Adherence, Invasion and Cytotoxicity of Some Bacterial Pathogens

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Abstract: One hundred and sixty two bacterial isolates recovered from different collected clinical specimens were screened for adherence, invasion and cytotoxicity against Vero cells. All these isolates were able to adhere to Vero cells by variable degrees. Concerning invasion, the *Staphylococcus* spp were found to have the highest levels of invasion reaching 2.3% while a few *Pseudomonas* spp showed invasion levels between 1 % and 2 % but the rest of the *Pseudomonas* isolates and all the other Gram negative rods showed invasion levels <1%. Upon screening for cytotoxicity by the MTT assay, about 38% of the *Staphylococcus* species showed only low cytotoxicity (<50%) after 24 h of incubation while the rest of the isolates showed no cytotoxic effects on Vero cells. In case of *Pseudomonas* species, after 6 h, 38.5% of the isolates showed low cytotoxicity (<50%), 7.7% showed moderate cytotoxicity (50-85%) while 11.5% of these isolates exhibited high cytotoxic effects (>85%). In case of the other Gram negative rods, 44.4% of the isolates showed low cytotoxicity, 3.2% showed moderate cytotoxicity while 6.3% showed high cytotoxic effects after 6 h of Vero cell infection. Moreover, statistical data (excluding the highly cytotoxic isolates) showed a significant positive correlation between adherence and invasion of all the tested isolates. In addition, a statistically significant positive correlation was found between the adherence and cytotoxicity of the *Staphylococcus* and *Pseudomonas* species. Concerning the statistical data for invasion and cytotoxicity, a significant positive correlation was found in case of *Pseudomonas* isolates only. Taken together, these results indicate that invasion is a post adherence effect, and that cytotoxicity of both *Staphylococcus* and *Pseudomonas* species is associated with a higher adherence to epithelial cells. Moreover, the results suggest that invasive *Pseudomonas* species can induce cytotoxicity but at low levels. [Journal of American Science. 2010;6(10):260-268]. (ISSN: 1545-1003).

Keywords: Adherence, Invasion, Cytotoxicity, Vero cells, *Pseudomonas*, *Staphylococcus*, MTT

1. Introduction

Bacteria are among the most diverse living organisms and have adapted to a great variety of environments including the human body. These bacteria use a number of different virulence mechanisms that allow them to conquer many different niches throughout the course of infection. Bacterial virulence is a multifactorial process that requires the use of a variety of components, many of which are coordinately regulated to allow the organism to adapt to the host environment and become successful pathogens. Bacteria uses different strategies to adhere to, in some cases invade and/or kill cells within their hosts (Pizarro-Cerda and Cossart, 2006).

Bacterial adherence is the establishment of the bacterial pathogen at the appropriate portal of entry. It corresponds to a specific interaction between a ligand expressed on the bacterial surface (adhesin) and a receptor on the epithelial cell surface. The process of bacterial adherence to host cells is an important step in the initiation of bacterial infection (Sansonetti, 1993).

In some bacterial species, invasion may follow the adherence step. Different bacterial pathogens have evolved different strategies to gain access to the intracellular compartment. Once intracellular, invasive bacteria can survive, multiply and spread (Sansonetti, 1993). Invasion is aided by the production of extracellular substances called invasins which are protein enzymes that act locally to facilitate growth and spread of the pathogen. The complexity of the bacterial tools used for cell adherence and invasion ranges from single monomeric proteins to intricate multimeric macromolecules that perform highly sophisticated functions and can be truly considered as nanomachines (Pizarro-Cerda and Cossart, 2006).

Certain bacterial species can establish locally and cause infections that remain extracellular by secreting toxins with local or systemic effects (Sansonetti, 1993). These toxins have a central role in the pathogenesis of bacterial disease and may damage or kill host cells by different mechanisms. Studying the virulence of bacterial

pathogens will help control disease and develop new strategies to prevent bacterial infection. The present study was undertaken to investigate three different virulence mechanisms (adherence, invasion & cytotoxicity) of bacterial pathogens isolated from different clinical specimens and the relation between these different mechanisms.

