Cytotoxic Activities of Some *Escherichia coli* Isolates: Possible Mechanisms and Approaches for Inhibition

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Abstract: The cytotoxicity of four highly cytotoxic *E.coli* isolates against Vero cells was studied and found to be cell associated. High cytotoxicity was observed at a multiplicity of infection of 200:1 and this activity gradually decreased as the bacterial count decreased. This cytotoxicity was affected by changes in pH and caused altered Vero cell morphology and detachment by different degrees which was apparent after 2 h of infection. In addition, viable metabolically active bacteria were necessary for Vero cell killing. Moreover, the four isolates showed very high adherence levels (>5%) while they showed very poor invasion (<0.01%) which implies that there is a strong relationship between the adherence and cytotoxicity of these isolates while cell invasion is probably not responsible for the observed cytotoxic effects. Upon quantitation of the hemolytic activity of the bacterial cells of these isolates, a clear relation could be seen between the hemolytic activity of the tested isolates and their cytotoxic effects on Vero cells. In addition, results confirmed that no cell free α -hemolysin was released under the tested conditions which imply that a cell associated hemolysin (β-hemolysin) rather than α -hemolysin was responsible for the observed cytotoxic effects. To further confirm this conclusion, the hemolytic activities of heat treated bacterial cells, gentamicin killed cells, bacterial cell lysates and metabolically inactive cells (bacteria cooled to 4° C) were measured and results showed that these treated bacterial suspensions lost their hemolytic activities which ensures the association between the hemolytic and cytotoxic activities of the tested isolates. In an attempt to inhibit bacterial cytotoxicity, agents with reported antiadherent effects like glucose, galactose and mannose reduced the cytotoxicities of the tested isolates. Mannose caused the greatest reduction in cytotoxicity followed by galactose, and then glucose . Dextran 70 also reduced the cytotoxicities of the tested isolates. Of the tested pharmaceutical excipients, poloxamer 407 had the greatest inhibitory effect on *E. coli* cytotoxicity while tween 80 caused a smaller effect. In addition, the metal ions Fe^{3+} and Zn^{2+} caused a marked reduction in the cytotoxicities of the tested isolates, where 5 mM nearly abolished the observed cytotoxic effects while Ca^{2+} had little or nearly no effect. [Journal of American Science. 2010;6(10):269-283]. (ISSN: 1545-1003).

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1. Introduction

E. coli is a Gram negative pathogen causing infections such as urinary tract infections, gastroenteritis and neonatal meningitis. *E. coli* was reported to produce a number of virulence factors with cytotoxic effects on host cells. The most important exported toxin is the α-hemolysin. This is a cytolytic poreforming toxin with a wide target cell spectrum including erythrocytes, leukocytes, endothelial and renal epithelial cells (Emody *et al.*, 2003). Alpha hemolysin was reported to be cytotoxic to a range of cell types within short infection periods (Island *et al*., 1998; Trifillis *et al*., 1994; Mobley *et al.*, 1990). Another hemolysin produced by *E. coli* is the cell associated β-hemolysin (Smith, 1963; Johnson, 1991; Mittal and Sharma, 1991; Jacobsen *et al.*, 2008). This is a cell bound hemolysin that is detectable during the exponential and stationary growth phases (Sobieszczan *et al.*, 2006) and whose activity was found to be dependent on the bacterial metabolic status (Short and Kurtz, 1971). The serine protease secreted autotransporter toxin (SAT) is another virulence determinant which contributes to the pathogenicity of *E. coli* (Maroncle *et al*., 2006) and has toxic activity against cell lines of bladder/kidney origin (Emody *et al.*, 2003). Guyer *et al.* (2002) showed that after only 1 h of incubation of SAT containing filtered supernatants with Vero cells, cells displayed cellular elongation, cytoplasmic shrinkage and the monolayer was no longer intact. Cytolethal distending toxin (CDT) is another toxin reported to be exported by *E. coli* (Johnson and Lior, 1988; Emody *et al.*, 2003). CDT enters into eukaryotic cells and breaks down double stranded DNA resulting in arrest of cell cycle (Emody *et al*., 2003; Heywood *et al*., 2005). Scott and Kaper (1994)

reported that *E. coli* supernatants containing CDT caused elongation of mammalian cells at 24 h followed by progressive cellular distention and cytotoxicity for up to 120 h. Cytotoxic necrotizing factor (CNF) was also reported to be exported by *E. coli* (Emody *et al*., 2003) and causes enlargement and multinucleation of mammalian cells after 24 h of incubation (Hostacka, 1994). Accordingly, the present study aimed at studying the cytotoxic mechanisms of four highly cytotoxic *E. coli* isolates. These isolates were obtained through a screening program directed for distinguishing isolates with cytotoxic activities (El-Housseiny *et al.*, submitted). Attempts for inhibiting this cytotoxic effect were also carried out.

2. Materials and Methods

Bacterial isolates and culture conditions

E. coli isolates E22, E35, E36 & E73 are highly cytotoxic organisms obtained through screening of different clinical isolates for their cytotoxicities against Vero cells in a previous study. These isolates were stored as bacterial stocks at -20°C in 50% glycerol solution for long term preservation. For conducting experiments, cytotoxic isolates were obtained from frozen stocks and subcultured twice on blood agar. Colonies were then cultured in test tubes containing 10 ml aliquots of TSB and incubated for 18-20 h at 37^{o}C under static conditions. This culture was kept at 4^{o}C and used for inoculum preparation which was prepared by transferring 50 µl of this culture into 10 ml TSB and incubation at 37^{o}C for 18-20 h under static conditions.

Cell line and growth conditions

African green monkey kidney epithelial cells (Vero Cell Line, ATCC No. CCL-81) were used in this study. Vero cells were maintained in Eagle's minimum essential medium with Earl's balanced salts (MEM Earl's; Sigma) supplemented with 2% Fetal bovine serum (FBS; Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5 % CO_2 and subcultured every 3-4 days. For experiments, Vero cells suspended in MEM Earl's supplemented with 10 % FBS were seeded in 96-well tissue culture plates and kept at 37°C and 5 % CO_2 for 24 h to form a confluent monolayer (5 x 10^4 cells/well).

Cytotoxicity assay

Crystal violet (CV) cytotoxicity assay was carried out as described by Kueng *et al.* (1989). Vero monolayer was infected with aliquots of the bacterial culture adjusted to contain 1 x 10^8 cfu/ml for 3 h. The cells were then washed with PBS and fixed by adding 110 µl of 1% glutaraldehyde solution to each well for 15 min. The plate was decanted and then washed 3 times by immersing in tap water and decantation. After air drying, 100 µl of 0.1% crystal violet solution was added to each well for 20 min. The excess stain was then removed and the plate submerged in tap water and subjected to a continuous slow flow of water for 15 min. After air drying, the stain was eluted with 100 µl of 10% acetic acid solution. The A_{540} of supernatants from infected and control cells were determined using an automatic microplate scanning spectrophotometer (Microreader 4 plus, Hyperion).The percentage cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \left[1 - \left\{\frac{A_{540} \text{ OF INFECTED CULTURE}}{A_{540} \text{ OF CONTROL}}\right\} X100\right]$$

Determination of the cytotoxicities of bacterial cells and cell free supernatants

For preparation of bacterial cells and cell free supernatants, bacteria were harvested by centrifugation at 12000 rpm for 5 min. The supernatants were separated and sterilized by filtration through 0.22-µm-pore-size membrane filters (Millex, Millipore Corporation, Bedford, USA). The bacterial pellets were washed once with PBS, then resuspended in MEM Earl's to an absorbance corresponding to 1×10^8 cfu/ml.

After washing the Vero monolayer twice with PBS, 100 µl of the bacterial cell suspension prepared above was added to each well and incubated at 37^{o}C (Multiplicity of infection of 200:1) for 3 h. Then, the crystal violet cytotoxicity assay was completed as described before. Control wells were incubated with 100 µl of MEM Earl's only. Additional experiments were performed using bacterial counts of 10^7,10^6,10^5 and 10^4 cfu/ml.

