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# Detection of virulence associated genes, haemolysin and protease amongst *Vibrio cholerae* isolated in Malaysia

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## SUMMARY

Eighty-four strains of *Vibrio cholerae* O1, O139 and non-O1/non-O139 from clinical and environmental sources were investigated for the presence of the toxin co-regulated pilus gene, *tcpA*, the virulence cassette genes *ctxA*, *zot*, *ace* and *cep* and also for their ability to elaborate haemolysin and protease. The *ctxA* and *zot* genes were detected using DNA–DNA hybridization while the *ace*, *cep* and *tcpA* genes were detected using PCR. Production of haemolysin and protease was detected using mammalian erythrocytes and an agar diffusion assay respectively. Analysis of their virulence profiles showed six different groups designated Type I to Type VI and the major distinguishing factor among these profiles was in the *in vitro* production of haemolysin and/or protease. Clinical O1, O139 and environmental O1 strains were similar with regard to presence of the virulence cassette genes. All environmental O1 strains with the exception of one were found to possess *ctxA*, *zot* and *ace* giving rise to the probability that these strains may actually be of clinical origin. One strain which had only *cep* but none of the toxin genes may be a true environmental isolate. The virulence cassette and colonization factor genes were absent in all non-O1/non-O139 environmental strains but production of both the haemolysin and protease was present, indicating that these may be putative virulence factors. These findings suggest that with regard to its pathogenic potential, only strains of the O1 and O139 serogroup that possess the *tcpA* gene which encodes the phage receptor, have the potential to acquire the CTX genetic element and become choleragenic.

## INTRODUCTION

Epidemics of cholera have been caused by *Vibrio cholerae* serogroup O1 of the classical and El Tor biotypes and more recently by serogroup O139 [1]. Other *V. cholerae* serogroups (O2–O138) have been associated with sporadic cases of gastroenteritis and extraintestinal infections in man [2]. The major virulence factor, cholera toxin (CT), produced by the O1 and O139 serogroups is responsible for the pathogenesis of secretory diarrhoea. In addition, a 20·5 kDa pilin subunit TcpA of the toxin co-regulated pilus (TCP), has been described to be essential for the successful colonization of the intestinal epithelium by *V. cholerae* [3].

On a unique 4·5 kb region of the *V. cholerae* genome termed the 'virulence cassette' in association with the CT gene *ctxAB*, other genes encoding for factors such as the zonula occludens toxin (Zot) [4], acessory cholera enterotoxin (Ace) [5] and the core-encoded pilus (Cep) [6] which is a putative colonization factor, have recently been identified in toxigenic *V. cholerae* O1. However, the role of these factors in the pathogenesis of cholera is not clearly understood [4, 5, 7]. Yet another significant advancement in cholera pathogenesis is the recent finding that the *V. cholerae* virulence cassette or CTX element corresponds to the genome of CTXΦ, a

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lysogenic filamentous bacteriophage which is involved in a novel mechanism of horizontal transfer of virulence genes [8]. Thus, this provides a basis for the emergence of new clones of toxigenic *V. cholerae* strains.

Other putative virulence factors identified to date include the haemolysin and protease. The haemolysin of El Tor strains, a 65 kDa cytolysin encoded by the *hlyA* gene, is considered to damage target cells by generating pores in their membranes and cause the production of blood and mucous in addition to fluid accumulation in ligated rabbit ileal loops [9, 10]. However, vaccine strains with deleted *hlyA* genes were still found to induce diarrhoea in volunteers, indicating that the haemolysin/cytolysin may not play a role in causing diarrhoea [11].

The *V. cholerae* soluble haemagglutinin/protease (HA/protease), a 32 kDa metalloenzyme has been demonstrated to be capable of 'nicking' the cholera toxin and has a 'detachase' activity enabling the detachment of vibrios from cultured human intestinal epithelial cells and thus may play a role in environmental transmission [12, 13].