2. Materials and Methods

Bacterial isolates and culture conditions

A total of 162 bacterial isolates including 47 *Staphylococcus* species, 52 *Pseudomonas* species and 63 other Gram negative rods were used in this study. These isolates were recovered from different collected clinical specimens including pus, sputum, urine, blood and stool specimens. Bacterial isolates used in the study are listed in Table 1. All the recovered clinical isolates were maintained onto nutrient agar slants at 4°C and subcultured every month. For conducting experiments, these bacterial isolates were cultured for 18 to 20 h at 37°C in Trypticase Soy Broth (TSB) under static conditions.

Table 1. Clinical sources and microscopical characters of the collected isolates

Specimen	Number of isolates	Group Category		
		Staphylococcus species	Gram negative rods	
			Pseudomonas species	Other species
Pus	74	22	30	22
Sputum	46	18	11	17
Urine	35	5	10	20
Blood	4	1	1	2
Stool	3	1	0	2
	Σ 162	Σ 47	Σ 52	Σ63

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).

Adherence and Invasion assays

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

After washing the Vero monolayer, aliquots of 100 µl of the bacterial suspensions (harvested by centrifugation, washed and resuspended in MEM Earl's to an absorbance corresponding to 1×10^8 cfu/ml) was added to each well (8 wells for each isolate). Following 2 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. 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 2 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.

Cytotoxicity assay

The MTT assay was carried out as described by Saliba *et al*. (2002). Confluent monolayers of Vero cells cultured in 96-well tissue culture plates were washed with PBS and fifty microliter aliquots of MEM Earl's was added to each well. After culturing the tested isolates for 18-20 h at 37°C in TSB, 50 µl aliquots of this culture (adjusted to contain 2 x 10^8 cfu/ml) were added to the wells (8 wells for each isolate). Control wells contained 50 µl MEM Earl's & 50 µl sterile TSB instead of the added bacterial culture. Following 6 h incubation at 37°C, the cells were washed with PBS and

incubated for 1 h with MEM Earl's containing gentamicin at 300 µg/ml to kill the adherent bacteria. After washing with PBS, cells were exposed to 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) at 2 mg/ml in PBS for 1 h at 37°C. Supernatants were then removed and cells were treated with 100 µl absolute ethanol to dissolve the formazan crystals formed in the viable metabolically active cells. The eluates of the 8 wells of each tested isolate were collected and their absorbance was measured at 540 nm (Pharmacia Biotech Ultraspec 2000). The percentage cytotoxicity was calculated using the following formula (Murakami *et al.*, 2000):

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

In case of *Staphylococcus* species, the MTT assay was carried out after 24 h of Vero cell infection.

Identification of the highly cytotoxic isolates

Further identification was only carried out for the highly cytotoxic isolates by API 20E identification kits (BioMerieux, Inc., France). The tests were performed according to the manufacturer's instructions and incubated for 24 h at 37º C. After incubation, the color reactions were read (some with the aid of added reagents). The oxidase reaction was done separately at Ain Shams specialized Hospital using Oxidase Discs (HiMedia Laboratories Pvt. Limited, India). The identification was obtained using the API Identification Table.

Statistical analysis

Statistical analysis was performed using SPSS 12 for Windows Software. Spearman's correlation coefficient (r_s) & their significance (P) were calculated to determine the correlation between different pairs of the three studied virulence mechanisms. A correlation was considered strong as r_s approaches 1, intermediate if r_s was close to 0.5 and weak if close to zero. A value for $P < 0.05$ was taken to indicate a statistically significant correlation (Silva *et al.*, 2008).

3. Results

Adherence and invasion of the bacterial isolates to Vero cells

The ability of all the collected bacterial isolates to adhere to and invade the Vero cells was investigated and results were expressed as a percentage of the initial bacterial count. Ten of the isolates (6 *Pseudomonas* species and 4 other Gram negative rods) caused detachment of the Vero monolayer within the 2 h of the assay and therefore their abilities to adhere to and invade the Vero cells were not determined. Adherence of *Staphylococcus*, *Pseudomonas* and other Gram negative species were in the ranges 0.007 - 8.05 %, 0.0006 - 4.65 % and 0.0022 - 7.95% respectively. On the other hand, invasion values ranged from 0 - 3.3 %, 0 - 1.67% and 0 - 0.92 % for *Staphylococcus*, *Pseudomonas* and other Gram negative species respectively.

