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MICROBIOLOGY

Characterization of a bacteriocin produced by a clinical isolate of *Shigella flexneri* 2

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Abstract: This study was conducted to determine the emergence of antibiotic producer *Shigella* strains, focusing the efforts in the search of new antimicrobial compounds. The ability of 388 *Shigella* isolates, which were originated from 15,131 fecal samples from patients with acute diarrhea analyzed between 2015-2017, to produce antimicrobial peptides was determined by deferred-antagonism assay. Here, we observed that 9.02% of the *Shigella* isolates produced an antimicrobial agent able to inhibit the *E. coli* AB133 strain growth. The CI172 strain was selected as producer for its antimicrobial characterization. This antagonist compound (*Sh*pCI172) was produced during exponential growth phase and displayed a restricted action spectrum. It is also thermo-resistant and has about 3 kDa molecular mass. The *Sh*pCI172 can be classified as a microcin, since CI172 did not display cross immunity with other well-known microcins. This is the first report where the production of a microcin by a *Shigella flexneri* 2 strains is described. The use of *Sh*pCI172 microcin may contribute to preventing or controlling diarrheal diseases. This finding represents an important contribution in the biotechnology field for its potential in the development of new antibiotics and/or food preservatives agents.

Key words: bacteriocin producer, clinical isolates, infection disease, new microcin, *Shigella flexneri 2.*

INTRODUCTION

Throughout the evolution, the living organisms have constantly adapted to the environment and to the simultaneous presence of other organisms. The production of specific antimicrobial peptides is a mechanism by which pathogenic bacteria take action to the environmental challenges, resulting in antibiosis interactions within the host´s microbial community (Riley & Gordon 1999, Engevik & Versalovic 2017).

Several *Enterobacterieaceae* members have the ability to produce antimicrobial peptides called bacteriocins to survive into the intestinal microbiota (Duquesne et al. 2007, Hammami et al. 2013). The bacteriocins are characterized as peptides mainly produced during the stationary growth phase, induced by different stress conditions (Ganzle et al. 1999, Aasen et al. 2000). Also, bacteriocins have a restricted action spectrum, since generally they are only toxic to other closely related species (Tagg et al. 1976, Hammami et al. 2013). Furthermore, these antimicrobial peptides belong to a heterogeneous group of molecules ribosomally synthesized, that are characterized as having low toxicity and thermo-resistance . Additionally, these antimicrobial compounds can be classified according to their molecular weight into peptides weighing less than 10 kDa called microcins (Mcc), and peptides weighing more than 10 kDa called colicins (Duquesne et al. 2007, Fimland et al. 2005, Garneau et al. 2002). Previously, we carried out an epidemiological

study to identify the prevalent bacterial agent of gastroenteritis in the Northwest Argentina region (NWA). We reported that 88.57% of 1,794 clinical isolates corresponded to *Shigella* spp. strains, being *Shigella flexneri* the species most frequently isolated. *Shigella* spp. is a non-motile Gram-negative bacterium that belongs to the *Enterobacteriaceae* family. *Shigella* represents one of the principal intestinal pathogens causing dysentery in humans. Two relevant features of this genus to take into account in the infection frequency are their extremely low infectious dose (about 10^2 cells), and the person-to-person transmission through direct contact with infected fecal samples (Anderson et al. 2016, Calcuttawala et al. 2015, Ina et al. 2003).

Numerous bacteriocins produced by different members of the *Shigella* genus have been described (Calcuttawala et al. 2015, Kaewklom et al. 2013). All of these were classified as colicins. Colicins are generally encoded by plasmid determinants and have different mechanisms to eliminate their competitors (Cascales et al. 2007). The molecular mass of these peptides can oscillate between 25 and 80 kDa. In *S. sonnei* it has been described the production of different types of colicins, while there is no information of microcin production by species of *Shigella flexneri* (Sousa et al. 2013, 2010).