Cytotoxicity of cell free supernatants was determined by adding 50 µl of MEM Earl's and 50 µl of the cell free supernatant to each well for 3 h. After that, the wells were washed and the CV cytotoxicity assay was completed as described previously. Control wells were incubated with 50 µl of MEM Earl's and 50 µl of sterile TSB.

Detection of extracellular production of a cytotoxic agent by the tested isolates during the cytotoxicity assay

To determine whether cytotoxicity was due to toxic bacterial products released during the incubation period in the cytotoxicity assay (3 h), the bacterial supernatants of cells (1 x 10^7 cfu/ml) grown for 3 h at 37° C in MEM Earl's were tested for cytotoxic effect. The cell free supernatants were prepared by filter sterilization through 0.22 µm-pore

size membrane filters and these were then incubated with the Vero monolayer for 3 h.

Adherence and Invasion assays for the cytotoxic isolates

Bacterial adherence and invasion were determined according to Plotkowski *et al.* (1994) and carried out as follows:

After washing the monolayer, aliquots of 100 µl of the bacterial suspensions (1×10^7 cfu/ml) were added to the wells of a microtitre plate (8 wells for each isolate). Following 1 h of incubation at 37°C in 5% CO_2, the inocula were removed and the Vero cells were washed 3 times with PBS to remove non associated bacteria (the assay time was reduced to 1 h and the bacterial count adjusted to 1×10^7 cfu/ml since a higher count or a longer time lead to detachment of the monolayer due to high cytotoxicity).Vero cells were then treated with lysis solution (0.025% trypsin and 1% tween 20 in PBS) for 30 min at 37°C and the total number of associated bacteria (adherent and invaded) was assessed by the colony counting method.

Gentamicin survival assay was used to quantify invasion. This was determined using the same method described above except that after 1 h incubation, the infected monolayer was treated with gentamicin solution 300 µg/ml for 1 h to kill the extracellular bacteria just before the addition of the lysis solution. Adherent bacteria were calculated as the difference between the total number of associated bacteria and the number of invaded bacteria.

Effect of mid-interval removal of non-interacting bacteria

Bacterial suspensions (1×10^7 cfu/ml) were left in contact with Vero cells for only 1.5 h, then the remaining suspended bacterial cells were removed and the wells were washed once with PBS. The incubation was then continued up to 3 h after addition of fresh 100 µl MEM Earl's to the wells. After that the crystal violet assay was carried out. Incubation of bacterial cells (1×10^7 cfu/ml) with Vero cells for 3 h was also carried out in parallel for comparison.

Effect of pH and incubation temperature on bacterial cytotoxicity

The bacterial suspensions were prepared as previously described except that the pH of MEM Earl's was adjusted with 0.3 N of HCl or NaOH sterile solution. The pHs studied were 6, 7, 8 and 10 and the cytotoxicity assay was carried out as mentioned before.

To study the effect of incubation temperature, again the cytotoxicity assay was carried out as mentioned before except that the plate was incubated at different temperatures (30^o C, 37^o C & 40^oC).

Effect of metabolic state of the tested bacterial isolates on their cytotoxic activities

Cytotoxic activity of killed bacterial cells

The bacterial cells of the tested isolates (1×10^8 cfu/ml in PBS) were killed by treating with 300 µg/ml of gentamicin solution for 1 h. Gentamicin at 100 µg/ml was sufficient to kill *P. aeruginosa* at 10^8 cfu/ml in 1 h (Plotkowski *et al.*, 1994).

To heat kill bacterial cells, bacteria (1×10^8 cfu/ml in PBS) were placed in a boiling water bath for 30 min. Cultures of the heat killed bacteria confirmed that no viable bacteria persisted after this treatment. The experiment was repeated except that the bacteria were heat treated at mild conditions (heating in a water bath at 56^oC for 10 min).

To prepare whole cell lysates, bacterial cells (1×10^8 cfu/ml in PBS) were sonicated by a sonicator (Microson, U.S.A) 5 bursts at 20 W, one min each with 30 sec rests on ice in between the bursts (Prasad *et al.* ;1996).

In each case the cytotoxic activity of the killed suspensions produced was determined by adding 90 µl of MEM Earl's to each well followed by 10 µl of the killed bacterial suspension. The plate was then incubated for 3 h after which the CV cytotoxicity assay was carried out.

Cytotoxic activity of viable metabolically inactive bacterial cells

Bacterial cells (1×10^8 cfu/ml in PBS) were cooled to 4^oC. Ten microliters of the cooled suspensions were then added to Vero cells bathed in 90 µl of ice cold MEM Earl's and the plate was kept at 4^oC for 3 h. Cold treatment of Vero cells in the absence of bacteria had no effect on the viability of the monolayer (Apodaca *et al.*, 1995).

Vero cell morphology after infection by bacterial cells of the tested *E. coli* isolates

To test the effect of bacterial isolates on Vero cell morphology, the bacterial cells were prepared as described and adjusted to a count of 1×10^7 cfu/ml. Vero cell morphology was assessed by examination using an inverted microscope (Hund Wilovert, Model 30, Germany) 1, 2 and 3 h post infection. At 3 h post

infection (p.i.), the cells were washed, fixed with 1% glutaraldehyde and photographed using a camera (Canon powershot A650) attached to an inverted microscope (Optech, Germany).

Studying the hemolytic activity of *Escherichia coli* isolates

The hemolytic activity of the tested isolates was assessed as described by Sobieszczanska *et al.* (2006) with some modifications and carried out as follows.

A standardized red blood cell suspension was prepared in phosphate buffered saline (PBS containing 0.02% $CaCl_2$) and adjusted to contain 0.5 $\times 10^7$ RBC/ml. Bacterial isolates were grown at 37°C for 18-20 h in TSB. Bacterial cells were washed with PBS and resuspended to a concentration of 1 $\times 10^9$ and 1 x 10^8 cfu/ml in eppendorf tubes. Aliquots of the resulting bacterial suspensions were mixed with equal volumes of the RBC suspension to give a MOI of 200 and 20 respectively and incubated at 37°C for 3 h. Then, the cells were sedimented by centrifugation and the amount of released hemoglobin in the supernatant was determined spectrophotometrically at 412 nm (Cortajarena *et al.*, 2001). A control experiment was carried out as above except that the bacterial cell suspension was replaced with PBS.

The hemolytic activity was also measured in filter-sterilized supernatants obtained from the bacterial cell suspension in PBS (containing 0.02% $CaCl_2$) which was incubated at 37°C for 3 h to determine whether cell free hemolysin was released into the environment during the assay period.

The hemolytic activity was expressed as the mean percentage of hemoglobin released in three independent assays in comparison to total lysis of RBCs in water.

In a parallel experiment, the crystal violet cytotoxicity assay was carried out for the *E. coli* isolates at MOI of 200:1 and 20:1 for 3 h as described previously.

Factors affecting hemolytic activities of E. coli isolates

a. Heat treatment of bacterial cells

Bacterial cell suspensions (1 x 10^8 cfu/ml) were heated at 56° C for 10 min and then their hemolytic activity was quantified

b. Bacterial killing by gentamicin

Bacterial cell suspensions (1 x 10^8 cfu/ml) were killed by treating with 300 µg/ml gentamicin for 1 h before carrying out the hemolysis assay.

c. Bacterial cell lysates

Whole bacterial cell lysates were prepared by sonicating bacterial cell suspensions (1 x 10^8 cfu/ml) using a sonicator (Microson, U.S.A) 5 bursts at 20 W, one min each with 30 sec rests on ice in between the bursts. Hemolytic activity of these preparations were then quantified.

d. Bacteria cooled to 4°C

Bacterial cells adjusted to 1×10^8 cfu/ml in PBS were cooled to 4°C then incubated with RBC suspension for 3 h at the same temperature. Hemolytic activity was then quantified.