Although many putative virulence factors have been described, the knowledge of the presence and distribution of these factors amongst the different serogroups of *V. cholerae* is necessary for understanding the role of each in the pathogenesis of cholera and to track the emergence of new choleragenic strains. This study reports on the distribution of the virulence cassette genes, *tcpA*, and the elaboration of haemolysin and protease amongst *Vibrio cholerae* belonging to different serogroups isolated in Malaysia between 1992 and 1993.

## METHODS

### Bacterial strains

Eighty-four strains of *Vibrio cholerae* from clinical and environmental sources were used in this study of which 42 were kindly provided by the Department of Medical Microbiology, University Hospital, 23 by the Institute of Medical Research, Kuala Lumpur and 19 by the Department of Medical Microbiology, Universiti Sains Malaysia. Of these, 54 were serogroup O1, 25 serogroup O139 and 5 belonged to serogroup non-O1/non-O139. The clinical strains were isolated from stools of diarrhoeal patients while the environmental strains were isolated from water and soil during a cholera epidemic. *V. cholerae* 569B (O1 Classical, Inaba, Benares Hindu University, India), *V. cholerae* S154 (O1 El Tor, Ogawa, Benares Hindu University, India) and *V. cholerae* M-O45 (O139, Kyoto University, Japan) were used as positive controls. Clinical strains comprising *Pseudomonas aeruginosa* 839, enterotoxigenic *E. coli* 8031A, *Klebsiella pneumoniae* 189 (University Hospital, Kuala Lumpur) and *Burkholderia cepacia* EY425 (Osaka City University, Japan) were used as negative controls in the various assays.

### DNA–DNA colony hybridization

The *ctxA* and *zot* genes were detected in a DNA–DNA colony hybridization assay using non-radioactive digoxigenin- (DIG) labelled DNA probes according to the manufacturer's instructions (DIG-DNA labelling and detection kit; Boehringer–Mannheim, Germany). The CT–DNA probe consisted of a 554 base-pair (bp) *Eco*RI fragment of *ctxA*, encoding for the CT subunit A, derived from recombinant *E. coli* HB101 containing plasmid pKTN901 (Kyoto University, Japan), while the Zot–DNA probe consisted of a 575 bp *Stu*I–*Acc*I fragment of *zot* derived from recombinant *E. coli* HB101 containing plasmid pBB24 (University of Maryland, USA). Colony blots of all strains were prepared on Hybond-N nylon membranes (Amersham International, USA) as described by Said and colleagues [14]. The colonies were then lysed and the DNA denatured *in situ* as described by Sambrook and colleagues [15]. Prehybridization, hybridization with the specific denatured digoxigenin-labelled (DIG) DNA probes (15 ng/ml) and detection of the bound labelled DNA probe was performed according to the manufacturer's protocols (Boehringer–Mannheim, Germany).

### Polymerase chain reaction (PCR)

All template DNA were prepared as described by Fields and colleagues [16] for PCR to detect the presence of the *tcpA*, *ace* and *cep* genes. Primer pairs used were as follows: CTCP I (5′-CACGATAAGAAAACCGGTCAAGAG-3′)/CTCP II (5′-ACCAAATGCAACGCCGAATGGAGC-3′) which amplify a 617 bp fragment of classical *tcpA* and ETCP I (5′-GAAGAAGTTTGTAAAAGAAGAACA-3′)/ETCP II (5′-GAAAGGACCTTCTTTCACGTT-3′) which amplify a 471 bp fragment of El Tor *tcpA* [17]. For the amplification of the 284 bp fragment of *ace*, the primers were ACE I (5′-GCTTATGATGGACACCCTTTA-3′) and ACE II (5′-GTTTA-

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ACGCTCGCAGGGCAAA-3′), described previously by Colombo and colleagues [18]. For the amplification of the 246 bp fragment of *cep*, the primer pairs were CEP I (5′-ATGTTTAGCTCACTGAAAAAC-3′) and CEP II (5′-TTTAGCCTTACGAATTAA-GCC-3′), synthesized based on the published gene sequence [6].

