CYTOTOXICITY OF *VIBRIO CHOLERAE* ON MADIN DARBY BOVINE KIDNEY CELLS

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ABSTRACT

The cytotoxicity of cell-free culture filtrates of 31 isolates of *Vibrio cholerae* O1 and O139, 5 reference strains and 26 clinical isolates, was tested on Madin Darby Bovine Kidney (MDBK) cells and Vero cells. The 3-[4,5-dimethylthiazol-2-y]-2, 5-diphenyltetrazolium bromide (MTT) test was used to detect the effect of the filtrates on the proliferation and viability of cultured cell populations. The filtrates were prepared from serial ten-fold dilutions of inoculated AKI and APW broth media with and without the addition of polymyxin B. The APW culture filtrates of both *V. cholerae* O1 and O139 with and without added polymyxin B showed greater toxicity to MDBK cells as compared to AKI filtrates. The cytotoxicity of AKI-grown *V. cholerae* O139 to MDBK cells was greater than that of *V. cholerae* O1 grown in the same medium. The cytotoxicity of APW filtrates on Vero cells was low and only noted when polymyxin was added to the medium.

Keywords: *Vibrio cholerae,* MDBK cells, Vero cells, Cytotoxicity and MTT test.

INTRODUCTION

V. cholerae is well documented as the causative agent of cholera. Its pathogenicity is mainly associated with the ability to produce the cholera enterotoxin (CT) (Faruque *et al.*, 1998). The CT is a product of the *ctx* genes; *ctx*A encodes the A subunit and *ctx*B encodes the B subunit of the toxin (Shangkuan *et al.*, 1995). The *ctx*A and *ctx*B are part of a larger genetic element, on the *V. cholerae* chromosome, that was only recently recognized to be the genome of the filamentous phage *ctx*ϕ. The 'core' region of *ctx*ϕ carries several phage morphogenesis genes as well as the *ctx* genes and is flanked by repeated sequences (RSs) that are necessary for the replication and integration of the phage DNA (Claudia *et al.*, 2000).

V. cholerae has been found to produce a variety of other extracellular products, which may have deleterious effect on eukaryotic cells in addition to the CT. Zona occludens toxin (*zot*) is a toxin that disrupts the tight junction between the mucosal epithelial cells (Fasano *et al.*, 1991). It causes increased ion and water loss. Such losses normally occur through the brush border but the action of this toxin also exposes the lateral sides of the cells and this may increase the efficiency of other virulence factors. The accessory cholera enterotoxin (*ace*) is capable of increasing the potential difference across mounted epithelial cells (Trucksis *et al.*, 1993). The core-encoded pilus (*cep*) is a putative colonization factor (Pearson *et al.*, 1993). However, the role of these factors in the pathogenesis of cholera is not clearly understood. The *zot*, *ace* and *cep* genes are also believed to be encoded on the *ctx*ϕ genome.

Lately, there were frequent reports about renal failure occurring with *V. cholerae* infections (Matthew *et al.*, 1997). Uremia due to acute tubular necrosis was noted in these cases. Furthermore, *V. cholerae* has also been reported to produce Verotoxin (VT), which is analogous to the toxin produced by *Shigella dysenteriae*.

MDBK is an epithelial-like cell line derived from an apparently normal adult steer. It was first demonstrated by SH Madin and NB Darby in 1957 (Nagama *et al.*, 1996). This epithelial line is utilized in laboratories around the world for a variety of applications in bacterial toxin research as well as in growing attenuated viruses for vaccine production. MDBK cells were chosen in this study to detect cytotoxicity and effect on cell viability and proliferation after exposure to the crude extract of *V. cholerae.* The limited number of publications on this cell line as an *in vitro* model for *V. cholerae* cytopathology was another encouraging factor to do this study. Vero cells are widely used to study the cytotoxic effects of toxin in comparison to MDBK cells.