Attempts for inhibition of cytotoxic activities of the tested bacterial isolates

Testing the effect of bacterial antiadherent agents

The tested antiadherent agents and their applied concentrations are shown in Table 1. After preparation of the monolayer as described previously, the medium was discarded and the monolayer was washed with PBS. Then, treatment with the antiadherent agents was carried out as follows.

a. Sugars and Dextran 70: Three different sugars reported to reduce the adherence of P. aeruginosa isolates were tested (Hafez *et al.*, 2005). The bacterial suspension of the tested isolates was adjusted to 1 x 10^7 cfu/ml in MEM Earl's and the tested sugar was incorporated in this suspension at the concentrations listed in Table 1. The resulting bacterial suspension was incubated for 30 min at 37°C. One hundred microliter aliquots of this suspension was then added to the wells containing Vero cells. Control wells were treated similarly except that the bacterial cells were omitted. In case of Dextran 70, the Vero monolayer was pretreated for 30 min at 37°C with 90 µl MEM Earl's containing 3 % Dextran 70. After that 10 µl aliquots of the bacterial suspension (1x10^8 cfu/ml) was added to each well. Control wells were included and contained 10 µl PBS instead of the added bacterial suspension.

b. Pharmaceutical excipients: The tested excipients included 4 nonionic surfactants which were reported to have antiadherent activity (Hafez *et al.*, 2005). In this case the surfactant was added concomitantly with the bacterial suspension to Vero cells. Ninety microliter quantities of MEM Earl's containing the mentioned concentrations (Table 1) of the tested agents were added to Vero cell containing wells

followed by 10 µl of the bacterial suspension (1×10^8 cfu/ml in PBS). Control wells were included and contained 10 µl PBS instead of the added bacterial suspension.

For all the tested agents, the crystal violet cytotoxicity assay was carried out after 3 h of incubation at 37°C and the results were compared with Vero cells infected with the tested bacterial isolates without treatment with the antiadherent agents.

Testing the effect of metal ions

Metal ions have been reported to affect bacterial virulence (Alksne and Projan, 2000) and therefore the effect of three different metal ions on bacterial cytotoxicity was tested. The bacterial suspension was adjusted to 1 x 10^7 cfu/ml in MEM Earl's and the different metal ions were incorporated at the concentrations listed in Table 1. The resulting bacterial suspension was incubated for 30 min at 37°C. One hundred microliter aliquots of this suspension were then added to monolayer containing wells (Sugarman *et al.*, 1982). Control wells were treated similarly except that the bacterial cells were omitted. The crystal violet cytotoxicity assay was carried out after 3 h of incubation. Again, monolayer containing wells infected with the tested bacterial isolates without treatment with the metal ions were included for comparison.

Table 1. Agents studied for inhibiting the cytotoxic activities of the tested bacterial isolates and their applied concentrations

Class	Agent used	Concentrations applied
1- Antiadherents		
a. Sugars	D-Glucose	2, 5 %
	Galactose	
	Mannose	
	Dextran 70	3%
b. Pharmaceutical excipients	Tween 20	0.025, 0.05, 0.1%
	Tween 80	
	Poloxamer188	
	Poloxamer407	
2. Metal ions	CaCl2	2.5, 5, 10 mM
	FeCl3.H2O	
	ZnCl2	

3. Results

Characterizing the cytotoxic activities of the tested isolates for revealing the possible associated mechanisms

Cytotoxicity of bacterial cells and cell free supernatants

The whole cultures of the four tested *E. coli* isolates showed cytotoxicity percentages more than 85 % as determined by the CV assay after 3 h of Vero cell infection. To ascertain which component of the whole culture of the four *E. coli* isolates was responsible for the observed cytotoxic effects, both the bacterial cells as well as the cell free growth supernatants were tested for cytotoxic activity. The results showed that the cell free supernatants of the four *E. coli* isolates caused nearly no cytotoxicity after 3 h of Vero cell infection. On the other hand, the washed bacterial cells caused high cytotoxic effects similar to that obtained by the whole cultures (data not shown). In all the tested isolates, high cytotoxicity was obtained at high bacterial cell counts (10^8 cfu/ml) and the cytotoxicity gradually decreased by decreasing the bacterial count (Figure 1). A bacterial count of 10^5 cfu/ml had nearly no cytotoxicity for the two isolates E22 and E73 while the cytotoxic effect of the other two isolates (E35 and E36) continued up to a count of 10^4 cfu/ml. A count of 10^7 cfu/ml was used for all the tested bacterial isolates in subsequent experiments.

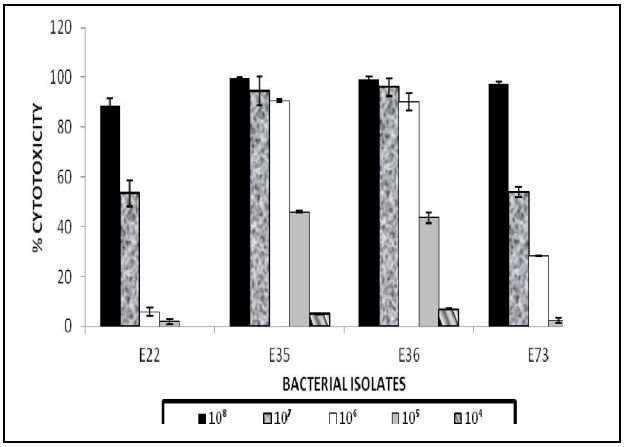

FIG 1. Effect of bacterial count on cytotoxicity of *E. coli* isolates The histograms represent the average percent cytotoxicity caused by the isolates and the error bars indicate the standard deviations of the data.

Detection of extracellular production of cytotoxic agents during the cytotoxicity assay

To test whether bacterial cells of the tested isolates released a cytotoxic product during the 3-h incubation period, cell free filtrates (0.22 µm filters) of 3-h bacterial cultures in MEM Earl's were prepared and incubated with Vero cells for 3 h. Results showed that the filtrates produced were nearly unable to cause Vero cell cytotoxicity (data not shown).

Adherence and Invasion of the tested isolates

As shown in Figure 2, although with different degrees, all the isolates showed very high adherence levels (>5%) while they showed very poor invasion (<0.01%).

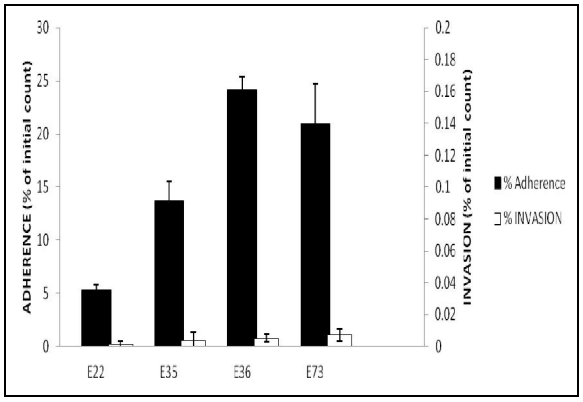

FIG 2. Adherence and Invasion of the tested *E. coli* isolates to Vero cells. The histograms represent the average percent adherence and invasion caused by the isolates and the error bars indicate the standard deviations of the data.

Effect of mid-interval removal of non-interacting bacteria, pH and incubation temperature on bacterial cytotoxicity

As shown in Figure 3, mid-interval removal of bacteria during the cytotoxicity assay period caused a decrease in the cytotoxicities of the tested isolates.