The *tcpA* genes were detected using a multiplex PCR assay described by Keasler and Hall [17]. The reaction mixtures were prepared in 50 $\mu$l volumes. Reagents and cycling conditions were as described by Fields and colleagues [16]. For the amplification of *ace* and *cep*, each reaction mixture consisted of the following reagents in their final concentrations: 2 $\mu$l of template DNA, 1 × *Taq* DNA polymerase buffer, 25 pmol of each primer, 0·2 mM of each, dATP, dCTP, dGTP and dTTP, 1·5 units of *Taq* DNA polymerase (Promega, USA), 1·5 mM $MgCl_2$ and sterile distilled water. The cycling conditions comprised of a pre-incubation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for *ace* and 50 °C for *cep* for 1 min and extension at 72 °C for 1 min. A final extension at 72 °C for 5 min was included for each PCR. The PCR products were electrophoresed on a 1·2% agarose gel. A reaction mixture containing all other components except template DNA was included as a negative control for each amplification. Recombinant plasmid DNA pCVD630 (University of Maryland, USA) containing the 290 bp *ace* and 249 bp *cep* genes was extracted from *E. coli* DHα and used as the positive control in the PCR for *ace* and *cep*.

### $GM_1$–enzyme linked immunosorbent assay

The $GM_1$–enzyme linked immunosorbent assay ($GM_1$–ELISA) was used for the detection of CT [19]. Broth cultures of all strains were prepared in AKI medium as described by Iwanaga and colleagues [20] and culture supernatants were harvested by centrifugation at 12000 ***g*** for 15 min. Wells of microtitre plates (Nunc, Denmark) coated with monosialoganglioside $GM_1$ were washed with phosphate buffered saline (PBS), blocked with 0·1% bovine serum albumin in PBS at 37 °C for 30 min and washed once with PBS, after which 100 $\mu$l of each culture supernatant was added to duplicate wells and incubated at room temperature for 2 h. The presence of CT was then detected by the sequential addition and incubation at room temperature for 90 min with anti-LT monoclonal antibody (Mab 1:3) (1:100 dilution, University of Goteborg, Sweden), goat anti-mouse Ig-HRP conjugate (1:4000 dilution, Bio-Rad, USA) and finally incubated in the dark for 10 min with the enzyme substrate orthophenyl diamine (OPD Sigma, USA). A positive result for CT was determined as an absorbance value greater than or equivalent to 0·1 above the mean background value at 490 nm [19]. Purified cholera toxin (1 $\mu$g/ml, Sigma, USA) was used as the positive control while culture supernatant of *E. coli* 8031A ($ST^+$) was used as the negative control.

### Detection of haemolysin

The haemolytic activity of *V. cholerae* strains was determined by a modified microtitre plate assay method [21]. Briefly, strains were inoculated into 3 ml of brain heart infusion broth containing 1% (v/v) glycerol (BHIGB) [22], and incubated at 37 °C for 24 h with agitation. One hundred microlitres of each broth culture and an equal volume of 1% rabbit, sheep or human group-O erythrocyte suspension in Alsever's solution was then added to duplicate wells of the microtitre plates. The plates were incubated at 37 °C for 1 h followed by further incubation at 4 °C for 18 h. Standards containing varying concentrations of lysed erythrocytes ranging from 0 to 100% haemolysis were used as controls. A cut-off of $\geqslant$ 50% haemolysis was used to indicate positive haemolytic activity.

### Detection of protease

Protease activity was detected using single-diffusion on a 0·75% (w/v) agar gel containing 1·5% (w/v) skimmed milk as the substrate [23]. Wells 4 mm in diameter were punched (6 wells/plate) on the agar plates. Culture supernatants from tryptic soy broth (TSB) cultures grown at 30 °C for 20 h were harvested by centrifugation at 40000 ***g*** for 20 min at 20 °C, after which 10 $\mu$l of each supernatant was added to the wells and incubated at 37 °C. The diameters of zones of clearing around the wells indicative of protease activity due to hydrolysis of casein was measured at 24 h post-incubation. Strains producing zone diameters of $\leqslant$ 8 mm, > 8 mm $\leqslant$ 12 mm or > 12 mm were categorized as low, average or high protease producers respectively.