Bacteria that cause gastrointestinal infections must overcome several barriers to achieve the host's colonization, developing several mechanisms to be settled in a new environment (Calcuttawala et al. 2015). *Shigella*´s capacity to produce bacteriocins could be one of the strategies that contribute to the successful colonization and infection at low doses . In this work, we identified and characterized a new low molecular mass bacteriocin produced by the *Shigella flexneri* 2 CI172 strain, not previously reported in the literature. This strain was isolated from a fecal sample of children suffering acute diarrhea, and display a multidrug resistances pattern (MDR). The search for new antimicrobial compounds is a growing field encouraged especially by the appearance of strains showing high rates of MDR worldwide. Thus, the characterization and identification of these antimicrobial compounds, produced by pathogenic microorganisms within the host, allow not only to understand the dynamic of the mechanisms of infection but will also the development of new tools applicable to the prevention, control and eradication of infectious diseases.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this work are listed in Table I, as described (Sanderson et al. 1995). *Shigella* strains were isolated from fecal samples collected in the Centro Provincial de Salud Infantil "Eva Perón" from Santiago del Estero, the Hospital Interzonal Eva Peron from Catamarca and the Hospital del Niño Jesus from Tucumán, from the northwest region of Argentine. The original epidemiological study was carried out using a protocol previously approved by the institutional review boards/ethics committees of each mentioned Argentina National Hospitals, informed by written consent. Ethical approval protocol was given by the Tucuman-Provincial Health System (SIPROSA) in agreement of Cooperation with Public Institutions (Protocol of Experimental Research #1137-410D-2019). The clinical isolates were obtained from fecal samples of pediatric patients with diarrhea, following the rules of the CLSI (The Clinical & Laboratory Standards Institute). Briefly, the samples were enriched in Selenite broth media for 18 h prior to inoculating onto Salmonella–Shigella agar plates. After 24 h of incubation at 37° C, isolates

Table I. Bacterial strains used in this study.

Strain	Description[a]	Reference or source
Escherichia coli		
MC4100 (pHK11)	ColV$^+$, pBR322, Ap^r	(Gilson et al. 1987)
H47	wild type isolation MccH47$^+$, InmH47$^+$	(Lavina et al. 1990)
RYC1000	MccB17$^+$ InmB17$^+$, MccB1+; pBR322;	
Ap^r	(Moreno et al. 1992)	
MC4100 (pTUC200)	MccJ25$^+$, Inm25$^+$, pBR322, Ap^r	(Salomon and Farias 1992)
MC4100	F$^-$ ***araD*** 139, D(***argF-lac***)205 l-***rpsL150*** (Sm^r) ***flbB5301 relAl deoC1 pstF25***	C.G.S.C.
DH5α	F$^-$ φ80d ***lacZ*** DM15 D(***lacZYA-argF)***U169 ***recA1endA1 supE44 hdR17*** (r_{k-} m_{k-}) ***thi-1 gyrA***	BRL
Salmonella enterica		
14028s		wild type ***S.*** Typhimurium
Clinical isolates	wild type ***S.*** Enteritidis	
Shigella		
Clinical isolates	wild type ***S. flexneri***	This work
Clinical isolates	wild type ***S. sonnei***	This work
Klebsiella pneumoniae		
RYC492	MccE492$^+$, InmE492$^+$	

Gene designations are summarized as described (Sanderson et al. 1995).

were identified using biochemical tests such as indole, motility, lysine decarboxylase, triple sugar iron agar, citrate, and urea. The clinical isolates (CI) were serologically classified, using antigen-specific serums from Malbrán Hospital. To confirm *Shigella* classification, we used the multiplex-PCR (mPCR) technique previously described (Farfan et al. 2010).

Selection of bacteriocin-producing strains

The bacteriocins production was tested by deferred antagonism assay on Luria Bertani plates (Sigma-Aldrich) (Fredericq 1948). Briefly, the potential producing strains were deposited by toothpicks on plates of Luria Bertani (LB) culture medium and incubated during 24 h at 37°C to allow the production and diffusion of the antibiotic peptide. Afterwards, the plates were exposed to chloroform vapors for 20 min to kill the possible producing strains, and then 4 ml of soft agar containing the indicator *E. coli* AB1133 strain were deposited in the top of the plate. Again, the plates were incubated 24 h at 37°C to allow the AB1133 strain growth. The production of bacteriocin was evidenced by the appearance of AB1133 growth inhibition halo.

Determination of numerous factors' effect on the bacteriocin production

The bacteriocin production time along the bacterial growth was determined at 30, 37 and 42°C. To this end, an overnight culture of the producer strain was 1:10 diluted into liquid LB medium and incubated at each mentioned temperature for 24 h. A culture aliquot was collected every hour to determine the growth achieved and the levels of the inhibitor compound present in the supernatant by deferred antagonism assay.

The temperature effect on the bacteriocin production was investigated in solid LB medium. Here, a colony of producer strain was deposited on LB agar medium plates and incubated at 30, 37 or 42°C for 24 h, killed by chloroform vapors, and then a soft agar containing the indicator AB133 strain was added. After 12 h of incubation at 37°C the growth inhibition halo size was measured.