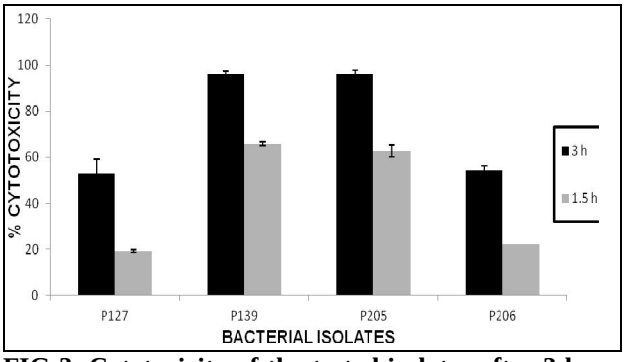

FIG 3. Cytotoxicity of the tested isolates after 3 h Vero cell infection time and mid-interval removal of non-interacting bacteria The histograms represent the average percent cytotoxicity caused by the isolates and the error bars indicate the standard deviations of the data.

In case of the effect of pH and in comparison to the effect at pH 7, pH 8 had nearly no effect on cytotoxicity whereas pH 10 significantly reduced the cytotoxicities of the two isolates E22 and 73. On the other hand, a pH of 6 increased the cytotoxicities of these two isolates (E22 and E73) (Figure 4).

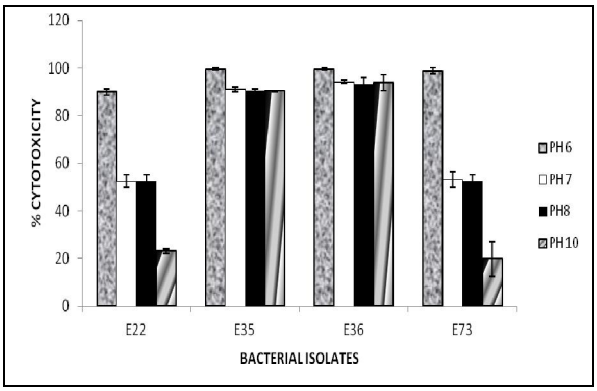

FIG 4. Effect of pH of MEM Earl's medium on bacterial cell cytotoxicity of *E. coli* isolates. The histograms represent the average percent cytotoxicity caused by the isolates and the error bars indicate the standard deviations of the data.

In case of incubation temperature, as compared to the original incubation temperature previously tested (37°C), the cytotoxicity of all the tested isolates did not greatly change at the different tested incubation temperatures (30° and 40°C)(data not shown).

Effect of bacterial metabolic state on their cytotoxic activities

To determine the role of bacterial metabolic activity of the tested isolates on their cytotoxicity, the cytotoxicities of killed bacterial cells (gentamicin treated, heat killed and sonicated bacterial cells) and viable metabolically inactive bacterial cells were tested. Results showed that all these treated cells lost their cytotoxic activities (data not shown).

Vero cell morphology after infection by bacterial cells of the tested *E. coli* isolates

Upon visual assessment of the Vero monolayer 1 h after infection with 1 x 10^7 cfu/ml of the tested isolates, no effect was seen on Vero cells (data not shown). Two hours post infection, undefined morphological changes were observed. However, 3 h post infection, E35 and E36 caused almost complete detachment of the Vero cells while the two other isolates E22 and E73 caused a lower degree of Vero cell detachment. The control cells showed the typical elongated shape of fibroblast-like cells (Figure 5).

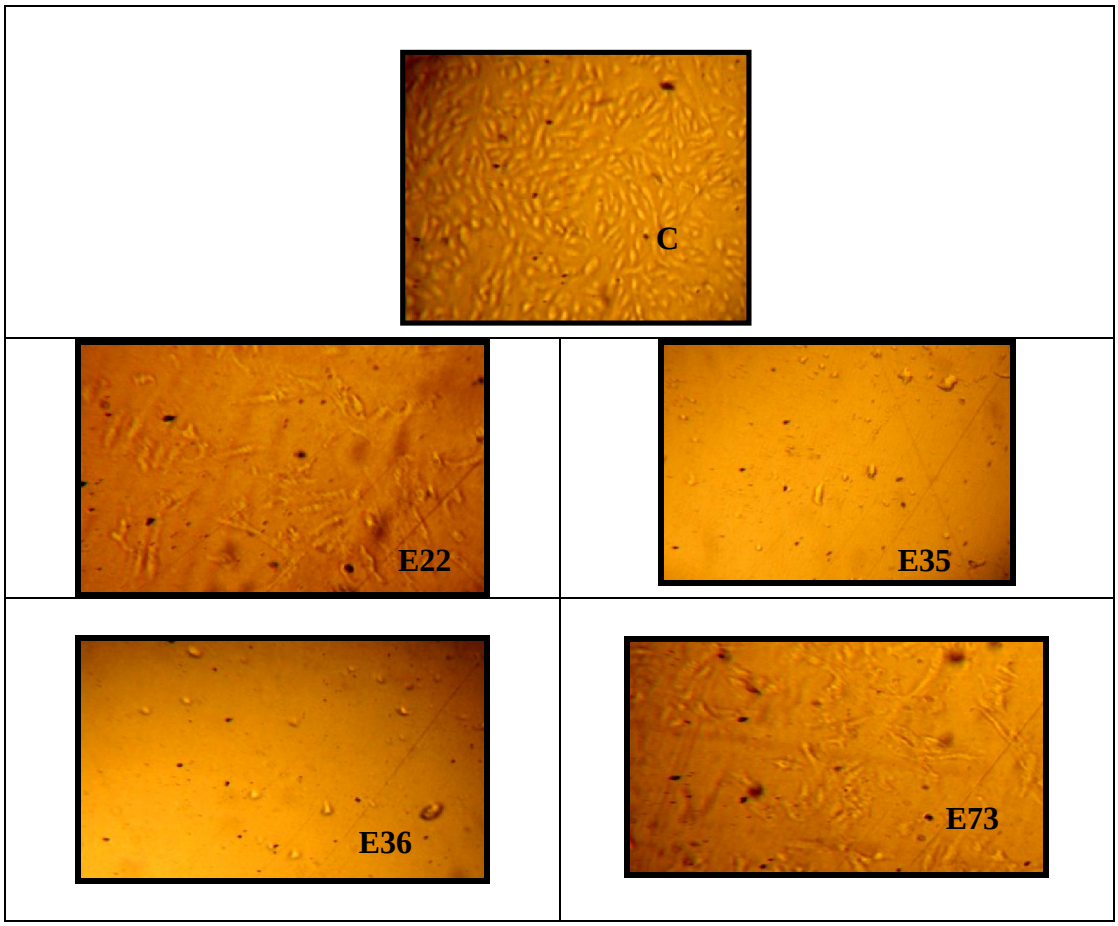

FIG 5. The cytotoxic effect of the tested isolates on Vero cells as revealedby microscopical examination after 3 h of infection. Images were captured using an inverted microscope(Optech, Germany) attached to a camera(Canon powershot A650). Mag x 400. C= control (uninfected cells)

Hemolytic activities of *E. coli* isolates

Equal volumes of bacterial cell suspensions and standardized RBC suspensions were mixed as mentioned previously and incubated at 37°C. At a MOI of 200:1, all the *Escherichia coli* cell suspensions showed high hemolytic activities (> 75%) after 3 h of incubation. Reducing the bacterial count (MOI of 20:1) led to lower hemolytic activities on the RBCs (Table 2). The crystal violet cytotoxicity assay was also carried out in parallel for the tested isolates. Similarly, a high cytotoxic effect was observed at MOI of 200:1, and such effect decreased at MOI of 20:1 (Table 2).

On the other hand, no hemolytic activity was observed in the filtrates obtained from bacteria suspended in PBS after being incubated at 37°C for 3 h, indicating that no free hemolysin was released from these isolates under the tested conditions (data not shown).