## RESULTS

The virulence cassette genes comprising *ctxA*, *zot*, *ace* and *cep* as well as the El Tor *tcpA* were found to be

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Table 1. *Distribution of virulence cassette and* tcpA *genes*

| | Virulence cassette genes | | | | Toxin co-regulated pilus gene | | |
|---|---|---|---|---|---|---|---|
| Source and serogroup | *ctxA* | *zot* | *ace* | *cep* | Classical *tcpA* | El Tor *tcpA* | No. (%) |
| Clinical O1 ($n = 33$) | + | + | + | + | − | + | 33 (100) |
| Environmental O1 ($n = 21$) | + | + | + | + | − | + | 20 (95·2) |
| | − | − | − | + | − | + | 1 (4·8) |
| Clinical O139 ($n = 25$) | + | + | + | + | − | + | 25 (100) |
| Environmental non-O1/non-O139 ($n = 5$) | − | − | − | − | − | − | 5 (100) |

Table 2. *Haemolytic activity of* V. cholerae

| | No. (%) of haemolytic *V. cholerae* strains | | | | |
|---|---|---|---|---|---|
| | Clinical | | Environmental | | |
| Erythrocyte | O1 ($n = 33$) | O139 ($n = 25$) | O1 ($n = 21$) | non-O1/ non-O139 ($n = 5$) | Total (%) ($n = 84$) |
| Rabbit | 30 (90·9) | 24 (96·0) | 10 (47·6) | 5 (100) | 69 (82·1) |
| Human group-O | 27 (81·8) | 24 (96·0) | 8 (38·1) | 5 (100) | 64 (76·2) |
| Sheep | 8 (24·2) | 0 (0) | 2 (14·3) | 3 (60·0) | 14 (16·7) |

present in all the 33 clinical and 20 of the 21 environmental O1 strains (Table 1). One environmental O1 strain was found to possess only the *cep* and El Tor *tcpA* genes. All 25 of the O139 strains were found to be similar to the clinical O1 strains in possessing the virulence cassette as well as the El Tor *tcpA* gene. The five environmental non-O1/non-O139 strains were negative for all the above genes. Of the 78 *ctxA* positive strains, 77 elaborated CT, a 98·7% agreement between the DNA–DNA hybridization assay and the $GM_1$–ELISA [95% confidence interval (CI): 97–100%]. One O1 strain of clinical origin was CT-negative. The non-O1/non-O139 strains were negative for both *ctxA* and CT production.

Production of haemolysin, where at least one type of erythrocyte was haemolysed was found amongst 69 (82·1%) strains (Table 2). Fifteen strains were non-haemolytic and these consisted of 3 clinical O1, 1 clinical O139 and 11 environmental O1 strains. Detection of haemolysin was best using rabbit RBCs (82·1%) followed by human group-O RBCs (76·2%). Production of the El Tor haemolysin as detected using sheep erythrocytes was found only amongst 14 (16·7%) strains. Among the O139 strains, 96% were haemolytic using rabbit and human RBCs but none haemolysed sheep erythrocytes. Less than 50% of environmental O1 strains produced haemolysin in comparison to the clinical strains although strains from both sources haemolysed the erythrocytes in a similar order of rabbit > human group-O > sheep. All five environmental non-O1/non-O139 strains haemolysed rabbit and human group-O RBCs and three of these strains also produced an El Tor-like haemolysin against sheep erythrocytes.

Production of extracellular protease was observed amongst 76 (90·5%) strains, of which 42 (55·3%) were high producers, 15 (19·7%) were average and 19 (25·0%) were low producers (Table 3). The 8 protease negative strains consisted of 2 (6·1%) clinical and 6 (28·6%) environmental O1 strains. High protease production was found amongst 54·5% of the clinical O1 strains while the environmental O1 strains consisted mainly of low protease producers (38·1%). Furthermore, among the environmental O1 strains a higher percentage (28·6%) were protease-negative as compared to only 6·1% among clinical O1 strains.