The effect of culture medium was tested, using LB medium at pH 4.5 or 7; M9 minimal medium containing high (0.2%) or low (0.02%) glucose concentration as carbon source; LAPTg and BHI medium. The presence of the *Sh*pCI172 was determined in the supernatants of the producer strain growing in the mentioned medium after 24 h at 37°C. In order to compare the levels of *Sh*pCI172 production we normalized the cultures at same OD_{600}. The *Sh*pCI172 production levels were determined measuring the growth inhibition halo size.

Here, to determine if the bacteriocin gathered the microcin`s characteristics (Duquesne et al. 2007, Fimland et al. 2005, Garneau et al. 2002), 10 µl of cell-free supernatant was autoclaved for 10 min at 120°C or incubated with proteinase K (1 µg μl^{-1} final concentration) for 1 h at 37°C. The remaining antibiotic activity was determined by the growth inhibition of AB1133 indicator strain by serial double-dilution of treated-bacteriocin.

Bacteriocin purification protocol

The bacteriocin partial purification was carried out as previously described (Acuna et al. 2012), using M9 medium (Sigma-Aldrich) containing 0.2% glucose. The supernatant activity was determined by double dilution methods. The solution containing the precipitated bacteriocin was preserved at 4°C until its use.

The high-performance chromatography analysis (HPLC) was performed using as stationary phase a Waters XBridge C18 column (5 µm, 4.6 x 250 mm; loop 50 µl) and a gradient of A)-TFA 0.1% and B)- CH_3CN /0.1% TFA as mobile phases. The cell-free supernatant ammonium sulfate precipitate was injected into a linear gradient of TFA/acetonitrile at a starting point of 75% TFA, for 50 min to a final amount of 5% TFA. The peak with antimicrobial activity was subjected to HPLC MS/MS spectroscopy analysis. The electrospray ionization (ESI) was set to positive ion mode with a source voltage of +3000 V, running at a vaporizer temperature of 220°C, a capillary temperature of 260°C, at gas pressure of 30 psi and an auxiliary gas pressure of 2 psi. The system was adjusted to scan a range of ion mode products between 1450–1750 *m/z*.

Determination of the molecular nature

The peptide nature was determined by the treatment with proteinase K (1mg/ml, Sigma-Aldrich) at 37°C for 1 h (Huang et al. 2016, Mukherjee et al. 2016). Serial dilutions of the precipitated antimicrobial compound within protease treatment were used as a control. The remaining activity after the treatment was determined by the double dilution method on the indicator strain. Three independent experiments were carried out to validate the results.

Determination of the charge

The net charge of the bacteriocin was determined by agarose gel electrophoresis (1% w/v), using the protocol described . After finished the run, 20 µl of the precipitated antimicrobial compound was deposited in the top left of the gel as a positive antimicrobial activity control. The gel was revealed by antibiotic activity against the AB1133 indicator strain. The assay was carried out three times, to validate the results.

Molecular weight determination

The molecular mass was estimated using cellophane (12 KDa cutoffs) and cellulose membrane (10 KDa cutoffs) (Sigma-Aldrich). In the top of each membrane a colony of the tested strain was deposited and the plate was incubated at 37°C overnight to allow the antibiotic production and its diffusion through the membrane pore. The membrane was removed and a soft agar containing the indicator strain was added and incubated at 37°C to observe the appearance of a growth inhibition halo.

The molecular mass was confirmed by a denaturing polyacrylamide gel (12%) electrophoresis, using MccJ25 (2.1 kDa) and insulin B-chain (3.5 kDa) as a molecular weight markers (Sigma-Aldrich). The gel was fixed with a 50% methanol solution and washed with solutions of decreasing concentration of methanol. The gel results were revealed by antimicrobial activity, as above described. Prior to development by activity, the proteins were stained with coomasie blue. The assay was carried out three times, to validate the results.

Antimicrobial spectrum determination

The sensitivity of different group of bacteria (*Salmonella*, *Shigella*, *E. coli*, *Klebsiella*, *Vibrio*, *Listeria*, *Clostridium*, *Enterococcus* and *Staphylococcus*) to bacteriocin was determined by crossed streak or spot sensitivity tests, as previously described (Salomon & Farias 1992). In the spot sensitivity tests, 10 µl of precipitated antimicrobial compound were deposited in the specific medium and covered with a soft agar (0.6%) inoculated with the tested strain. Here, the appearance of a growth inhibition halo indicates sensitivity.

RESULTS

Isolation of antimicrobial peptide producer strains

In this paper, we focus our study on characterization of *Shigella*–bacteriocin producers. These strains were previously isolated from fecal samples of patients with gastroenteritis in the Northwest region of Argentina, during the summer seasons of 2015-2017.