TABLE 2. Hemolytic activity of *E. coli* isolates and their cytotoxic effect on Vero cells

E. coli isolate	Hemolysis (%)		Cytotoxicity (%)	
	Bacterial suspension at MOI of		Bacterial suspension at MOI of	
	200:1	20:1	200:1	20:1
E22	76.67 ± 0.7	27.44 ± 1.39	85.72 ± 2.21	51.34 ± 6.8
E35	90.97± 3.37	82.72 ± 2.25	99.72 ± 0.48	94.79 ± 0.53
E36	93.72 ± 5.5	86.09 ± 1.59	99.19 ± 1.40	96.21 ± 1.46
E73	82.52 ±1.43	32.52 ± 1.43	95.81 ± 0.61	54.09 2.11

Factors affecting hemolytic activities of *E. coli* isolates

Heating at 56°C for 10 min, treatment with gentamicin or sonication of bacterial cell suspensions of the tested isolates nearly abolished their hemolytic activities (data not shown).

Moreover, cooling bacterial cells to 4°C then incubation with the RBC suspension for 3 h at 4°C also completely inhibited hemolytic activities of the four tested isolates (data not shown).

Inhibition of cytotoxic activities of the tested bacterial isolates

Use of antiadherents

The different antiadherent agents used included three sugars (glucose, galactose & mannose), Dextran 70 and four pharmaceutical excipients (tween 20, tween 80, poloxamer 407 & poloxamer 188)

A. Sugars

In case of glucose, a concentration of 2% reduced the cytotoxicity of the two *E. coli* isolates E22 and E73 and had nearly no appreciable effect on the other two isolates (E35 and E36), while 5% glucose greatly reduced the cytotoxicity of all the *E. coli* isolates (Figure 6).

For galactose, a dose dependant reduction in the cytotoxicity of *E. coli* isolates was obtained (Figure 6). Five percent galactose caused complete inhibition of the cytotoxicity of E22.

Similar to galactose, mannnose caused a dose dependant reduction in the cytotoxic activity but the effect was much more apparent where 5% mannose nearly abolished the cytotoxicities of all the tested isolates (Figure 6).

The effect of dextran 70 on bacterial cytotoxicity was also tested. As shown in Figure 6, dextran 70 (3 %) reduced the cytotoxicities of E22, E35, E36 and E73 by 59.5%, 52.2%, 46.3% and 55.6%, respectively.

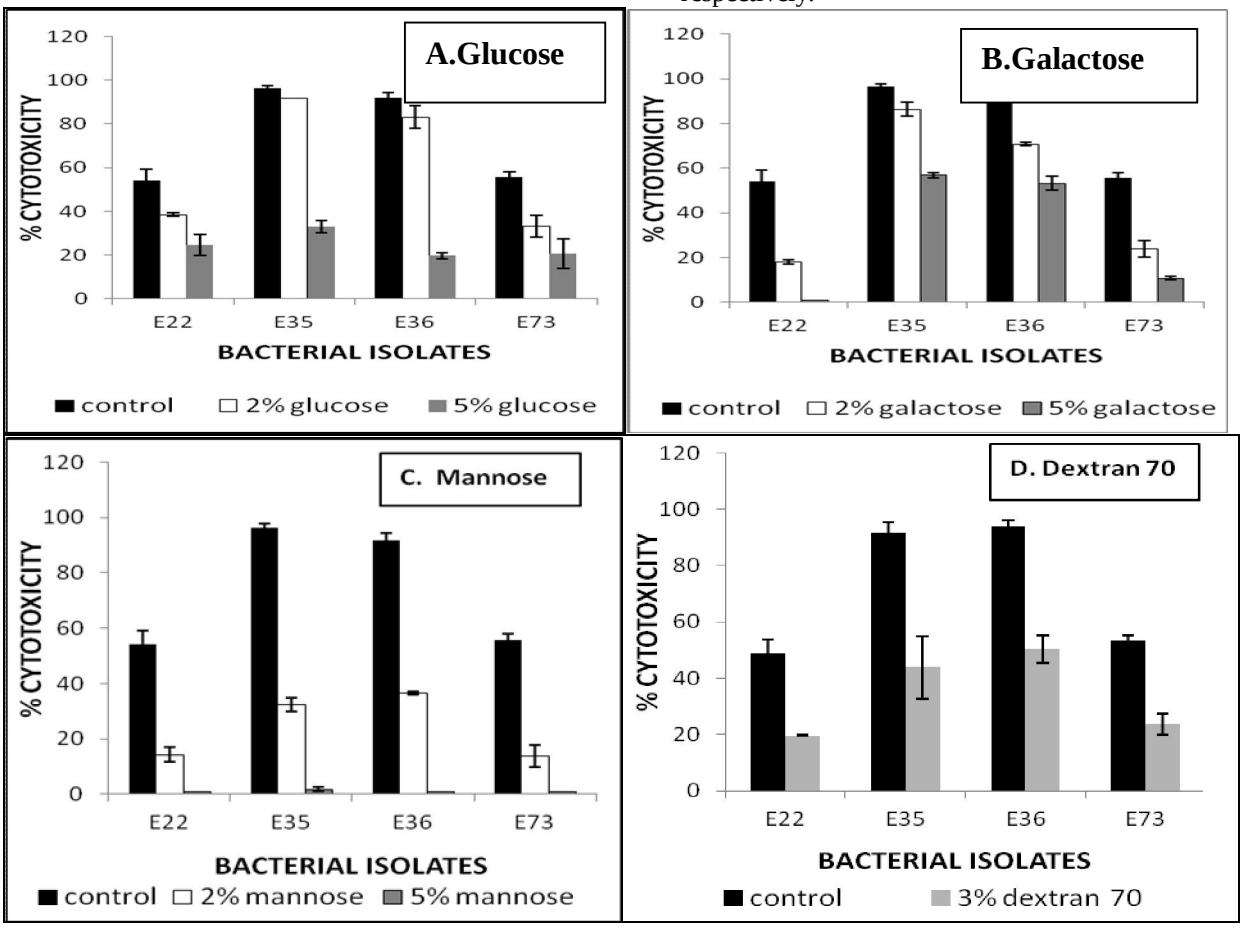

FIG 6. Effect of different concentrations of Glucose (A), Galactose (B), Mannose (C), Dextran 70 (D) on the cytotoxicity of *E. coli* isolates after 3 h of Vero cell infection. The histrograms represent the average percent cytotoxicity caused by the isolates and the error bars indicate the standard deviations of the data.

B. Pharmaceutical excipients

The four nonionic surfactants (tween 20, tween 80, poloxamer 188 and poloxamer 407) were tested at three different concentration levels (0.025, 0.05 and 0.1 %). In case of tween 20, 0.025 % had nearly no effect on bacterial cytotoxicity whereas 0.05 and 0.1 % affected the Vero monolayer integrity (data not shown). In case of tween 80, Figure 7 shows that it caused a dose dependant reduction in the cytotoxicities of two *E. coli* isolates (E22 and E73) while it showed a minimal effect on the other two isolates. Tween 80 at 0.1% was found to affect the Vero monolayer integrity (data not shown). On the other hand, poloxamer 188 had nearly no effect on the cytotoxicities of most *E. coli* isolates (data not shown). Regarding poloxamer 407, Figure 7 shows that this surfactant markedly reduced the cytotoxicities of the tested isolates. It was noteworthy that the highest reduction in cytotoxicity was observed with this agent compared to other tested surfactants. A concentration of 0.025 % nearly abolished the cytotoxic activities of E22 and E73 while double this concentration was required for reducing the cytotoxicities of the other two isolates E35 & E36. Again, at a concentration of 0.1 %, the Vero monolayer integrity was affected.