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Table 3. *Protease production by* V. cholerae

| Source and serogroup | No. (%) strains producing protease L* | A† | H‡ | N§ | Total |
|---|---|---|---|---|---|
| Clinical O1 | 9 (27·3) | 4 (12·1) | 18 (54·5) | 2 (6·1) | 33 |
| Environmental O1 | 8 (38·1) | 3 (14·3) | 4 (19·0) | 6 (28·6) | 21 |
| Clinical O139 | 1 (4·0) | 6 (24·0) | 18 (72·0) | 0 (0) | 25 |
| Environmental non-O1/non-O139 | 1 (20·0) | 2 (40·0) | 2 (40·0) | 0 (0) | 5 |
| Total (%) | 19 (22·6) | 15 (17·9) | 42 (50·0) | 8 (9·5) | 84 (100) |

* L, low producers, † A, average producers, ‡ H, high producers, § N, protease negative.

Table 4. *Composite virulence profile of the different* V. cholerae *serogroups*

| Type | Virulence profile*: Virulence cassette genes/*tcpA*/CT | Haemolysin and protease | No. (%) of strains: Clinical O1 | Clinical O139 | Environmental O1 | Environmental Non-O1/ Non-O139 |
|---|---|---|---|---|---|---|
| I | Czac⁺/C*tcpA*⁻/E*tcpA*⁺/CT⁺ | Hly⁺/Prot⁺ | 27 (81·8) | 24 (96·0) | 4 (19·0) | – |
| II | Czac⁺/C*tcpA*⁻/E*tcpA*⁺/CT⁺ | Hly⁺/Prot⁻ | 2 (6·1) | – | 6 (28·6) | – |
| III | Czac⁺/C*tcpA*⁻/E*tcpA*⁺/CT⁺ | Hly⁻/Prot⁺ | 3 (9·1) | 1 (4·0) | 10 (47·6) | – |
| IV | Czac⁺/C*tcpA*⁻/E*tcpA*⁺/CT⁻ | Hly⁺/Prot⁺ | 1 (3·0) | – | – | – |
| V | Cza⁻c⁺/C*tcpA*⁻/E*tcpA*⁺/CT⁻ | Hly⁻/Prot⁺ | – | – | 1 (4·8) | – |
| VI | Czac⁻/C*tcpA*⁻/E*tcpA*⁻/CT⁻ | Hly⁺/Prot⁺ | – | – | – | 5 (100·0) |
| | Total | | 33 | 25 | 21 | 5 |

* Presence (+) or absence (–) of the listed virulence factor; Czac (virulence cassette genes): C, *ctxA*; z, *zot*; a, *ace*; c, *cep*; C*tcpA*, classical biotype toxin co-regulated pilus gene; *EtcpA*, El Tor biotype toxin co-regulated pilus gene; CT, cholera toxin; Hly, haemolysin, against rabbit erythrocytes; Prot, protease.

The majority (72%) of O139 strains were high producers, similar to the clinical O1 strains. Amongst the 5 environmental non-O1/non-O139 strains 1 was a low producer, 2 were high producers and 2 were average producers of protease.

Based on the distribution of the virulence-associated genes, production of protease and haemolysin against rabbit erythrocytes, the strains were grouped into their composite virulence profile and a comparison amongst the different serogroups and source of isolation was made. Six different profiles, designated Type I to Type VI were encountered (Table 4). The O1 strains of both clinical and environmental origin exhibited four different profiles. The majority of clinical O1 strains (81·8%) belonged to Type I, whereas the majority of environmental O1 strains (47·6%) belonged to Type III. On further analysis, although four different profiles were identified amongst the O1 strains, they differed mainly in the presence or absence of haemolysin and protease. Only one environmental O1 strain belonged to the Type V profile and in addition to being haemolysin-negative, was found to be negative for the toxin genes *ctxA*, *zot* and *ace*. The O139 strains were found to be more homogenous and comprised of only two profiles, with the majority (96%) belonging to Type I, similar to the clinical O1 strains and the remaining 4% belonging to Type III. All five of the non-O1/non-O139 strains belonged to the Type VI profile being consistently negative for the virulence cassette genes, *tcpA* and CT but positive for haemolysin and protease.