MATERIALS AND METHODS

Bacterial strains

Twenty-six local clinical isolates of *V. cholerae* , 15 of O1 serotype and 11 of O139 serotype, were tested in this study. Among them 20 were provided by the Institute of Medical Research, Kuala Lumpur and the rest by the Microbiology Laboratory of Tengku Ampuan Afzan

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hospital (HTAA), Kuantan, Pahang. Five control strains of *V. cholerae* O1 and O139 were kindly provided by the Department of Microbiology, School of Medicine, University Science Malaysia Hospital, Kubang Kerian, Kelantan.

MDBK cells

The cells used in the present study were provided by Dr. Noor Rain Abdullah from Department of Herbal Medicine Research Centre, Institute of Medical Research (IMR), Kuala Lumpur and were prepared from a stock stored in liquid nitrogen, at Bioassay Laboratory, Institute of Medical Research, Kuala Lumpur.

Vero cells

Vero cells were obtained from the same laboratory as MDBK cells.

Cell Line expansion

The cryovials containing cryo-preserved MDBK cells or Vero cells were thawed. Then, the cell lines were adapted to a single, standard complete culture medium, RPMI 1640 (Gibco Brl. Life Technology, USA,). The culture medium was supplemented with 5% heat-inactivated fetal bovine serum (Gibco Brl Life Technology, USA), 2 mM L-Glutamine (Gibco Brl. Life Technology, USA.), 5.94 g of HEPES (Gibco Brl. Life Technology, USA), 20.0 g sodium bicarbonate (Gibco Brl. Life Technology, USA) and antibiotics, 100 IU/ml penicillin and 100 µg/ml of streptomycin. The cell suspension is centrifuged at 3000 rpm for 10 minutes at 20-24°C (KUBOTA 6800, Japan) and the cell pellets were resuspended in 1 ml of complete culture medium (CM + 5% FBS). One drop of the resuspended cells was then added to 10 ml of complete culture medium in 25 mm^2 culture flasks. The loosely capped flasks were then incubated in 5% CO_2 at 37°C.

Established adherent cell monolayers approaching 80% confluence were harvested with 2 ml of 0.025% trypsin (Gibco Brl. Life Technology, USA). The cells were washed 3 times in complete medium and then sub-cultured into 75 cm^2 culture flasks in a CO_2 incubator at 37°C. An 80% confluent cell monolayer was selected for MTT test.

MTT Test (3-['4, '5-dimethylthiazol-2 -y]-2, 5-diphenyltetrazolium bromide Test)

The MTT test is used to study the proliferation and metabolic activity of cell populations in culture. The test is also done for quantitative cytotoxicity assays as in this study.

MTT solution

MTT solution was prepared as follows: 500 mg MTT reagent 2128 (SIGMA) was dissolved in 100 ml of PBS using sonifier. This was followed by sterilization through a 0.45 µm cellulose-acetate filter (Whatman Inc.) into a universal bottle. The bottles were wrapped with aluminum foil and stored at - 20°C.

MTT test procedure

The test was performed using both Vero and MDBK cells. *V. cholera* O1, *V. cholera* O139 *and E. coli* were tested as follows: MDBK cells or Vero cells were harvested from exponential phase maintenance cultures. Then, viable cells were counted by trypan blue exclusion and 180 µl of the cell suspension was dispensed to 96-well cell culture plates (NUNC™, Nalge Nunc International, Denmark). Twenty µl of serial 10-fold dilutions (1:1, 1:10, 1:100 and 1:1000) of bacterial culture filtrates were added in triplicate. Peripheral wells, lacking cells, of each plate were utilized as blank. Plates were then incubated at 37°C in 5% CO_2 for 7 days prior to addition of the tetrazolium reagent. After the incubation period, 50 µl of diluted MTT was added to all wells and the assay plates were reincubated for 3 hours. After incubation the cell monolayers were inspected microscopically for purple formazan formation. All supernatants were removed carefully and replaced with 200 µl of DMSO (Wako Pure Chemical Industrial) (Romijin *et al.,* 1988). The plates were rocked on a plate shaker for 15 minutes. The absorbance of each well was measured at a wavelength of 570 nm by ELISA reader (Thermomax™, Molecular devices, Menlo Park, CA, USA). Cells treated with 1.0 % Triton X100 (Roche, Eng Lab) were used as the positive control while cells grown in culture media containing uninoculated APW and AKI were used as the negative control. Culture media alone were used as blanks to negate their influence on assay results.