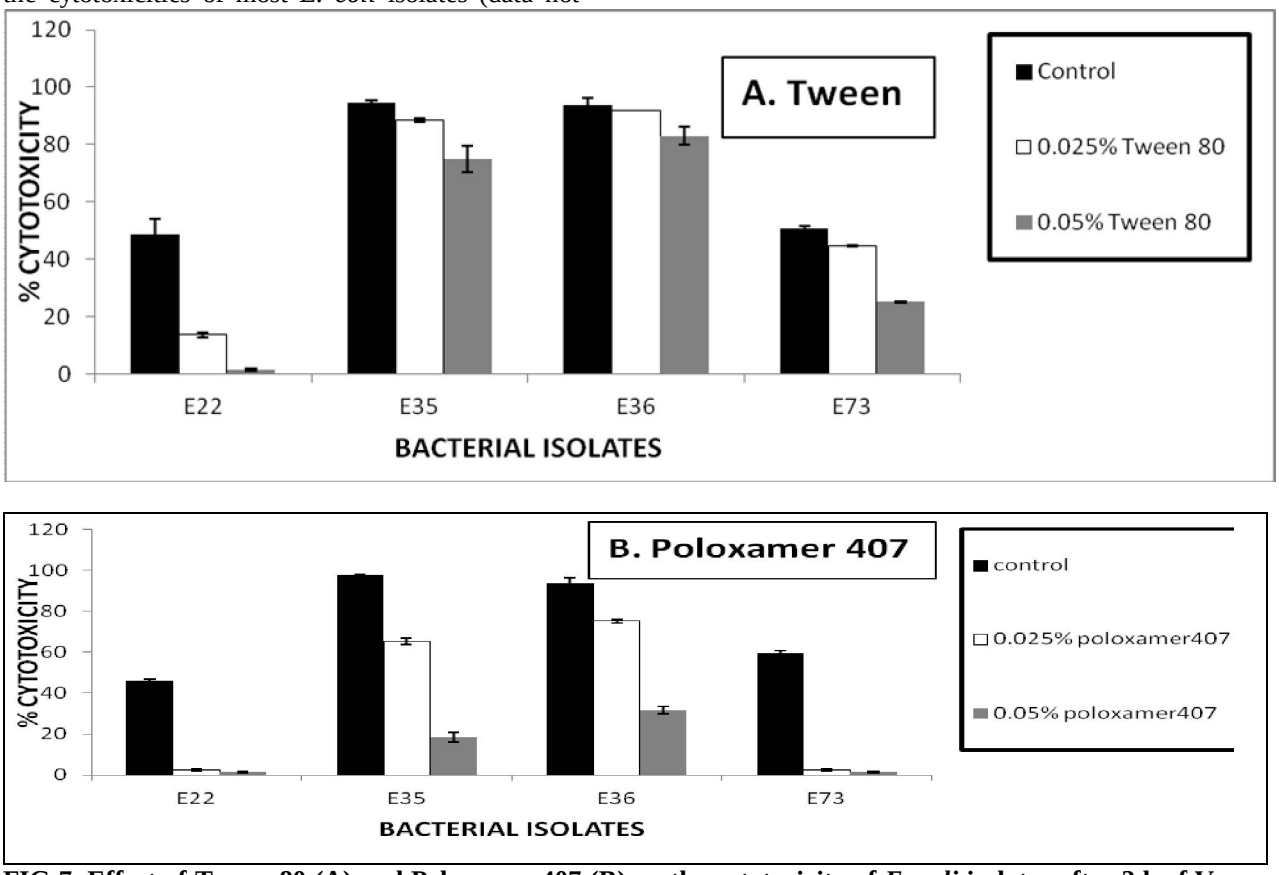

FIG 7. Effect of Tween 80 (A) and Poloxamer 407 (B) on the cytotoxicity of *E. coli* isolates after 3 h of Vero cell infection. The histograms represent the average percent cytotoxicity caused by the isolates and the error bars indicate the standard deviations of the data.

Use of metal ions

In addition to antiadherent agents, the metal ions listed in M & M (Table 1) were also tested for their effect on the cytotoxicities of the bacterial isolates using 3 different concentration levels (2.5, 5 and 10 mM). Concentrations of 2.5 and 5 mM but not 10 mM of the tested metal ions did not affect Vero cell viability as confirmed by staining with trypan blue.

Calcium ions had a minimum effect on the cytotoxicity of most *E. coli* isolates at the two tested concentrations (data not shown). On the other hand both iron and zinc ions dramatically reduced the cytotoxicities of all *E. coli* isolates at a concentration of 2.5 mM while 5 mM caused nearly complete inhibition of the cytotoxicities of these isolates in both cases (Figure 8).

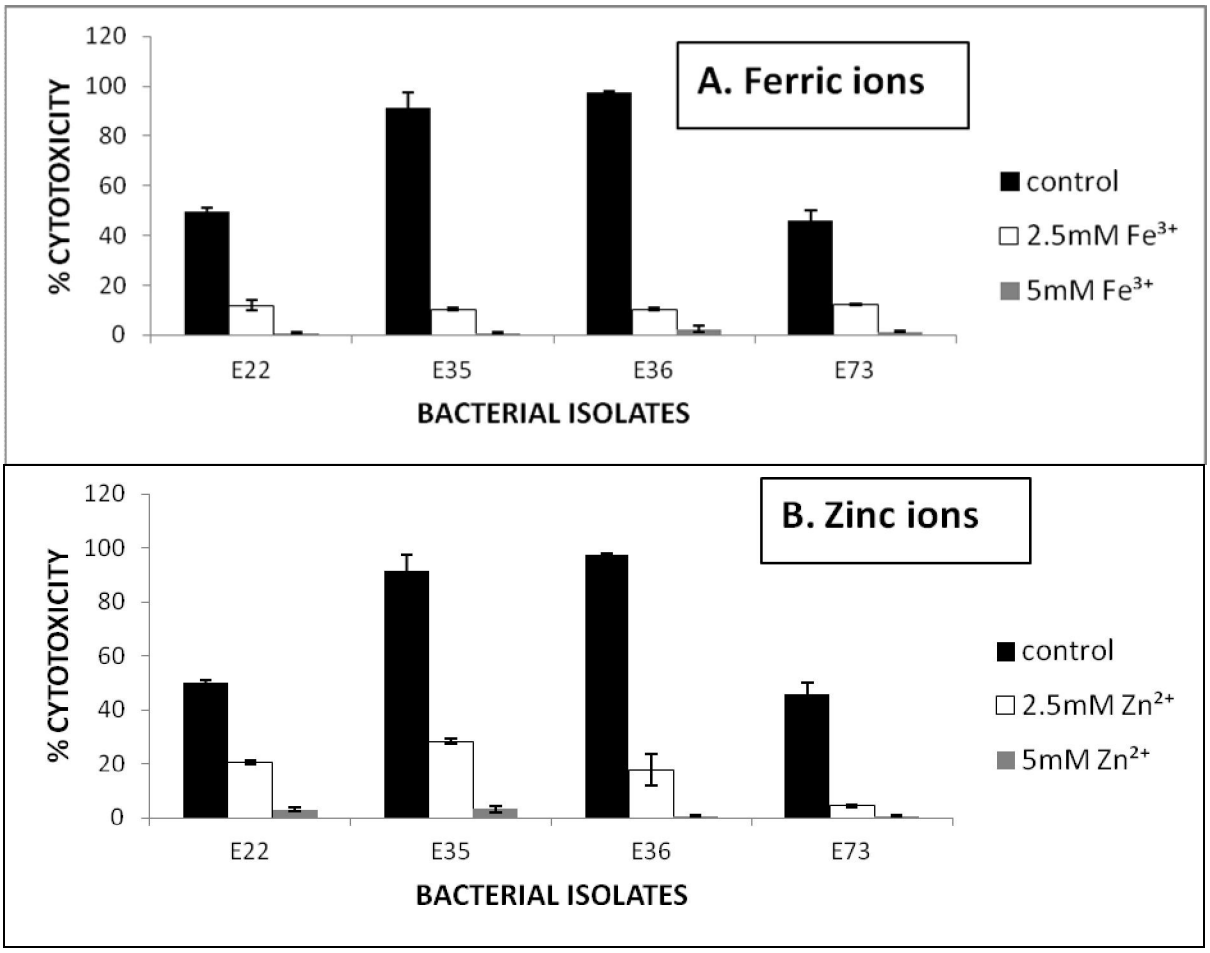

FIG 8. Effect of different concentrations of Iron (A) and Zinc ions (B) on the cytotoxicity of *E. coli* isolates after 3 h of Vero cell infection the histograms represent the average percent cytotoxicity caused by the isolates and the error bars indicate the standard deviations of the data.