The profiles of adherence and invasion of the different bacterial categories to Vero cells are shown in Figures 1 & 2. As shown in the Figures, the percentage of *Staphylococcus* isolates showing adherence in the range >0.1-≤1% was the highest while about 15% of the *Staphylococcus* isolates showed high adherence values (> 5%). On the other hand, the percentage of these isolates showing invasion in the range >0.01-≤1% was the highest while 4.3% showed high invasion levels (> 2%). In case of *Pseudomonas* species, the percentage of isolates showing adherence ≤0.1% was the highest. In addition, 21.74% of the isolates showed adherence between 2-5% but none of these isolates showed adherence >5%. Concerning invasion, most of these isolates showed invasion ≤0.01%, while a few of the *Pseudomonas* isolates showed invasion levels between 1-2% and none of these isolates showed high invasion (>2%). In case of the other Gram negative rods, again the percentage of isolates showing adherence in the range >0.1-≤1% was the highest while only one isolate showed high (>5%) adherence. On the other hand, these isolates showed variable levels of invasion while none of these isolates showed invasion >1%. Moreover, about 15% of *Staphylococcus* species, 24% of the *Pseudomonas* species & 23.7% of the other gram negative rods did not invade the Vero cells.

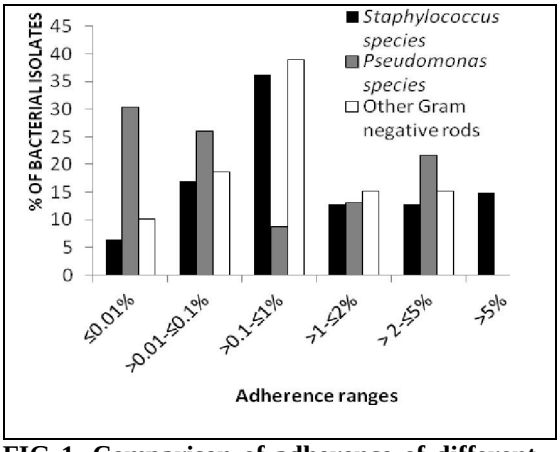

FIG 1. Comparison of adherence of different to Vero cells categories to Vero cells

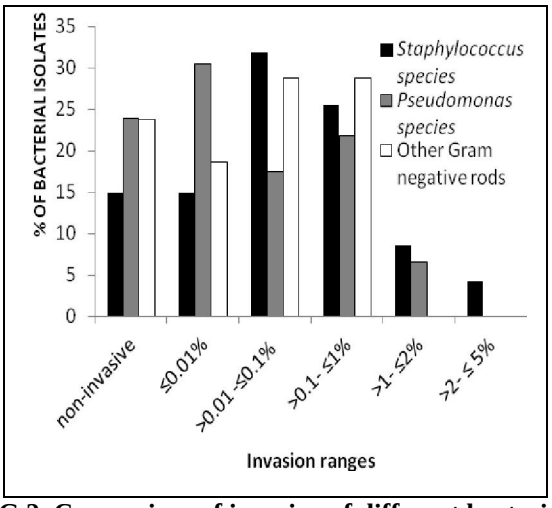

FIG 2. Comparison of invasion of different bacterial categories

Cytotoxicity of the bacterial isolates to Vero cells

The cytotoxicity of all the collected isolates was tested. Results were expressed as percentage cytotoxicity and were summarized in Table 2. For *Staphylococcus* species, none of the isolates showed cytotoxic activity after 6 h of Vero cell infection (data not shown). When the assay was repeated after 24 h of infection, again 29 isolates showed no cytotoxixity and only low cytotoxicity was exhibited by 18 isolates. In case of the *Pseudomonas* isolates, after 6 h of infection, 20 isolates showed low cytotoxicity, 4 isolates showed moderate cytotoxicity while 6 isolates (having the codes P127, P139, P205, P206, P217 & P231) showed high cytotoxicity. In case of the other Gram negative rods, 28 isolates showed low cytotoxicity, 2 isolates showed moderate cytotoxicity & 4 isolates (having the codes E22, E35, E36 & E73) showed high cytotoxic effects. It is noteworthy that the 6 *Pseudomonas* isolates and the 4 other Gram negative rods showing high cytotoxicity were the same isolates that caused Vero cell detachment during the adherence and invasion assays.