Cytotoxicity was determined using the following formula:

$$\text{Cytotoxicity} = \frac{\text{Optical density of the negative control} - \text{Optical density of the test}}{\text{Optical density of the negative control} - \text{Optical density of the positive control}} \times 100$$

Enzyme Linked Immunosorbent Assay (ELISA)

CT production by *V. cholerae* isolates was detected by the Protein Detector™ ELISA Kit, HRP, ABTS System (Kirkegaard & Perry Laboratories, Inc. USA). This test was done for both the detection of cholera toxin, as well as for the confirmation of the identity of these strains (Abe *et al.*, 19871).

RESULTS AND DISCUSSION

The cytotoxic effect of *V. cholerae* serotypes O1 and O139 was tested on two different cell lines, Vero and MDBK, using culture filtrates of organisms grown on AKI and/or APW media with and without added polymyxin B. No detectable impairment of proliferation or metabolic activity of Vero or MDBK cells was noted

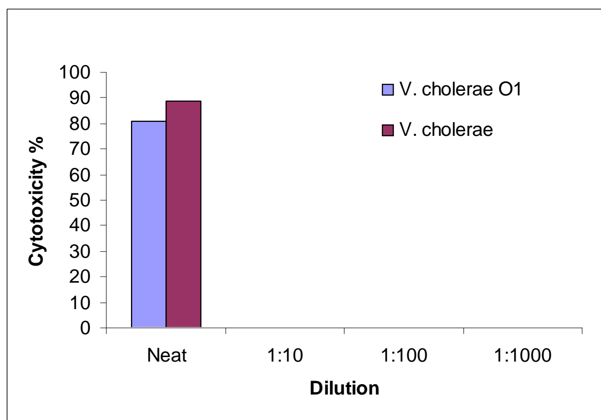

Fig. 1: Cytotoxicity of *V. cholerae* Culture Filterate on Vero Cells using MTT Test (APW medium with Polymyxin B).

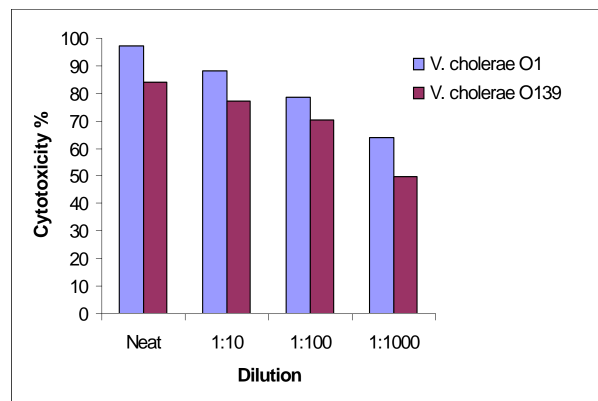

Fig. 2: Cytotoxicity of *V. cholerae* Culture Filterate on MDBK Cells using MTT Test (APW medium with Polymyxin B)

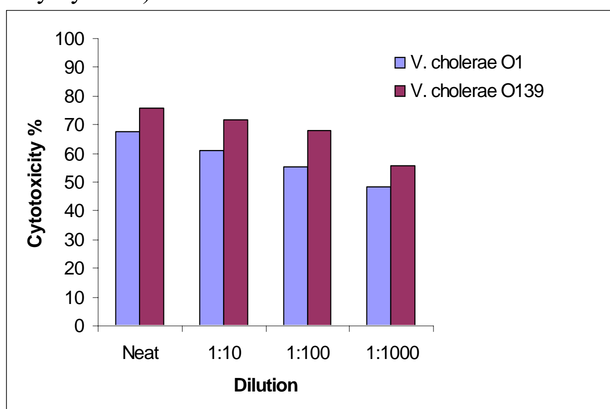

Fig. 3: Cytotoxicity of *V. cholerae* Culture Filterate on MDBK Cells using MTT Test (APW medium without Polymyxin B)