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## DISCUSSION

Although CT is the major virulence determinant in the pathogenesis of cholera, *V. cholerae* has been found to produce a variety of other extracellular products that may have deleterious effects on eukaryotic cells. In addition, it is known that strains belonging to non-O1 serogroups have also been associated with cholera-like disease. Furthermore, the recent finding of the CTXΦ phage mechanism [8] in the transfer of virulence genes, increases the potential for the emergence of new toxigenic strains. In this study, we investigated the distribution of *tcpA*, the virulence cassette genes, production of haemolysin and protease among *V. cholerae* O1, O139 and non-O1/non-O139 of clinical and environmental origin to assess the pathogenic potential of these strains.

In relation to the toxin genes of the virulence cassette, the concurrent presence of *zot* and *ace* with *ctxA* was identified in all clinical O1, O139 and all but one environmental O1 strain and none of these genes were present in the environmental non-O1/non-O139 strains. This finding is in agreement with two recent studies which also reported the *ctx* dependent occurrence of *zot* and *ace* among clinical and environmental O1 and O139 strains and their absence among environmental non-O1/non-O139 strains from diverse geographical origins [18, 24]. However, the occurrence of combinations containing natural deletions of *zot*, *ctx*, and/or *ace* among O1 strains has also been reported [24] but such strains were not found in this study. Our findings reaffirm the suggestion that *zot* and *ace* do not exist independently and therefore the Zot and Ace toxins may have a synergistic role with CT in the pathogenesis of cholera [25]. Additionally, the possible role of *zot* in the morphogenesis of the CTXΦ bacteriophage has been reported [8].

The distribution of *cep*, the fourth gene of the virulence cassette was found among all strains that possessed *ctxA*, *zot* and *ace* but its presence was independent of these genes in one environmental strain. The absence of *ctxA*, *zot* and *ace* in this strain may be due to natural deletion of the genes [24]. The *cep* gene which encodes Cep appears to be highly conserved among O1 and O139 strains and absent among non-O1/non-O139 strains. It has been reported that deletion of the *cep* locus reduced colonization in mice but not in humans [7]. The Cep protein is reported to share high amino acid sequence homology not only with the flexible pilin (Fxp) of *Aeromonas hydrophila*, involved in colonization [6] but also with the M13 coliphage virion capsid protein [8]. Therefore, it has been proposed that *cep* encodes the virion capsid protein of CTXΦ and may therefore be a filamentous phage particle rather than a pilus [8]. However, the possibility of Cep as a putative colonization factor among the O1 and O139 serogroups cannot be ruled out altogether and needs to be explored further. To date there are no reports on the distribution of *cep* in association with the other virulence cassette genes and a pilus structure has yet to be identified.

The El Tor *tcpA* gene which encodes the major subunit of the TCP was identified amongst all clinical and environmental O1 and O139 strains studied including the environmental O1 strain negative for *ctxA*, *zot* and *ace*. In addition to its role in colonization, the TCP also acts as a receptor for CTXΦ that carries the CTX genetic element [8]. The presence of *tcpA* among all CT producing O139 strains and even among the non-CT producing environmental O1 strain demonstrates that these strains have the potential to horizontally acquire the CTX genetic element.

An interesting observation in this study was that 20 of the 21 environmental O1 strains demonstrated the presence of the virulence cassette genes, *tcpA* and CT production. This finding is in contrast with two other studies that reported the absence of these genes amongst the majority of environmental O1 strains [18, 24] as well as to that of Johnson and colleagues [25] who reported that environmental O1 strains were generally non-toxigenic. However, Colombo and colleagues [18] and Kurazono and colleagues [24] also reported the presence of toxigenic strains among 25% of their environmental O1 strains studied which confirms that toxigenic strains do exist in the environment. The presence of these genes amongst our environmental strains has to be interpreted with caution due to the non-availability of detailed documentation on the collection of these strains. Based on the information that these strains were isolated from the environment during an outbreak, in all probability they may have been present in the environment due to faecal contamination. Alternatively, it is possible that these were true environmental strains that may have acquired the virulence genes through the phage mechanism of horizontal transfer since they possessed *tcpA*. The identification of one environmental O1 strain in this study that only possessed the colonization factor genes *cep* and *tcpA* demonstrates that

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non-toxigenic O1 strains are naturally present in the environment as reported.

