turn leads to dehydration [2,3]. Without access to proper treatment the death rate of cholera is estimated to 50-60% [1].

As of today 206 serogroups of *V. cholerae* have been determined [4]. At first it was believed that only *V. cholerae* belonging to serogroup O1 were able to cause epidemics or pandemics, while other serogroups were believed to be connected to sporadic cholera-like outbreaks [5]. Therefore strains of serogroups other than O1 were labeled non-O1. This changed when a cholera epidemic that started 1992 in Madras, India, was caused by a non-O1 strain [6]. This serogroup was labeled O139, and all other serogroups were labeled non-O1, non-O139.

The mechanisms for how O1, O139 *V. cholerae* cause cholera are known [5,7], while further studies on non-O1, non-O139 *V. cholerae* are needed.

The cholera toxin gene (*ctx*) is the gene encoding CT [2] which causes diarrhea in the *V. cholerae* host cells. CT consists of a single A-subunit which links to five B-subunits [8]. CT enters the cell through the plasma membrane of intestinal cells by absorbing a glycolipid. Then it enters the endoplasmatic reticulum (ER). Inside the ER the A-subunit of CT dissolves from the B pentamer. The A subunit enters the cytosol where it causes fluid loss by activating adenyly cyclase. Adenyl cyclase induces massive cAMP production which initiates chloride secretion and water movement in the intestine, thus causing the loss of fluid. 95% of O1, O139 strains test positive for cholera toxin (CT) while 95% of the non-O1, non-O139 test negative for CT [7]. Strains of *V. cholerae* that do not produce CT can still produce toxic secretes containing hemolysins, metalloproteases etc., which are completely diverse from CT produced by toxigenic *V. cholerae* O1 and O139 strains [9]. Studies have shown that hemolysin might be the cause of gastroenteritis when it comes to non-O1 *V. cholerae* [10].

The toxin hemolysin A (hlyA) is a water-soluble pore-forming toxin produced by *V. cholerae* and causes lysis of red blood cells (RBC) [11]. The toxin is commonly called the El Tor hemolysin and was initially isolated by Honda and Finkelstein (1979) [12]. Different strains express a range of different hlyA toxins, some virulent and some non-virulent [13].

The serogroups O139 and non-O1, non-O139 are encapsulated whereas O1 strains have no capsule. This makes it easier for non-O1, non-O139 *V. cholerae* to survive in the human body during bactericidal activity. The capsule works as a shield against the defense mechanisms within the host. This causes non-O1, non-O139 to be responsible for more extraintestinal diseases than serogroup O1 [12]. Strains of non-O1, non-O139 *V. cholerae* can cause oedema, vomiting, diarrhea and also contribute to necrosis [14].

Other virulence factors are PrtV and Hap. PrtV is a metalloprotease, secreted into the culture supernatant of *V. cholerae*. The bacterial protease appears to have cytotoxic effects which lead to cell death [15]. The PrtV protease damages important elements in the blood plasma [16]. Fibrinogen, fibronectin and plasminogen are some of the elements degraded by the protease [16]. Most enteric pathogens need to overcome the protective barrier, which cover the intestinal epithelium, in order to cause disease. The *hap* gene in *V. cholerae* encodes a hemagglutinating zinc-dependent metalloprotease (HA/P). HA/P can activate cholera toxin and El Tor cytolsin and hemolysin by proteolysis [17]. It also hydrolyzes proteins such as fibronectin, mucin and lactoferrin, making it a potential virulence factor [18].

Bacteria have surface or envelop proteins that are able to agglutinate human or animal RBC and bind to its N-acetylmuramic acid. The RBC will form a type of lattice in this case. The agglutination of RBC can be seen as clumping of large particles [19].

The serum of humans and most animals have the ability to kill gram-negative bacteria for protection. The serum uses three ways of killing the bacteria through complement-mediated pathways: the classical, the alternative and the lectin pathway. All three pathways lead to the lysis of the bacteria by a membrane attack complex (MAC) [21]. Only gram-negative bacteria are able to form MAC. Capsules, LPS and outer-membrane proteins on the cell surface grant the bacteria resistance against the lethal effects of complement-mediated serum killing [22].