4. Discussion

In the present study, the results concerning the *E. coli* cytotoxicity mechanisms could be summarized as follows:

1. Cytotoxicity was found to be cell associated since neither filtered supernatants obtained from 18-h cultures in TSB nor those obtained from 3-h cultures in MEM Earl's caused Vero cell cytotoxicity.
2. High cytotoxicity was observed using $1x10^8$ in 3 h only and this activity gradually decreased as the bacterial count decreased.
3. Upon microscopical examination, *E. coli* isolates caused altered morphologies and detachment of the Vero monolayer by different degrees that were apparent after 2 h.
4. Viable metabolically active bacteria were necessary for Vero cell killing for the tested isolates.
5. *E. coli* cytotoxicity was affected by changes in pH but not incubation temperature.
6. High adherence but poor invasion levels were obtained for all the tested isolates which indicates a strong relationship between adherence and cytotoxicity. However, this cytotoxicity was apparently not due to invasion.

Upon reviewing the literature accumulated on the cytotoxicity caused by *E. coli* isolates involved in sepsis or urinary tract infections (since E22 and E73 recovered from pus while E35 and E36 from urine specimens), it was found that *E. coli* produces a number of virulence factors with cytotoxic effects on cultured mammalian cells (Emody *et al.*, 2003). Of these virulence factors, hemolysins were reported to cause rapid cytotoxic effects within short infection periods similar to the one observed in the present study (Island *et al.*, 1998; Trifillis *et al.*, 1994). As

mentioned previously, *E. coli* produces 2 types of hemolysins, α-hemolysin which is found free in the supernatant and β-hemolysin which is cell associated (Smith, 1963; Short and Kurtz 1971; Jacobsen *et al.*, 2008; Mittal and Sharma, 1991). The results obtained in the present study show that cytotoxicity was cell associated which implies that β-hemolysin is the virulence factor involved in causing cytotoxicity of the tested bacterial isolates. In order to determine the role of β-hemolysin in the cytotoxic activity of the tested isolates, the hemolytic activity of the bacterial cells of these isolates was quantitated.

Hemolytic activities of *E. coli* isolates. As shown in the results (Table 2), at MOI of 200:1, high hemolytic activity and high cytotoxic effect were observed. Decreasing the bacterial count to a MOI of 20:1 lead to a decrease in the hemolytic activity on RBC and a corresponding decrease in the cytotoxic effect observed on the Vero cells for all the isolates. A clear relation could be seen between the hemolytic activity of the tested isolates and their cytotoxic effects on Vero cells. To ensure that no α-hemolysin was released during the 3 h incubation period, hemolytic activity of filter sterilized supernatants was measured and results confirmed that no cell free hemolysin was released under the tested conditions. Taken together, these findings imply that a cell associated hemolysin (β-hemolysin) was responsible for the observed cytotoxic effects. To further confirm this conclusion, the hemolytic activities of heat treated bacterial cells, gentamicin killed cells, bacterial cell lysates and metabolically inactive cells (bacteria cooled to 4° C) were measured. All these treatments have been shown to abolish the cytotoxic activities of the tested isolates. The results showed that these treated bacterial suspensions also lost their hemolytic activities which ensures the association between the hemolytic and cytotoxic activities of the tested isolates. The conclusion is that the β-hemolysin rather than the cell free α -hemolysin is the virulence factor involved in causing cytotoxicity of the tested *E. coli* isolates and therefore the findings obtained in the present study can be explained as follows:

1. Filtered supernatants from 18 h cultures were devoid of cytotoxic activity while the washed cells showed marked cytotoxicity after 3 h of incubation. This can be explained because β-hemolysins are cell associated and not secreted into the supernatant. Similar findings were obtained by Sobieszczanska *et al.* (2006) who suggested that cell bound hemolysin rather than cell free hemolysin was responsible for the cell detaching activity of *E. coli* strains tested to epithelial cells. However, this finding may also have been due to the fact that α-hemolysins are secreted only during the exponential phase of bacterial growth (Oropeza-Wekerle *et al.*, 1989). Mobley *et al.* (1990) demonstrated that marked cytotoxicity was noted at 3 to 6 h of incubation of epithelial cells with cells of hemolysin positive strains which coincided with the secretion of hemolysin during the mid-exponential phase of growth and which is switched off during the late exponential phase with hemolytic activity rapidly diminishing thereafter. In addition, Trifillis *et al.* (1994) observed significant killing of epithelial cells by the cells of a hemolysin producing *E. coli* strain after 1 h of incubation and showed that α-hemolysin was the primary factor responsible for this cytolethal effect. This was confirmed by the presence of cytolethal activity in bacterial filtrates obtained from bacterial dilutions (10^6 cfu/ml) allowed to grow for 5 h to maximize hemolysin production. Therefore, in order to determine if α-hemolysin was the factor responsible for the observed cytotoxic effects in the present study (whether α-hemolysin was secreted during the 3 h incubation period), bacteria were allowed to grow for 3 h in MEM Earl's medium, then these suspensions were filter sterilized prior to incubation with Vero cells. As shown in the results, these filtrates failed to cause cytotoxic effect to Vero cells which indicate that α-hemolysin is most probably not the factor responsible for the observed cytotoxic effects. In contrast to α-hemolysins, β-hemolysins are detectable during the exponential and stationary growth phases (Sobieszczanska *et al.*, 2006).

2. High cytotoxicity was observed after 3 h only and this cytotoxicity gradually decreased as the bacterial count decreased since β-hemolysins were reported to cause rapid cytotoxic effects on cultured mammalian cells. Sobieszczanska *et al.* (2006) reported cytotoxic activity of *E. coli* isolates (producing β-hemolysin) on epithelial cells after 90 min of incubation only. Cytotoxicity gradually decreased as the bacterial count decreased due to a corresponding decrease in the β-hemolytic activity of these isolates. A clear relation was seen between the hemolytic and cytotoxic activities of the tested isolates. A similar correlation was obtained by Peerbooms *et al.* (1984) between the hemolytic activities of the tested *Proteus* strains and their cytotoxic effects on Vero cells due to a hemolysin which resembles β-hemolysin of *E. coli*. Moreover, results obtained by Sobieszczanska *et al.* (2006) indicated a close relationship between the hemolytic and cell detaching activities of *E. coli* cells. In addition, 3 h incubation with mid interval removal of bacteria led to a decrease in the observed cytotoxic effects which indicates that more adherence probably leads to stronger cytotoxic effects.

It was noteworthy that E35 and E36 isolates which were obtained from urine showed stronger β-hemolytic activities and cytotoxicities than E22 and

E73 isolates which were obtained from pus. This may be due to the potency of the cell associated toxin since a correspondingly high hemolytic activity was also obtained for these two isolates rather than their affinity to Vero cells which are similar to the cells of the infection site from which they were isolated (UPEC).

3. Upon microscopical examination, *E. coli* isolates caused altered morphology and detachment of the Vero monolayer by different degrees which was apparent after 2 h. Similar cell detachment after 90 min of infection of epithelial cells by *E. coli* isolates due to β-hemolysin was reported by Sobieszczanska *et al.* (2006).

