Detection of cytotoxic activity on Vero cells in clinical isolates of *Serratia marcescens*

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Abstract

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Cytotoxin production was studied in 60 *Serratia marcescens* strains isolated from hospitalized patients. Association of cytotoxic activity with serotype, source of isolation and presence of plasmids was also evaluated. Thirteen of the 60 *S. marcescens* strains produced a cytotoxic effect on Vero cells. These strains were isolated from distinct clinical sources and classified into seven different serotypes (O1:H7; O4:NM; O10:NT; O19:NM; O6,14:H4; O6,14:NM and O6,14:H1). No relationship was observed between cytotoxic activity and clinical source or serotypes of the strains. Plasmids from five cytotoxin-producing *S. marcescens* strains were transferred to *E. coli* K12/711. The transconjugants did not exhibit cytotoxicity, indicating that the cytotoxic effect is not plasmid-mediated among these strains. Although a cytotoxic activity was demonstrated in filtrates of some *S. marcescens* strains, further studies should be performed to assess the role of this toxin in pathogenesis.

Introduction

Serratia marcescens has been recognized as an important nosocomial pathogen causing respiratory and urinary tract infections (1), bacteremia (2), meningitis (3,4), peritonitis (5), and other disorders (6,7). This bacterium has been considered to be an important agent of nosocomial infections, particularly among newborns and patients submitted to invasive procedures at the University Hospital of the School of Medicine of Ribeirão Preto, SP, Brazil.

Reports in the literature regarding virulence of this bacterium have shown characteristics such as resistance to serum bactericidal activity (8), cell-bound hemolysin (9,10), presence of fimbriae and adherence to uroepithelial cells (11,12), and production of extracellular proteases (13) and nucleases (14). Cytotoxin production has been considered to be an important virulence factor shown by several bacteria (15-18), but as far as we know there are no data on cytotoxin production by *S. marcescens* strains.

Bacterial toxins are currently detected according to action on mammalian cell lines, evidenced by changes in cell shape. These

morphological changes are defined as cyto-pathic effects and can be useful to classify a cytotoxin. Most bacterial toxins cause initially cytopathic effects but only those denominated cytotoxin lead to death of cultured cells (19). In addition, viability of the cell monolayer treated with a cytotoxin can be determined using the neutral red assay (20,21).

The purpose of the present study was to investigate the ability of clinical isolates of *S. marcescens* to produce cytotoxin. The correlation of cytotoxin production with clinical source, serotype of strains and presence of plasmids was studied. Characteristics of the cytotoxic activity such as heat stability and the type of cytopathic effect were also evaluated.

Material and Methods

Bacterial strains

A total of 60 *S. marcescens* strains supplied by the Microbiology Laboratory of the University Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo, Brazil, were studied. The strains were isolated from surgical wounds (11 isolates), urinary infections (12 isolates), respiratory tract (4 isolates), catheter (3 isolates), abscesses (3 isolates), ocular secretions (4 isolates), bacteremias (7 isolates) and others. *E. coli* H30 (serogroup O26), which produces verocytotoxin, was used as a positive control in cytotoxicity assays and *E. coli* K12/711 (nontoxigenic) was used as a negative control and as a plasmid recipient in conjugation experiments.

Characterization of *Serratia marcescens*

The strains were serotyped at Instituto Adolfo Lutz, SP, Brazil, according to standard methods (22). Resistance to 10 antimicrobial agents (amikacin, ampicillin, chloramphenicol, gentamicin, kanamycin, nali-

dixic acid, tetracycline, sulfamethoxazole-trimethoprim, carbenicillin and streptomycin) was determined by the method of Bauer et al. (23).

Clinical data

The clinical records of all patients were reviewed. Only those patients who became infected 48 h after admission to the hospital were considered to have nosocomial infections. Colonization is defined as the situation where isolation of *S. marcescens* occurred without evidence of overt disease.

Bacterial filtrates

The strains were cultured in 10 ml of Trypticase Soy Broth (TSB, Difco Lab., Detroit, MI) at 37ºC for 18 h, with shaking. Bacterial culture filtrates were obtained by centrifugation (15,000 rpm for 10 min at 4ºC) and the supernatants were sterilized by filtration through 0.2-μm filters (Millipore).