In this study we use various techniques to analyze virulence factors and serum resistance in six different strains of *V. cholerae*: L1, L4, C6706, K1, USS and V5.
<table>
<thead>
<tr>
<th>V. cholerae strains</th>
<th>Genotype</th>
<th>Hemagglutinating activity</th>
<th>Hemolytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ctx</td>
<td>hlyA</td>
<td>hap</td>
</tr>
<tr>
<td>C6706</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L1</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L4</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KI</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>USS</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V5</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

TABLE 1: Overview of the different V. cholerae strains. Our definition of the levels of hemagglutinating activity can be seen in figure 1. The hemolytic activity with horse RBC was performed with undiluted bacterial supernatant.

Materials and methods

Six different V. cholerae strains of different serogroups were obtained from Mitesh Dongre at the Department of Molecular Biology, Umeå University, Umeå, Sweden. The different strains were C6706, L1, L4, USS, KI, and V5.

Bacterial growth. Strains were streaked on Luria agar plates and single colonies were then inoculated in Luria broth and incubated at 37°C or 30°C overnight. The bacterial cultures were measured with optical density (OD)(Ultrospec 10, Amersham Biosciences) to determine the concentration of the bacteria. The concentrations of the different strains varied between $2.64 \times 10^8$ – $3.28 \times 10^9$ cells per ml.

Hemagglutination test. Human blood was used for the hemagglutination test. The samples obtained were already prepared; the serum removed and red blood cells (RBC) diluted to 50% (v/v) in PBS, pH 7.4. The RBC were diluted once more to 8% for use in the hemagglutination test. The bacterial cultures were concentrated to $10^{10}$ cells per ml. Five microliters of RBC and 5 μl of the bacterial suspension were mixed on a glass slide with a loop. The slides were then swirled by hand to further mix the samples. Agglutination could in some cases be seen with the naked eye after just a few seconds. The hemagglutinating activity was estimated in three levels; +, ++ and ++++, from low to high activity.

Hemolytic assay. Human RBC and horse RBC were used for the hemolysis test. The human RBC were already prepared as above. The horse RBC were prepared by centrifuging 1 ml blood at 3 250 rpm for 5 min and then removing the supernatant. After that, 1 ml of PBS, pH 7.4, was added. This process was repeated four times and thereby diluting the serum. After the fourth centrifugation, the horse RBC were diluted to 50% with PBS and stored at 4°C in 100 μl aliquots. Both the human and the horse RBC were then further diluted to 8% in PBS with 0.1% gelatin (v/v). After incubating 1 ml of overnight bacterial cultures at 37°C, they were centrifuged in 14 000 rpm for 5 min. From the supernatant, 900 μl was removed and centrifuged once more in the same conditions. A final amount of 800 μl was transferred to a new tube and was used for the hemolysis assay. Seven wells were filled with 100 μl PBS each in a V-bottomed 96-well assay plate (Falcon) and 200 μl of the supernatant was added to a separate well. By taking 100 μl from the well with the supernatant and then add it to the first well with PBS a dilution of two times was obtained. This was repeated with the following wells, always taking from the previous and adding to the next, until the last well. Since the last well then contained 200 μl, 100 μl was discarded. This resulted in eight wells with 1, 2, 4, 8, 16, 32, 64 and 128 times dilution of the supernatant. Of the RBC, 100 μl was added to each well and was thoroughly mixed. The plate was then incubated for 1h at 37°C. Two controls were also made, one with 100 μl blood and 100 μl PBS (representing 0% hemolysis), and one with 100 μl RBC and 100 μl 0.01% (v/v) Triton-X100 (representing 100% hemolysis). After incubation, the plate was centrifuged at 1800×g for 10 min in a swinging bucket centrifuge. From each supernatant, 90 μl was transferred to the wells of a flat bottomed 96-well assay plate (Falcon) and the absorbance of the released hemoglobin was measured at 540 nm to quantify the hemolytic activity.

Colonies of the different strains were streaked on blood agar plates and these were incubated overnight. The hemolytic activity was determined by measuring the clear halo that had formed around the colonies. The activity was classified as + (1-2
mm), ++ (3-5 mm) and +++ (6-10 mm).