4. Viable metabolically active bacteria were found to be necessary for cell cytotoxicity since gentamicin treated bacteria, heat treated bacteria, bacterial cell lysates and metabolically inactive cells (cooled to 4°C) failed to cause Vero cell cytotoxicity. This could be explained because cell bound hemolysin activity was reported to be closely associated with cell viability (Snyder and Koch, 1966). Short and Kurtz (1971) showed that disruption of cells by sonic treatment failed to liberate β-hemolytic activity into the medium and that this cell associated activity declined as the number of viable cells declined. Furthermore, they reported that agents that interfere with cellular metabolism e.g. streptomycin completely inhibited β-hemolytic activity. In addition, Sobieszczanska *et al.* (2006) showed that inactivation of the cell bound hemolysin by heating *E. coli* cells at 56°C for 10 min, or UV irradiation was associated with loss of their mammalian cell detaching activity. Moreover, Kaca and Rozalski (1991) showed that cell bound hemolysin does not show hemolytic activity at 4°C which explains the loss of cytotoxicity at this temperature.

5. Cytotoxicity of *E. coli* isolates was affected by changes in pH but not temperature. Concerning pH, a greater cytotoxic activity was obtained at a pH of 6 for the E22 & E73. This could be explained since Snyder and Koch (1966) reported that β-hemolysin produced during the early period of production (1, 2 and 3 h cultures) had a pH optimum of 6 or less. In case of temperature, similar to our findings but with a different pathogen, Kaca and Rozalski (1991) showed that *Proteus* strains exhibited similar cell bound hemolytic activities at 25, 37 and 42°C.

6. Highly cytotoxic isolates were found to be highly adherent to Vero cells which indicates a relation between adherence and cytotoxicity of these isolates. This may be because β-hemolysin is cell bound and so greater contact with host cells is accompanied with a higher cytotoxic effect. It has been reported previously that cytotoxicity caused by bacteria-associated hemolysin requires some degree of contact or close proximity between the bacteria and host cells (Gadeberg and Orskov, 1984). However, this β-hemolytic activity apparently does not require invasion of bacteria into the epithelial cells. In addition, as mentioned earlier, invasion was reported to be an unimportant aspect in the pathogenesis of some *E. coli* isolates (Peerbooms *et al.*, 1984).

In 2000, Guignot *et al.* investigated the pathogenicity of a pyelonephritogenic *E. coli* strain and found that the cytotoxicity of this strain to mammalian cultures is apparent at 2 h post infection (p.i.) and completely detached the cell monolayer if left more than 3 h post infection. They identified the virulence factor to be hemolysin (*hly*A) although surprisingly, sterile supernatants of 2.5, 7 or 18-h cultures failed to induce cell lysis. They therefore suggested that this strain does not secrete a functional hemolysin and/or that cell contact is important for efficient cell lysis. This might be another possibility for the results obtained in the present study.

Inhibition of cytotoxic activities of the tested bacterial isolates

Bacterial virulence mechanisms have been recently explored as attractive targets for the development of new therapeutic agents (Felise *et al.*, 2008). Inhibition of cytotoxic effects caused by bacterial isolates serves as a potential mode of treatment for infections caused by these isolates. Several approaches have been proposed in an attempt to inhibit bacterial cytotoxicity. These approaches include the use of agents that reduce bacterial adhesion (Apodaca *et al.*, 1995) since inhibition of adherence of bacteria leads to a pronounced effect on the pathology by directly decreasing toxin loads (Zaidi *et al.*, 1997).

Another approach includes the use of metal ions. Bacterial toxins are one of the virulence factors produced by bacteria that can be regulated by environmental conditions. For example, there is direct evidence which shows that iron operates at the molecular level and acts as a regulatory molecule, controlling not only the iron acquisition machinery but the expression of toxins (Sritharan, 2006). There are a number of reports regarding the effect of iron on the virulence of *P. aeruginosa* available (Mittal *et al.*, 2008). In the present study, the effect of both antiadherent agents and metal ions on bacterial cytotoxicity was tested.

The results show that the sugars tested caused an inhibition in the cytotoxicities of the tested isolates. These results may be attributed to a corresponding reduction in the adherence of these isolates. Hafez *et al.* (2005) previously reported that glucose and galactose showed a dose dependant reduction in the

adherence of *E. coli* isolates to Vero cells. In addition, a greater reduction in adherence was obtained with galactose compared to glucose which was in accordance with the results obtained in the present study. Mannose was also tested by the authors against *E. coli* isolates and showed maximum reduction in adherence which was greater than that obtained by glucose and galactose (reaching >75% at 5% mannose). This may explain why 5% mannose nearly abolished the cytotoxicities of the *E. coli* isolates used in the present study. In addition, Guggenbichler (1983) and Salit & Gotschlich (1977) also reported that mannose inhibited the adherence of *E. coli*. Dextrans are α-1,6-linked glucose polymers that have been shown by other investigators to nonspecifically coat eukaryotic cells (Barghouthi *et al.*, 1996) and inhibit the adherence of several bacterial pathogens (Thomas and Brooks 2004; Bryan *et al.*, 1999). Therefore, its effect on bacterial cytotoxicity was investigated by pretreatment of Vero monolayer for 30 min without its removal during the assay. As shown in the results (Figure 6), dextran 70 reduced the cytotoxicities of all the tested isolates. Again, this effect may be attributed to dextran's antiadherent action. Because they are inexpensive and nontoxic, dextrans might be ideal therapeutic agents for prevention of bacterial infections.

The four nonionic surfactants (tween 20, tween 80, poloxamer 188 and poloxamer 407) have been previously reported to nonspecifically reduce the adherence of *E. coli* isolates to Vero cells by decreasing the surface hydrophobicity of isolates (Hafez *et al.*, 2005). Therefore, their effect on bacterial cytotoxicity was investigated. As shown in the results, tween 80 reduced the cytotoxicities of the *E. coli* isolates. Poloxamer 407 was found to cause a greater reduction in the cytotoxicities of the tested isolates. The effect of these excipients on cytotoxicity may be attributed to their nonspecific effect on adherence of the tested isolates. Hafez *et al.* (2005) reported that these nonionic surfactants caused a significant reduction in the adherence of *E. coli* isolates to Vero cells. In addition, they reported that the highest antiadherent effect was observed with poloxamer 407 where the reduction in adherence reached about 98% of that of the control which explains why a concentration of 0.05% nearly abolished the cytotoxicities of some isolates in the present study. Taken together, the obtained results strongly suggested that the use of such excipients especially poloxamers in formulating preparations could possibly be a prophylactic approach against *E. coli* infections.

Since metal ions were known to affect bacterial virulence (Alksne and Projan, 2000), the effect of three metal ions on bacterial cytotoxicity was investigated. As shown in the results, calcium ions had little or no effect on the cytotoxicity of the tested *E. coli* isolates at the concentrations tested (2.5 and 5 mM). In case of iron ions, the results showed that at 2.5 mM, Fe^{3+} decreased the cytotoxicities of all the isolates while a concentration of 5 mM nearly abolished the cytotoxicities of the isolates. Litwin and Calderwood (1993) reported that several bacterial toxins, including toxins from *E. coli* and *C. diphtheria* were produced in lesser amounts when an excess of Fe^{3+} was present in the culture media.

Regarding the effect of zinc ions, results showed that Zn^{2+} ions caused a pronounced reduction in the cytotoxicities of all the tested isolates and 5 mM nearly abolished these cytotoxicities. Crane *et al.* (2007) reported that zinc causes a decrease in the expression of *E. coli* protein virulence factors and reduces their adherence to tissue culture cells due to inhibition of adhesins. In addition, Roselli *et al.* (2003) reported that Zn^{2+} protected intestinal cells from enterotoxigenic *E. coli*-induced damage by inhibiting bacterial adhesion, preventing the disruption of barrier integrity and modulating cytokine expression.

5. Conclusion,

The tested agents which were shown to inhibit cytotoxicity of the tested isolates could be used in the prevention and/or treatment of many *E. coli* infections.

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