Table 2. Cytotoxicity of bacterial isolates to Vero cells

Bacterial isolates		No. of bacterial isolates			
		NO CYTOTO-XICITY	LOW **	MODERATE **	HIGH **
Staphylococcus* species		29	18	0	0
Gram negative rods	***Pseudom-onas* species**	22	20	4	6
	Other species	29	28	2	4

* Results after 24 h of Vero cell infection

** Low cytotoxicity (<50%), moderate (50-85%) & high (>85%)

Identification of the highly cytotoxic isolates

The ten highly cytotoxic isolates were further identified by API 20E Identification Kits. The isolates having the codes P127, P139, P205, P206, P217 and P231 were identified as *Pseudomonas aeruginosa* while the isolates having the codes E22, E35, E36 and E73 were identified as *Escherichia coli*.

Relationship between adherence, invasion and cytotoxicity of the tested isolates

After screening all the collected isolates for adherence, invasion and cytotoxicity, the results (excluding the highly cytotoxic isolates) were analyzed using Spearman's correlation coefficient (r_s) to determine whether a relation existed between these different virulence mechanisms. These results are summarized in Table 3. A strong and statistically significant positive correlation was observed between the adherence and invasion of the *Staphylococcus* & *Pseudomonas* species (r_s = 0.851 & r_s = 0.787 respectively, $P < 0.01$) while an intermediate but also significant correlation was found for the other Gram negative rods (r_s = 0.559, $P < 0.01$). On the other hand, the statistical data of adherence & cytotoxicity of the tested isolates showed a strong and significant positive correlation in case of *Pseudomonas* species (r_s = 0.765, $P < 0.01$) while an intermediate but also statistically significant positive correlation was found for the *Staphylococcus* species (r_s = 0.632, $P < 0.01$). However, a weak non significant correlation was obtained in case of the other Gram negative rods (r_s = 0.305, P =0.101). However, the statistical data of invasion and

cytotoxicity of the tested isolates showed an intermediate but significant positive correlation in case of the *Pseudomonas* species (r_s = 0.517, P < 0.01) whereas a weak non significant correlation was found in case of the rest of the isolates (r_s = 0.427, P =0.077 & r_s = 0.118, P = 0.534 for *Staphylococcus* species & other Gram negative rods respectively).

In case of invasion, results revealed that about 15% of the *Staphylococcus* species, 24% of the *Pseudomonas* species while 23.7% of the Gram negative rods other than *Pseudomonas* species were unable to invade Vero cells. The rest of the tested isolates invaded Vero cells by different degrees. It was noteworthy that the *Staphylococcus* species showed the highest levels of invasion (reaching 2.3%) while a few *Pseudomonas* species showed invasion levels >1%. On the other hand, none of the Gram negative rods other than *Pseudomonas* species showed invasion >1%. Probabaly, cell invasion is not an important aspect in the pathogenesis of most of the Gram negative rods tested.

TABLE 3. Spearman's correlation coefficient values between different pairs of virulence mechanisms of the different bacterial categories

Bacterial Category	Virulence Mechanisms		
	Adherence vs Invasion	Cytotoxicity vs Adherence	Invasion vs Cytotoxicity
***Staphylococcus* species**	0.851*	0.632*	0.427
***Pseudomonas* species**	0.787*	0.761*	0.517*
Other Gram negative rods	0.559*	0.305	0.118

Significance level : * P < 0.01

4. Discussion:

The Bacteria can have wide ranging & deleterious effects on the hosts (Heinzelmann *et al.*, 2002). In order to be able to infect hosts, bacteria need different virulence systems. Processes like adherence, invasion and evasion of host defense are crucial for the bacteria during infection (Kauppi, 2006). To overcome infections caused by bacterial pathogens, it is necessary to clarify the pathogenicity mechanisms that are used by these pathogens, and the relationship between these different virulence mechanisms. The results of the present study revealed that all the tested isolates were able to adhere to the Vero cell line but by variable degrees. However, no general relation between the origin of isolation and the ability of the tested isolates to interact with Vero cells could be established. In addition, Figure 1 shows that no pronounced difference was observed upon comparing the adherence of *Staphylococcus* species and Gram negative rods other than *Pseudomonas* species. The percentage of isolates in both categories that showed adherence in the range >0.1-≤1% was the highest. However, while 15% of *Staphylococcus* species showed adherence >5%, only 1.6% of the Gram negative rods other than *Pseudomonas* species showed adherence in this range. On the other hand, most of the *Pseudomonas* species showed low (≤0.1%) or high (>2-≤5%) adherence values while none showed adherence >5%. These adherence values were comparable to those reported by other investigators (Hafez *et al.*, 2005; Woolley *et al.*, 2008)