**Isolation of genomic DNA.** One milliliter of each overnight culture was put in Eppendorf tubes and centrifuged at 14 000 rpm for 5 min. Then the supernatant was removed and the pellet was kept in -20°C overnight. The pellet was then dissolved in 567 μl of TE-buffer (10mM Tris HCL, 1 mM EDTA) and 30 μl of SDS was added to lyse the cells. This was thoroughly mixed and then incubated at 37°C for 1.5h. To prevent CTAB from binding to the nucleic acids and form a precipitate, 100 μl of 5 M NaCl was added to each tube. To this, 80 μl of CTAB was added, and the solution was then mixed and incubated for 10 min at 65°C with further mixing 5 min into the incubation. After incubation, 700 μl of chloroform/isoamyl alcohol was added and mixed thoroughly. The solutions were then centrifuged at 14 000 rpm for 5 min. This caused the chloroform phase of the solution to go to the bottom of the tube. The alcohol phase containing the DNA was transferred to a new Eppendorf tube and once again centrifuged at 14 000 rpm for 5 min. The supernatant was transferred to a new tube. To precipitate the nucleic acids, 600 μl of isopropanol was added. The precipitated DNA was pulled up against the wall of the Eppendorf tube and the alcohol was removed. The DNA was washed a total of three times in 70% ethanol. After washing, the ethanol was removed, the DNA redissolved in 100 μl of TE-buffer and stored at -20°C.

**PCR assay.** Gene expressions of ctx, hlyA, prtV and hapA were examined using polymerase chain reaction (PCR). The DNA that was analyzed was both the genomic DNA (see previous section) and DNA aquired by a technique that will be described here. One colony from each strain was resuspended into 50 μl of MQ-water and then boiled for 10 min. After boiling, the samples were centrifuged in 14 000 rpm for 5 min. For the PCR-mixture, a master mix was made in a 1.5 ml Eppendorf tube. 1×Dream Taq buffer, 0.4 mM dNTPs, 10 μM of each primer and 0.25 units/reaction of Taq polymerase. From the master mix, 20 μl was transferred to 0.5 ml PCR tubes with 5 μl of bacterial DNA. These were spun quickly and then kept on ice. The PCR machine (Mastercycler Personal, Eppendorf) was loaded with the samples and the cycle (95°C for 30 sec, 56°C for 30 sec, 72°C for 1 min) was repeated 29 times followed by a final extension at 72°C for 10 min. After cooling the samples (4°C), 5 μl of each was loaded into a 0.7% agarose gel together with 1 μl of 6×DNA Loading Dye (Fermentas). As a reference, Gene Ruler 1 kb DNA Ladder Plus (Fermentas) was used. Electrophoresis was carried out at 120 V for 30 min. The gels were then stained in ethidium bro-mide for 10-15 min and analysed under UV-light.

**Protease assay.** The bacterial strains were inoculated into 10 ml of tryptic soy broth (TSB) media and incubated overnight at 37°C. Cell density was measured with OD (Ultrospec 10, Amersham Biosciences). One milliliter of each culture was transferred to an Eppendorf tube and centrifuged at 14 000 rpm for 5 min. Of the supernatant, 100 μl was mixed with 100 μl of azocasein solution (5 mg/ml azocasein in 100 mM Tris, pH 8.0). The solution was vortexed and then incubated at 37°C for 1 h. After incubation, 400 μl of protein precipitant (TCA, 10%) was added to stop the assay [8] and the solution was then centrifuged at 14 000 rpm for 10 min. Fresh Eppendorf tubes were prepared with 700 μl of 0.525 mM NaOH to enhance the coloration and the supernatant from the centrifugation was added to these tubes to obtain a final volume of 1300 μl. To quantify the protease activity, the solutions were
measured for absorbance at 442 nm. A blank, which contained all of the above, except the bacterial supernatant, was used as a reference. V5 was used as a positive control since it is known to produce proteases [23]. A mistake was made when V5 was prepared for the protease assay. Instead of adding 400 μl TCA, 400 μl PBS was added. This caused an unwanted dilution of the sample. 400 μl TCA was still added, but this caused the absorption of the V5 sample to be lower than it would probably have been if the additional dilution would not have occurred.