Cytotoxicity assay

The cytotoxicity assay was performed as described by Konowalchuk et al. (24). Vero (African green monkey kidney) cells were cultivated in tissue culture flasks with Eagle's modified essential medium (MEM; Gibco, Grand Island, NY), supplemented with 10% fetal calf serum, 0.75 mM L-glutamine, 40 μg/ml gentamicin and 1 μg/ml amphotericin B. Confluent monolayers were removed with trypsin EDTA, resuspended to approximately 4×10^5 cells/ml in MEM and 0.1-ml samples were pipetted into each well of a 96-well microtiter plate. After incubation at 37ºC in 5% CO_2 for 72 h, the medium was replaced with 180 μl MEM and 20 μl of the bacterial filtrate was added to each well. As negative controls, some wells received only MEM, TSB or *E. coli* K12/711 filtrates and *E. coli* H30 filtrates were used as positive control. Each sample was tested in triplicate. Vero

monolayer morphology was observed under the inverted microscope and checked for cytotoxic effect for five days.

Neutral red assay

Cell viability was quantified by the neutral red cytotoxicity assay (20). Briefly, after the cytotoxicity assay (incubation with filtrates for 24-96 h), the medium containing the bacterial culture filtrates was removed and the cultures were washed with phosphate-buffered saline, pH 7.4. Two hundred μl MEM containing 50 μg/ml neutral red was added to each well and the plate was incubated for 3 h at 37°C. The media containing the dye were removed and each well was washed for 2-3 min with formol-calcium (40% formaldehyde, 10% anhydrous calcium chloride) to remove non-incorporated neutral red. Finally, 0.2 ml of an acetic acid-ethanol mixture (1.0 ml glacial acetic acid in 100 ml 50% ethanol) was added to each well and the plate was kept for 15 min at room temperature in order to remove the dye from the viable cells. Plates were transferred to a spectrophotometer (Titertek Multiskan model 340) and read with a 540-nm filter. Two wells on the first row received medium without neutral red and served as blanks. Control cultures, 8 wells located in different areas of the plate, received normal medium without test filtrates. Cell viability was determined by comparison to the absorbance values obtained for control wells (without toxin), which were taken as 100% cell viability. The cytotoxicity assay and the quantitative colorimetric assay were carried out on the same cell culture plate.

Heat stability

Filtrates that induced morphological changes in Vero cells were tested for heat stability. One-ml samples of bacterial cul-ture filtrates were incubated for 30 min in sealed tubes in a thermostatically controlled water bath at 40, 50, 60, 70, 80 or 90°C, prior to the cytotoxicity assay. The cytotoxic activity of the filtrates was determined by cell viability measured by the neutral red assay, as described above.

Plasmid isolation and conjugation

The cytotoxin-producing *S. marcescens* strains selected for plasmid isolation and conjugation assays were susceptible to nalidixic acid and resistant to ampicillin, chloramphenicol and tetracycline, among other drugs. Plasmid DNA was isolated according to the extraction method described by Kado and Liu (25) and electrophoresed on 0.7% agarose gel. The molecular mass of each plasmid was determined by comparison with plasmids of known molecular masses: pR27 (110 MDa), pJPN11 (66 MDa) and pRK (13.2 MDa). The conjugation experiments were performed as described elsewhere (26) using *E. coli* K12/711 as recipient. Briefly, mating mixtures consisting of 10^7 donor cells/ml sensitive to nalidixic acid and 10^8 recipient cells/ml were incubated at 37°C. After incubation for 24 or 72 h, the bacterial mixtures were plated onto MacConkey's agar (Difco, Detroit, MI) containing nalidixic acid (50 μg/ml). Depending upon the antibiotic sensitivity of the donor strain, the bacterial mixture and appropriate controls were plated onto MacConkey's-nalidixic acid agar containing one of the following antibiotics: chloramphenicol (20 μg/ml), carbenicillin (20 μg/ml), tetracycline (20 μg/ml), streptomycin (25 μg/ml) or ampicillin (50 μg/ml). The isolated transconjugants were submitted to plasmid extraction and electrophoresis on agarose gels in order to confirm the plasmid transfer. All transconjugants were analyzed for antibiotic resistance and cytotoxicity.

Results

Cytotoxicity of the *Serratia marcescens* strains

Cytopathic effects were observed in 13 culture filtrates of *S. marcescens* strains. After incubation with culture filtrates there was a change from spindle-shaped cells char-
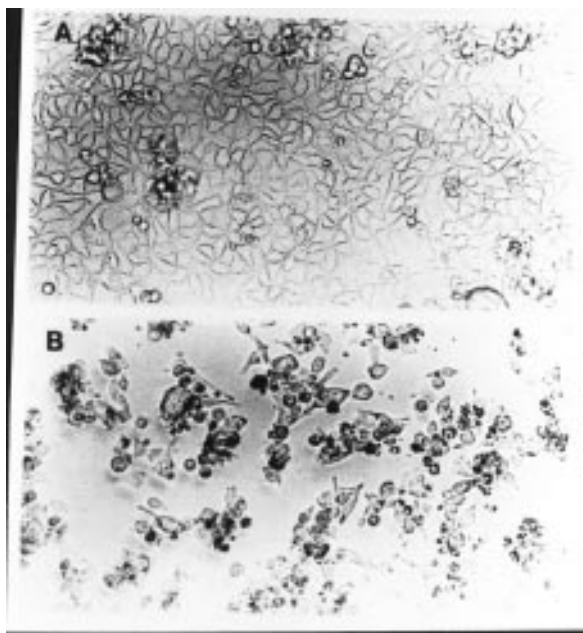
acteristic of normal Vero cells to round and shriveled cells, and these changes were followed by gradual destruction of the monolayer (Figure 1).