The exact role of the haemolysin in the pathogenesis of cholera is yet to be discerned. In a study of 1154 *V. cholerae* strains from diverse geographical origins Kurazano and colleagues [24] reported that the haemolysin gene *hlyA* was the most conserved genetic element independent of biotypes and serogroups. In the present study, it was found that different erythrocyte species differed in their detection ability and rabbit erythrocytes were the most suitable whereby 82·1% of the strains were haemolytic. The El Tor haemolysin, traditionally used to differentiate the El Tor and classical biotypes, was found only in 18·5% of the O1 strains. This observation is in agreement with findings of Barrett and colleagues [26] that recently isolated El Tor strains are poorly haemolytic against sheep RBCs, suggesting a genetic drift due to selection pressures and adaptation in newer niches.

Although *hlyA* is reported to be highly conserved, the expression of the gene was not consistent among the O1 strains. The lack of expression may be due to either the presence of mutations as a result of repeated subculturing and long term storage or due to these strains lacking the *hlyA* gene naturally. The production of haemolysin by O139 strains has not been widely investigated to date but 96% of our strains were haemolytic being similar in profile to the clinical O1 strains.

The haemolytic nature of the majority (81%) of O1 and O139 strains studied independent of its toxigenicity suggests a possible housekeeping role of the *V. cholerae* haemolysin, the acquisition of iron or in overcoming host defence mechanisms. However, among the non-O1/non-O139 strains the haemolysin may play a more significant role in virulence and pathogenesis since these strains were non-toxigenic and lacked colonization factor genes.

Variable amounts of protease were produced by 90% of the strains which may be due to the enzyme remaining cell-associated or to lower copy numbers of the gene in some strains. The *V. cholerae* protease has been suggested to enhance CT activity *in vivo*, induce degradation or inactivation of host mucosal defence proteins and may also be involved in bacterial colonization or transmission [12, 13, 23, 27]. The O139 and non-toxigenic environmental non-O1/non-O139 strains also produced high amounts of protease similar to the clinical O1 strains. We believe that this is the first report of protease production in O139 strains.

In conclusion, the 84 strains comprised six different composite virulence profiles. The clinical and environmental O1 strains were distributed over four different profiles (Table 4). These profiles were similar with regard to either the presence or absence of the virulence cassette genes (*Czac*), the El Tor toxin coregulated pilus gene (*EtcpA*) and cholera toxin (CT) which are the major virulence determinants in cholera but differed mainly in the production of extracellular haemolysin and/or protease. Only one environmental O1 strain belonging to the Type V profile was found to be naturally non-toxigenic, i.e. lacking all the toxin genes of the virulence cassette and therefore may represent a true environmental O1 strain. It is highly probable that the remaining environmental O1 strains may have been present due to faecal contamination as they were found to be toxigenic. The majority of O139 strains were similar to the Type I profile of the El Tor O1 clinical strains, giving credibility to the suggestion that the O139 serogroup may have evolved from the El Tor O1 serogroup [28]. The environmental non-O1/non-O139 strains comprised a distinctly different profile which consistently lacked the toxin and colonization factor genes. However, these strains produced haemolysin and protease suggesting that these may be putative virulence factors amongst this group since these strains have been associated with cases of diarrhoea, gastroenteritis and a variety of extraintestinal infections in humans [2]. Based on the findings of this study and in light of the phage mechanism of horizontal gene transfer, it may be postulated that only non-toxigenic strains of the O1 and O139 serogroup which possess *tcpA* are likely to acquire the CTX element via infection by CTXΦ.

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