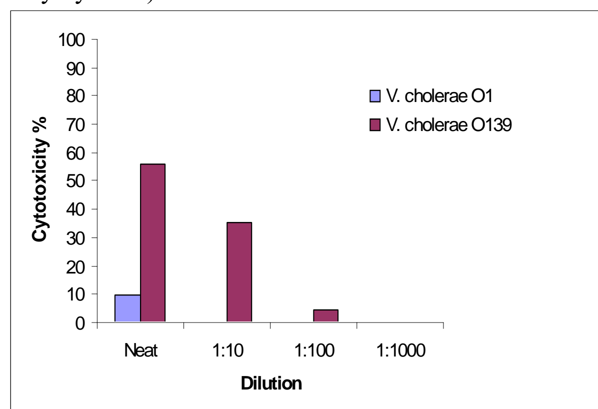

Fig. 4: Cytotoxicity of *V. cholerae* Culture Filterate on MDBK Cells using MTT Test (AKI medium with Polymyxin B)

when they were exposed to uninoculated APW or AKI media. In contrast, the culture filtrate of *V. cholerae* strains grown in either media exhibited variable cytotoxicity. The MTT test was used for quantitatively assessing the effect of the culture filtrates on the above cells in addition to direct observation of cytotoxicity development.

Both serotypes O1 and O139 exhibited marked cytotoxicity on MDBK cells when grown in APW medium with and without polymyxin B. The cytotoxicity was evident up to 10^{-3} dilution where it was 49.7 % for O139 strains and 63.7 % for O1 strains grown in medium with polymyxin B (fig. 2). Comparable results were obtained with filtrates of these organisms grown in APW medium without polymyxin B with lower toxicity (fig. 3).

However, when grown in AKI medium the O1 strains were cytotoxic to MDBK cells only when polymyxin B was added to the medium and even then it was a minimal 9.9 % when applied neat (fig. 4). The cytotoxicity of O139 strains grown in the same medium with polymyxin B was noticeable (35.1 %) at up to 10^{-1} dilution. O139 strains grown in AKI medium without polymyxin B had little cytotoxic effect ≤14.5 % on MDBK cells (fig. 5).

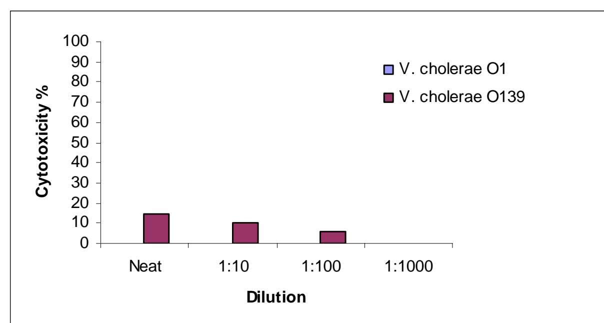

Fig. 5: Cytotoxicity of *V. cholerae* Culture Filterate on MDBK Cells using MTT Test (AKI medium without Polymyxin B).

On Vero cells, strains of both serotypes of *V. cholerae* grown in APW medium with polymyxin B caused cytotoxicity but not when grown in antibiotic-free medium (fig. 1).

In general, the severity of the visualized cytotoxic effect caused by the bacterial filtrates on cultured cells correlated quite well with their level of cytotoxicity in MTT tests and showed a discernible dose-response effect when various dilutions from neat to 10^{-3} were applied.

All the tested strains of *V. cholerae* in this study exhibited evidence of increased cytotoxicity to both Vero and MDBK cells when polymyxin B was added to the bacterial growth media. This may be due to the binding of polymyxin B to the cell membrane of *V. cholerae* destroying the membrane function as a permeability barrier and thus causes lyses of the cells and the liberation of more toxins (Mathur and Waldor, 2004 and Waldor, 1996). This enhancing effect of polymyxin B has also been noted for the liberation of CT quantified by ELISA test (Abe *et al.*, 1987). When Vero and MDBK cells are compared as to their susceptibility to the cytotoxic effect of *V. cholerae* culture filtrates, it was quite clear that the latter cells are the most sensitive (figs. 1 and 2). By analogy to the Vero cytotoxin of *Escherichia coli* O157:H7 that was shown not to be able to invade MDBK cells but HeLa cells (Riley *et al.*, 1990). The presence of higher affinity receptors for the cytotoxins of *V. cholerae* on MDBK cells as compared to Vero cells may be a speculative reason for their higher susceptibility.