Serum resistance. One colony from each strain, C6706, L1, L4, KI, USS and V5, was inoculated in 5 ml LB at 37°C overnight. Six falcon tubes were filled with 2.5 ml of LB and 50 μl bacterial solution from each strain. The falcon tubes were incubated and shaken at 37°C for 4 hours. A total of 30 ml blood was obtained from three volunteers. The blood was put on ice for 1 hour to clot. The coagulated blood was centrifuged at 1600×g at 4°C for 10 minutes. The supernatant, the blood serum, was transferred and put into a falcon tube. Then 800 μl blood serum was transferred into Eppendorf tubes and put into -80°C. Four different chelator solutions (50mM EDTA; 50mM EDTA, 0.005mM MgCl$_2^{+}$; 50mM EGTA, 0.005mM MgCl$_2^{+}$ and 50mM EGTA, 0.005mM MgCl$_2^{+}$, 0.0125mM CaCl$_2^{2+}$) were prepared in 4 different falcon tubes. Twenty microliters of each buffer was added into six Eppendorf tubes and mixed with 30 μl of blood serum. The Eppendorf tubes were incubated for 1 hour at room temperature. After four hours the incubated bacterial OD was measured and 10$^9$ cells of each strain were put into Eppendorf tubes and then centrifuged at 14 000 rpm for 2 minutes. The supernatant was discarded and then 1 ml of PBS was added. The procedure was repeated twice, and after the last centrifugation the supernatant was discarded and 500 μl PBS was added. The bacteria were diluted to ∼2 × 10$^7$ by mixing 10 μl of bacterial solution with 990 μl PBS. A solution of 60% blood serum was prepared by mixing 800 μl of blood serum with 533 μl PBS. The serum solution was added into 14 different Eppendorf tubes which of 7 were put into 60°C for 30 minutes to inactivate the serum. Then 50 μl of each bacterial solution was added to 6 tubes (one for each buffer (4); one for the active serum and one for the inactive serum). All tubes were incubated at 37°C for 1 hour. Then 900 μl of PBS and 11 μl resazurin were added to each tube. One hundred microliters from each Eppendorf tube was put into 3 wells on two 96-well plates and were then incubated for 1 hour at 37°C. The fluorescence was measured at 530 nm emission and 590 nm excitation.

The same bacterial solutions used for the fluorescence measurement in the serum resistance assay were also used for viable count. Ten microliters from each strain (with both active and inactive serum) were diluted in 990 μl PBS to yield a concentration of 10$^3$ bacteria per ml. One hundred microliters was then transferred to another Eppendorf tube and diluted in 900 μl PBS to yield a concentration of 10$^4$ bacteria per ml. One hundred microliters from each dilution were spread on luria agar plates. The plates were then incubated at 37°C overnight and colony forming units (CFU) were determined the next morning.
Results

In table 1 is an overview of the different strains and their characteristics. It is already known that C6706 belong to the O1 serogroup [24]. From the PCR using genomic DNA as template, it was concluded that C6706 and USS were the only strains that expressed the ctx gene. Furthermore, all strains expressed hlyA, prtV and hap genes (figure 4).

When streaked on blood agar plates, all selected cultures showed positive hemolysis. Further, hemolytic assay with horse RBC showed the presence of secreted hemolysin in the free culture supernatant of all selected strains. However, the hemolysin was detected only at the higher supernatant concentrations (figure 2a). On the other hand, none of the culture supernatants showed any detectable hemolysis with either rabbit or human RBC (figure 2c, 2b).

When tested for proteolytic degradation of azocasein, cell free culture supernatant of all the selected strains showed the presence of protease(s) (figure 5). However, the results for strain V5 i ambiguous due to an experimental error.

All selected strains showed varying levels of hemagglutination as summarized in table 1. Hemagglutination results were compared with the reference slides as shown in figure 1.

Figure 3a shows the survival of the different bacteria in active blood serum. Inactive serum was used as a negative control as the highest survival rate was found in the inactive serum. The figure shows that 87% of L1, 51% of L4, 1% of C6706, 97% of KI, 0% of USS and 58% of V5 survived in the active blood serum. The effect of the chelator solutions are shown in figure 3b.

A viable count was also performed which showed no difference between active and inactivated serum (data not shown).

Discussion

The PCR analysis of selected strains for the known virulence factor genes namely ctxA, hapA, prtV and hlyA showed that none of the strains except the two O1 strains C6706 and USS contain the ctxA gene, further corroborating the fact that all other strains belong to non-O1, non-O139 serogroups. Furthermore, all strains showed the presence of metallo-protease genes i.e. hapA and prtV, and the major hemolysin gene hlyA.