Cytotoxicity of the collected isolates was also investigated using the MTT assay. MTT assay is a non radioactive colorimetric assay system first described by Mosmann T (1983) and is used for measurement of cytotoxicity caused by bacterial pathogens (Saliba *et al.*, 2002) (Kubica *et al.*, 2008) (Couto *et al.*, 2007). It detects early interference with cell metabolic activity. After 6 h of incubation with Vero cells, none of the *Staphylococcus* species showed a cytotoxic effect. This indicates that these isolates were not cytotoxic to Vero cells or required a longer time to induce cytotoxicity. Therefore, the infection time was prolonged to 24 h for these isolates. Results show that after 24 h, 38.3% of the isolates showed only low cytotoxicity (<50%) while the rest of the isolates still showed no cytotoxic effects on Vero cells. These results are consistent with previous studies testing the effect of different *Staphylococcus* species against Vero cells. For instance, it has been reported that *Staphylococcus epidermidis* was devoid of cytotoxic activity to Vero cells (Allaker *et al.*, 2006). In addition, no cytotoxic effect was observed when *S. sciuri* was tested against Vero cell monolayers after 24 h (Stepanovic *et al.*, 2001). Moreover, when *S. aureus* cytotoxicity to Vero cells was examined, only 6 out of 20 tested

strains exhibited cytotoxic effects after 24 h of infection (Tao *et al.*, 1999). These findings could be explained because it has been shown that the cytotoxic effects of *Staphylococcus* species vary considerably depending on the cell type used in the study (Stepanovic *et al.*, 2001).

In case of *Pseudomonas* species, after 6 h of incubation with Vero cells, 20 isolates showed low cytotoxicity (<50%), only 4 isolates showed moderate cytotoxicity (50-85%) while 6 of these isolates which were later identified to be *P. aeruginosa* exhibited high cytotoxic effects (>85%). These cytotoxic effects may be attributed to various products since *Pseudomonas* species have been reported to produce many extracellular as well as cell associated virulence factors that are cytotoxic to mammalian cells including proteases (Zhang *et al.*, 2003), phospholipase C (Meyers *et al.*, 1992) (Rossignol *et al.*, 2008) exotoxin A (Bourke *et al.*, 1994) and exoenzymes S & U (Vallis *et al.*, 1999). Moreover, Sawa *et al.* (1998) found that the invasive strains of *P. aeruginosa* could also cause cytotoxicity in human epithelial cells after the application of a large inocula. In case of Gram negative rods other than *Pseudomonas* species, after 6 h of Vero cell infection, 28 isolates showed low cytotoxicity, 2 isolates showed moderate cytotoxicity while 4 isolates which were later identified to be *E. coli* showed high cytotoxic effects. Again, several bacterial products produced by different Gram negative species were reported to cause cytotoxic effects to cultured mammalian cells including hemolysins of *Proteus* species (Mobley *et al.*, 1991) shiga toxins of *Shigella* species (Sandvig, 2001), cytotoxins of *Salmonella* species (Ashkenazi *et al.*, 1988), shiga-like toxins of *E. coli* (Giraldi *et al.*, 1990) (Roberts *et al.*, 2001) & hemolysins of *E. coli* (Island *et al.*, 1998). Therefore the cytotoxic effects observed by the tested isolates in the present study might have been due to one or more of these cytotoxins.