Determination of cell viability

Cell viability was determined for all *S. marcescens* strains and the results obtained were closely similar. Figure 2 illustrates the results obtained for three *S. marcescens* strains, *Serratia* 1 (serotype O19:NM, isolated from urinary infection), *Serratia* 4 (serotype O6,14:H4, isolated from the respiratory tract) and *Serratia* 5 (serotype O1:H7, isolated from a surgical wound), and the negative control *E. coli* K12.

Heat stability

Tests for heat stability showed that the cell monolayer lost approximately 30% cell viability when the bacterial filtrates were heated to 60ºC, and no cytopathic effect was observed at 70ºC, as shown by maintenance of the viability of Vero cells (Figure 3).

Clinical data

The clinical data correlating the patients and the cytotoxin-producing *S. marcescens* strains are shown in Table 1. The strains were obtained from ocular infection (2 strains), pneumonia (2 strains), urinary infection (3 strains), abscess (1 strain), surgical wound (3 strains) and catheter (2 strains). The majority of these infections (92%) were considered to be nosocomially acquired, and one was only colonization. The age of patients ranged from newborn to 72 years.

Serotyping

The serotypes of the *S. marcescens* isolates are shown in Table 2. Bacterial strains that showed untyped flagellar antigen or that were non-motile are referred to as NT and

Figure 1 - Cytopathic effect of *S. marcescens* filtrates on Vero cells. *A,* Negative control (*E. coli* K12); *B,* cytopathic effect after 24 h. Magnification: 100X.
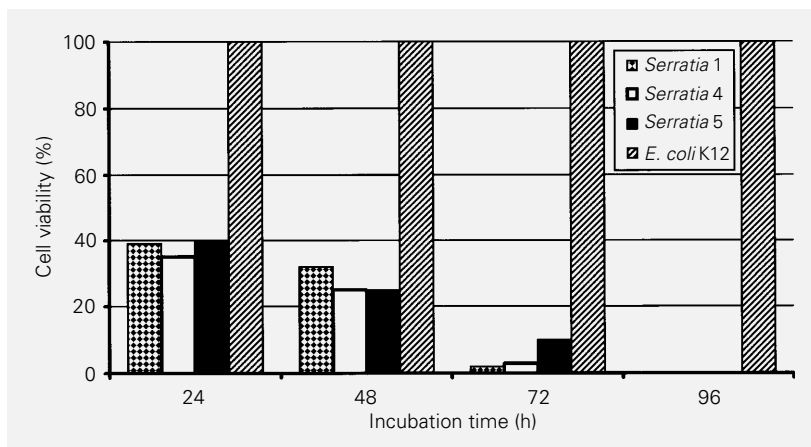

Figure 2 - Determination of the viability of Vero cells treated with filtrates of the *S. marcescens* strains numbers 1, 4 and 5, and negative control (*E. coli* K12) after different incubation times.

NM, respectively. The cytotoxin-producing strains were classified into 7 different serotypes: O6,14:H4 (7 strains), O1:H7 (1 strain), O4:NM (1 strain), O10:NT (1 strain), O19:NM (1 strain), O6,14:NM (1 strain) and O6,14:H1 (1 strain).