Earlier researches have established the hemagglutination, hemolytic and serum resistance properties of V. cholerae when tested against blood obtained from various sources, including humans. We tested all the selected strains for bacterial hemagglutination of the human RBCs. All the strains showed varying levels of hemagglutination. It has been reported that V. cholerae can also produce and secrete active hemagglutinins and hemolysins as well. A study by Iyer, et al. (2000), demonstrated that 100% of the examined non-O1, non-O139 strains lysed both rabbit and human RBC. We tested the cell free supernatant of all the selected strains for hemolysis of horse, rabbit and human RBCs. The hemolysis assay with horse RBC showed a concentrations yielded higher hemolytic activity which decreased upon dilution of the supernatant (figure 2a). However, we observed almost negligible hemolysis
FIGURE 4: (4a) The genomic DNA from the different strains and the expression of (4b) ctx and (4c) hlyA genes. In (4b) there seems to be insufficient staining. (4a) contains only genomic DNA. (4b) contains DNA isolated by the other method described (see PCR assay in materials and methods), except for C6706* which is genomic DNA. (4c) contains only genomic DNA. In (4d) the expressions of hap, prtV and hlyA have been investigated. All of the wells contain genomic DNA, and the wells marked V5* contain the genomic DNA from V5 that was obtained from Mitesh Dongre, Department of Molecular Biology, Umeå, Sweden. The Gene Ruler marks the following sizes (from top to bottom): 20kb, 10kb, 7kb, 5kb, 4kb, 3kb, 2kb, 1kb, 700b, 500b, 400b, 300b, 200b, 75b.
with the rabbit and human RBCs even at higher supernatant concentration (figure 2c, 2b). We argued this problem could be due to a non-responsive blood since we obtained blood from a single source instead of pooling it from different donors, or could be due to very low hemolysin production by the tested bacterial samples. Nevertheless, since even the reference O1 strains C6706 and USS did not show any significant activity with the human and rabbit RBCs, a possible experimental error cannot be ruled out.

In the proteolytic activity test the C6706 strain was used as reference since it is known to produce hemagglutinin as well as PrtV proteases [15]. A dilution error when preparing V5 rendered it inadequate for drawing any conclusions. All other strains showed proteolytic activity. Previous studies indicate that this ability is essential for killing organisms and bacterial survival [24].

When only EDTA was added, all complement mediated pathways were inhibited but the survival rate was lowered. This was unexpected since no bacteriolytic activity should have occurred. Serum resistance could not explain why the rate of survival was highest when all pathways were active and lowered when inhibited. When EDTA and EGTA were saturated with Mg\(^{2+}\), the alternative pathway was active and all strains showed resistance. When EGTA, Mg\(^{2+}\) and Ca\(^{2+}\) were added, the bacteria showed a rate of survival similar to figure 3a. This was expected since the conditions were the same and all pathways were active. USS and C6706 showed a low rate of survival which indicated that they did not bestow resistance to the classical pathway. In a study by Li, et. al (2008), where bacterial activity in humoral fluids were investigated, EDTA increased the survival rate by 42 percentage points while the other chelator solutions did not increase survival. If we put the data from EDTA aside, the result showed that all strains displayed resistance against the alternative pathway. L1, L4, K1 and V5 were resistant to the classical pathway while C6706 and V5 were not.

Since the non-O1, non-O139 bacteria can survive in the blood serum, the blood might be a possible invasive pathway for those bacteria. No positive control with 100% survival of bacteria was used. A positive control might have yielded a better result. The survival rate of KI was decreased by all chelator solutions, which is strange as the chelator solutions should inhibit the complement and increase survival rate.

The result from the viable count was omitted because there were too many colonies to count.

More replicates of every assay should be performed to ensure the quality of the experimental methods and the following results.

**Proposed future studies.** In the serum resistance assay only EDTA should inhibit lysing of the bacteria, but it did not. There is a possibility that it actually inhibited the lysing of cells and also inhibited the resazurin coloring of the living bacteria. A proposed future study would be to further investigate the effect of EDTA on strains of *V. cholerae* to see if it inhibits resazurin coloring or not.

**Acknowledgements**

Thanks to Mitesh Dongre, Sun Nyunt Wai and Barbro Lindmark for their support and for proof-reading our report.
References