After screening of all the bacterial isolates for adherence, invasion and cytotoxicity, it was interesting to find out whether a relationship existed between these different virulence mechanisms. As shown in the results, the statistical data showed a significant positive correlation between adherence and invasion of the tested isolates (*Staphylococcus* species, *Pseudomonas* species and other Gram negative rods). This indicates that invasion is a post adherence event and that bacterial adherence to the host cells is a prerequisite for invasion to take place. These findings are in agreement with those obtained by Hensen *et al.* (2000) who reported that the percent of invasion of *S. aureus* isolates was highly correlated with their percent of adherence to epithelial cells. In addition, Schmidt *et al.* (1989) also demonstrated a strong correlation between adherence and invasion of *Staphylococcus* species to epithelial cells. Moreover, Martinez *et al.* (2000) reported that the type I pilus adhesion FimH in some *E. coli* mediates not only adhesion but also invasion of epithelial cells. Regarding *Pseudomonas* species, Plotkowski *et al.* (1994) demonstrated that pilated *P. aeruginosa* was taken up by endothelial cells at a higher rate than the nonpilated *P. aeruginosa* since adherence is the first necessary step in the process of cell endocytosis.

The statistical data for adherence and cytotoxicity of the tested isolates showed a significant positive correlation in case of *Staphylococcus* and *Pseudomonas* species. This indicates that cytotoxicity of these isolates may be associated with higher adherence to epithelial cells. Enhanced adherence might enable a greater number of cells to attach to the host cell surface which may collectively produce a stronger cytotoxic effect. These findings are in agreement with those obtained by Zaborina *et al.* (2006) who reported that adherence significantly correlated with epithelial barrier disruption caused by *Pseudomonas aeruginosa*. Martino *et al.* (2002) & Silva *et al.* (2008) also demonstrated a strong and statistically significant correlation between *Pseudomonas aeruginosa* cytotoxicity and adherence. Moreover, Rajan *et al.* (2000) recently demonstrated that only fully virulent *P. aeruginosa* capable of coordinately expressing both adhesins and cytotoxins were able to induce apoptosis in epithelial cells. Concerning *Staphylococcus* spp, Cifrian *et al.* (1996) previously reported that *Staphylococcus* strains expressing toxins showed enhanced adherence. No significant correlation was observed in case of other Gram negative rods which indicates that cytotoxicity caused by these isolates may be due to extracellular products that do not require adherence of the bacterial isolates to Vero cells.

On the other hand, the statistical data for invasion and cytotoxicity showed a significant correlation in case of *Pseudomonas* isolates only. This indicates that invasive *Pseudomonas* species can induce low levels of cytotoxicity. In 1998, Sawa *et al.* reported that application of a large inocula of an invasive *P. aeruginosa* strain caused cytotoxicity in cultured epithelial cells. This indicates that some *Pseudomonas* isolates are invasive and induce low levels of cytotoxicity after prolonged incubation. Similar results were obtained by Zhu *et al.* (2000) who reported that invasive *Pseudomonas aeruginosa* can induce

cytotoxicity in epithelial cells at high bacterial numbers. This cytotoxicity was related to bacterial density and the mechanism maybe different from the one induced by strains causing acute cytotoxicity. In addition, Cowell *et al.* (1999) reported that *P. aeruginosa* may be grouped into 2 phenotypes: invasive and cytotoxic. Cytotoxic strains remain outside the host cells and induce acute cytotoxicity from this extracellular location within 3 h. In contrast, invasive *P. aeruginosa* enter epithelial cells and may kill cells only after prolonged incubation. No relation was observed in case of the rest of the isolates (*Staphylococcus* and other Gram negative species) which indicates that cytotoxicity caused by these isolates was independent of their ability to invade Vero cells.

5. Conclusion,

The results of the present study reveal that all the collected isolates were able to adhere to Vero cells by variable degrees. Concerning invasion, some *Staphylococcus* & *Pseudomonas* species were able to efficiently invade Vero cells whereas others were not. On the other hand, cell invasion was probably not an important aspect in the pathogenesis of most of the Gram negative rods tested. Upon screening for cytotoxicity, a few of the *Staphylococcus* species showed only low cytotoxicity after prolonged incubation, while the *Pseudomonas* species and other Gram negative rods showed variable levels of cytotoxicity after 6 h of incubation. In addition, the statistical analysis revealed that bacterial adherence to the host cells is a prerequisite for invasion to take place. Moreover, the percentage cytotoxicity of the *Staphylococcus* and *Pseudomonas* isolates was highly correlated with their percent of adherence to epithelial cells. In addition, the invasive *Pseudomonas* species can induce low levels of cytotoxicity while there was no correlation between invasion and cytotoxicity for the rest of the tested isolates.

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1. 6. References

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