Plasmids and transconjugants

Plasmids were found in 8 cytotoxin-producing *S. marcescens* strains, but only 5 of these strains transferred plasmids to *E. coli* K12/711 (Figure 4). *Serratia* isolates numbers 15, 20 and 27 showed the same antibiotic resistance pattern. These isolates were resistant to ampicillin, carbenicillin, gentamicin, streptomycin, sulfamethoxazole-trimethoprim, kanamycin and tobramycin and all transferred one plasmid (~60 MDa) that carries resistance to carbenicillin, ampicillin, streptomycin and tobramycin. Strain number 45 was resistant to ampicillin, carbenicillin, gentamicin, streptomycin, sulfamethoxazole-trimethoprim, kanamycin, tobramycin and chloramphenicol and transferred another plasmid of ~66 MDa that showed resistance to ampicillin, carbenicillin, kanamycin, streptomycin, and sulfamethoxazole-trimethoprim. Strain number 1 showed a resistance pattern similar to that of number 45, with the addition of tetracycline. Only this isolate transferred two plasmids to *E. coli* K12/711: one plasmid (~54 MDa) that carries resistance to tetracycline and chloramphenicol and another (~66 MDa) carrying resistance to ampicillin, carbenicillin, streptomycin, kanamycin and sulfamethoxazole-trimethoprim. Transconjugants are reported as donor number/recipient number. Culture filtrates obtained from transconjugants did not produce morphological changes of Vero cells and cell viability always reached 100%.

Discussion

In the present study, 13 culture filtrates of 60 (21.7%) *Serratia marcescens* strains showed cytotoxic activity on Vero cells. These strains were isolated from various clinical sources, and from patients of different ages. Although no association between cytotoxic activity and isolation source was observed, most cytotoxin-producing strains (92%) were isolated from nosocomial infections.

The cytopathic effects of the *S. marcescens* isolates on Vero cells were characterized by cell rounding and detachment. Cell rounding without swelling can occur when
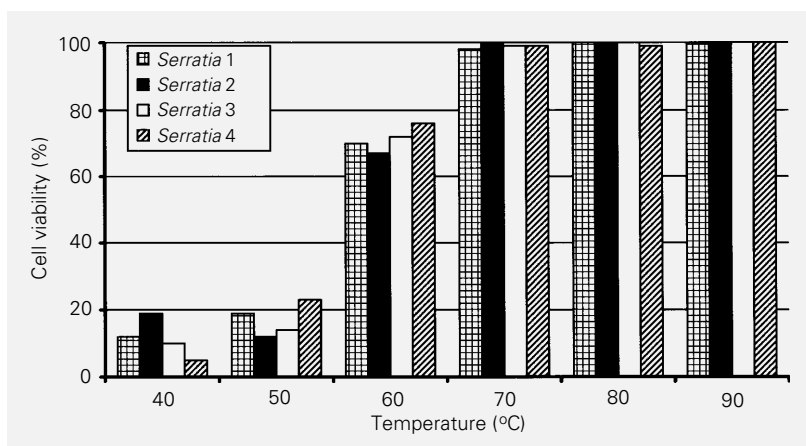

Figure 3 - Effect of heat treatment of *Serratia marcescens* cytotoxin. The filtrates were submitted to different temperatures and tested on Vero cells. The viability of the cell culture was determined by the neutral red assay.

Table 1 - Clinical data of patients with cytotoxin-producing *S. marcescens* and serotypes of the strains.

HI = Hospital infection; CO = colonization; NT = untyped flagellar antigen; NM = non-motile.

Strain (number)	Clinical source	Patient age (years)	Type	Serotype
1	urinary tract	72	HI	O19:NM
4	respiratory tract	34	HI	O6,14:H4
5	surgical wound	47	HI	O1:H7
6	surgical wound	35	HI	O6,14:H4
13	umbilical catheter	newborn	HI	O6,14:H4
14	subclavian catheter	21	HI	O6,14:H4
15	ocular secretion	newborn	HI	O6,14:H4
20	ocular secretion	newborn	HI	O6,14:H4
27	urinary tract	35	HI	O4:NM
45	urinary tract	61	HI	O10:NT
47	subphrenic abscess	20	HI	O6,14:H4
52	surgical wound	34	HI	O6,14:NM
60	respiratory tract	39	CO	O6,14:H1

the cytoskeleton is altered in the absence of plasma membrane damage. Toxins that produce this effect are classified as cytoskeleton-altering toxins (18).

The criterion used here to define cell

Table 2 - Serotypes and cytotoxin production of the *Serratia marcescens* strains studied.

NT = Untyped flagellar antigen; NM = non-motile.

Serotypes	Number of isolates	Number of cytotoxin-producing strains
O1:H4	2	0
O1:H7	2	1
O3:H1	2	0
O4:NM	1	1
O4:H4	1	0
O5:H13	1	0
O5:H8	2	0
O10:NT	1	1
O13:H17	1	0
O16:NM	1	0
O19:NM	1	1
O19:H1	1	0
O6,14:H4	34	7
O6,14:H12	3	0
O6,14:NT	4	0
O6,14:H12	1	0
O6,14:H8	1	0
O6,14:H1	1	1
O6,14:NM	1	1

injury was the loss of cell culture viability measured by the neutral red assay. The results showed that the monolayer of Vero cells treated with *S. marcescens* culture filtrates was completely damaged after 72-96 h, causing the death of the cell culture, when compared to the negative control.