This study demonstrated the better suitability of APW medium as compared to AKI medium in inducing the expression of cytotoxic factors and that this ability was enhanced by the presence of polymyxin B (figs. 1 and 2). The reason for the enhancement of the cytotoxin production needs further evaluation. A previous study in our laboratory on the quantitation of CT production by ELISA confirmed its higher production in polymyxin B containing APW medium as compared to APW alone and to AKI medium.

Both O1 and O139 serotypes produced CT in AKI medium and the level was even slightly higher for the former, a result which is in contrast to the MTT test that showed either negligible or no cytotoxicity of O1 strains grown in AKI medium with or without polymyxin B respectively (Sugiyama *et al.,* 1996). Thus it is apparent that the toxicity to cell cultures is independent or not directly linked to the production of CT. Other substances are probably secreted that have a more direct effect on cultured mammalian cells. The limitation of the study is that the detected cytotoxicity and the MTT toxicity cannot be attributed to a particular product or molecule due to the crude nature of the culture filtrates. However, one can speculate that these effects might be the results of toxins such as zonula occludens toxin (*zot*) and accessory cholera enterotoxin (*ace*). The cytotoxic effect of *V. cholerae* O1 on Vero cells may be related to *ace* factor and the cytotoxicity on MDBK cells might be related to *zot* (Rodriguez-Angeles *et al.,* 1994).

A study about *Clostridium difficile ace* show some common features with that of *V. cholerae* (Borriello, 1998). A growing number of toxins of *C. difficile* have been reported to act by affecting the host cell cytoskeleton. *C. difficile* infections can result in severe diarrhea and the virulence of this pathogen is dependent on its elaboration of two related toxins, TxA and TxB. These toxins are among the largest monomeric toxins described, with molecular weights of 308,000 for TxA and 270,000 for TxB. Although TxA has traditionally been referred to as an enterotoxin and TxB as a cytotoxin (Borriello, 1998), they both exert a cytotoxic effect *in vitro*. Both TxA and TxB are glucosyltransferases using uridine diphosphate glucose as a substrate to inactivate monoglucosylation members of the Rho family of small GTPase at Thr, an amino acid residue located within the putative effector domain of the Rho proteins (Fasano, 1999).

CTx shows similar pathological effect on epithelial cells that involves the cytoskeletal structure. Rho GTPases regulate a variety of cytoskeleton-dependent cellular functions such as cell adhesion and motility, growth factor-mediated signaling, cellular transformation and induction of apoptosis (Narumija *et al.,* 1997). The dramatic effects of TxA and TxB on tissues and cells, including cytoskeletal depolymerization, increased intestinal permeability and caused diarrhea, cellular reaction and rounding, disruption of cell adhesion, chemotaxis and activation of apoptosis (Pothoulakis, 1996). Besides the inactivation of Rho proteins, their activation is also associated with increased intestinal permeability and diarrhea. Zonula occludens toxin (*zot*), a toxin elaborated by *V. cholerae* (Fasano *et al.,* 1991 and 1995) is a single-polypeptide chain of 44.8 kD encoded by the bacteriophage CTX[phi] present in toxigenic strains of *V. cholerae* (Waldor, 1996). The mechanism of action of *zot* involves the rearrangement of the epithelial cell cytoskeleton due to protein kinase C [alpha]-dependent polymerization of actin filament strategically located to modulate intercellular tight junction (Fasano, 1998).

As the MDBK cells showed more cytotoxicity to crude extract of *V. cholerae*, in comparison to Vero cells, this study may demonstrate an easier and efficient selection of cell line for cytotoxicity study.

Further investigations are needed to elucidate the specific virulence factor(s) and susceptibility that cause cytotoxicity on MDBK cells.

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