The cytotoxic activity of the *S. marcescens* culture filtrates was inactivated at 70ºC, a result similar to that reported for some *E. coli* cytotoxins such as verocytotoxins (27) and Shiga toxin (28).

In *E. coli*, some serogroups are generally isolated from verocytotoxin-producing strains (29). In the present study, no relationship between serotype and cytotoxin production was demonstrable. *S. marcescens* strains were classified into seven different serotypes and the O6,14:H4 serotype was the most common among the isolates studied, independent of the production of cytotoxin.

Furthermore, other virulence characteristics such as hemolysin production (10) and resistance to serum bactericidal activity (8) were detected in 97% and 92% of the strains, respectively. However, no association with cytotoxin production was observed, since

Figure 4 - Agarose gel electrophoresis of plasmids from cytotoxin-producing *Serratia marcescens* transferred to *E. coli* K12/711. *A, E. coli* K12/711 (recipient); *B, Serratia* 1 (donor); *C*, 711/1a (transconjugant); *D*, 711/1b; *E, Serratia* 15; *F*, 711/15; *G, Serratia* 20; *H*, 711/20; *I, Serratia* 27; *J*, 711/27; *K, Serratia* 45; *L*, 711/45; *M*, pR27; *N*, pJPN11; *O*, pRK. pR27, pJPN11 and pRK are molecular mass standards.
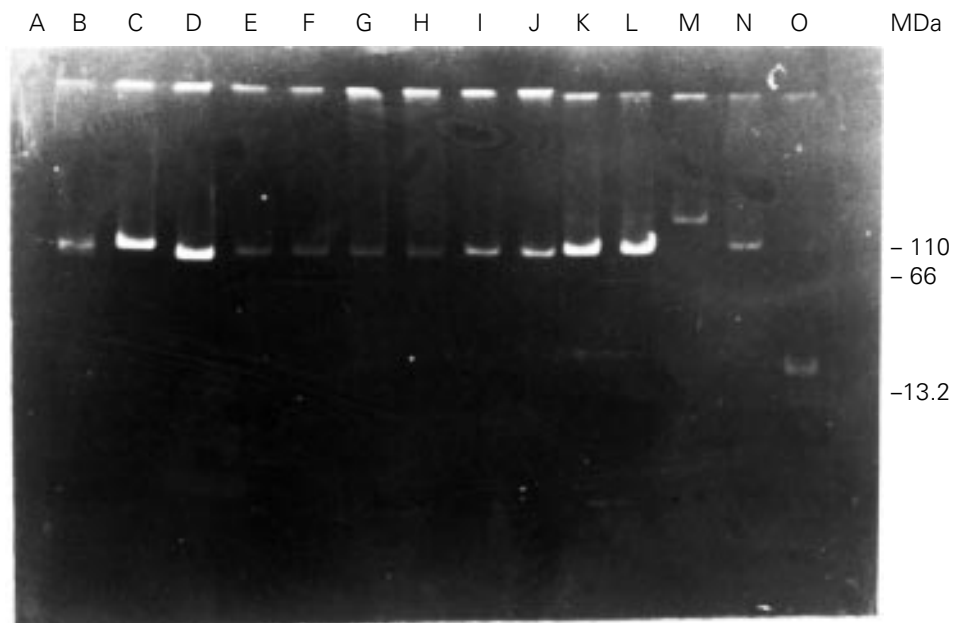
these characteristics were also found in most of the non-producing strains.

Five cytotoxin-producing strains transferred plasmids to *E. coli* K12/711. The transconjugants received plasmids carrying antibiotic resistance but they did not produce cytotoxins, suggesting that the genetic information for this activity is not carried on these plasmids.

Although most bacterial toxins seem to be located on the chromosome, toxins such as CNF2 and heat-labile (LT) and heat-stable (ST) enterotoxins of *E. coli* (17,30,31) have been associated with plasmid presence. However, the controlling genes for verocytotoxin production are phage-encoded in several *E. coli* strains (32).

Some cytotoxins are homologous between different bacterial species, as observed for Shiga-like toxins produced by *E. coli* and Shiga toxin (33), or for Shiga-like toxin II-related toxins produced by *Citrobacter freundii* (34). Thus, characteristics such as source of isolation, heat stability and type of cytopathic effect are preliminary tests that can be useful for differentiation of a cytotoxin.

In conclusion, the present results describe a cytotoxic activity on Vero cells induced by clinical isolates of *S. marcescens,* and may provide impetus for subsequent work on the characterization of this cytotoxin.

Acknowledgments

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