Here, we analyzed the ability of 388 *Shigella* isolates to produce antimicrobial peptides using the deferred antagonism assay. We report here that 35 of 388 *Shigella* isolates were able to produce an antimicrobial agent that inhibits the growth of sensitive *E. coli* AB133 strain (Figure 1a). These 35 clinical isolates (CI) were serotyped and their identity was confirmed by mPCR. The results demonstrated that the 62% of these 35 CI producer strains belonged to *Shigella sonnei* species, while the remaining 38% belonged to different *Shigella flexneri* serovars (Figure 1b).

Due to above observation, we selected the CI172 strain for the following studies. CI172 was obtained in Santiago del Estero province, from a sample of a six years old female patient, during the 2017 summer season and was classified as *Shigella flexneri* 2 (Figure 1a, spot 5).

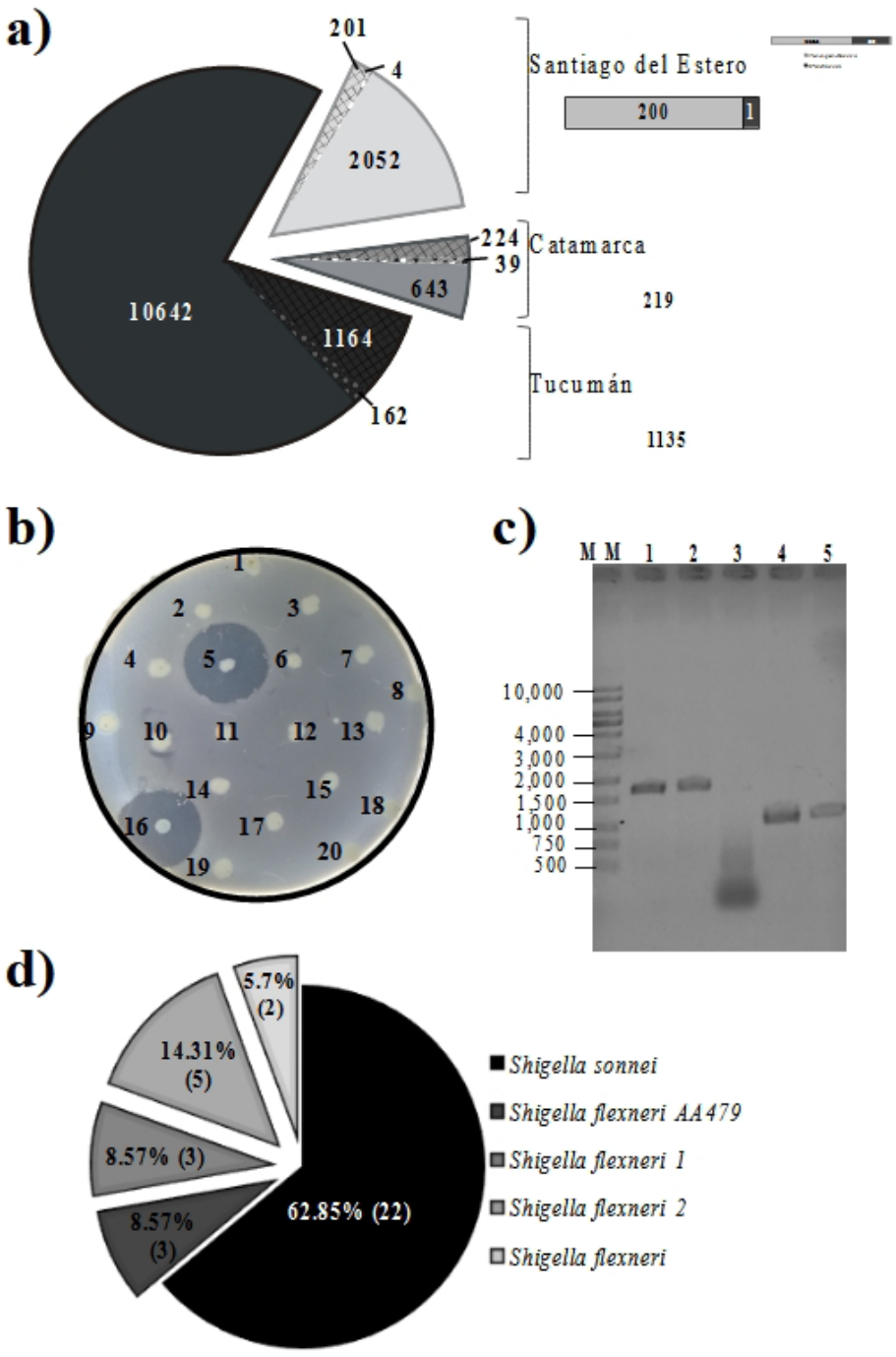
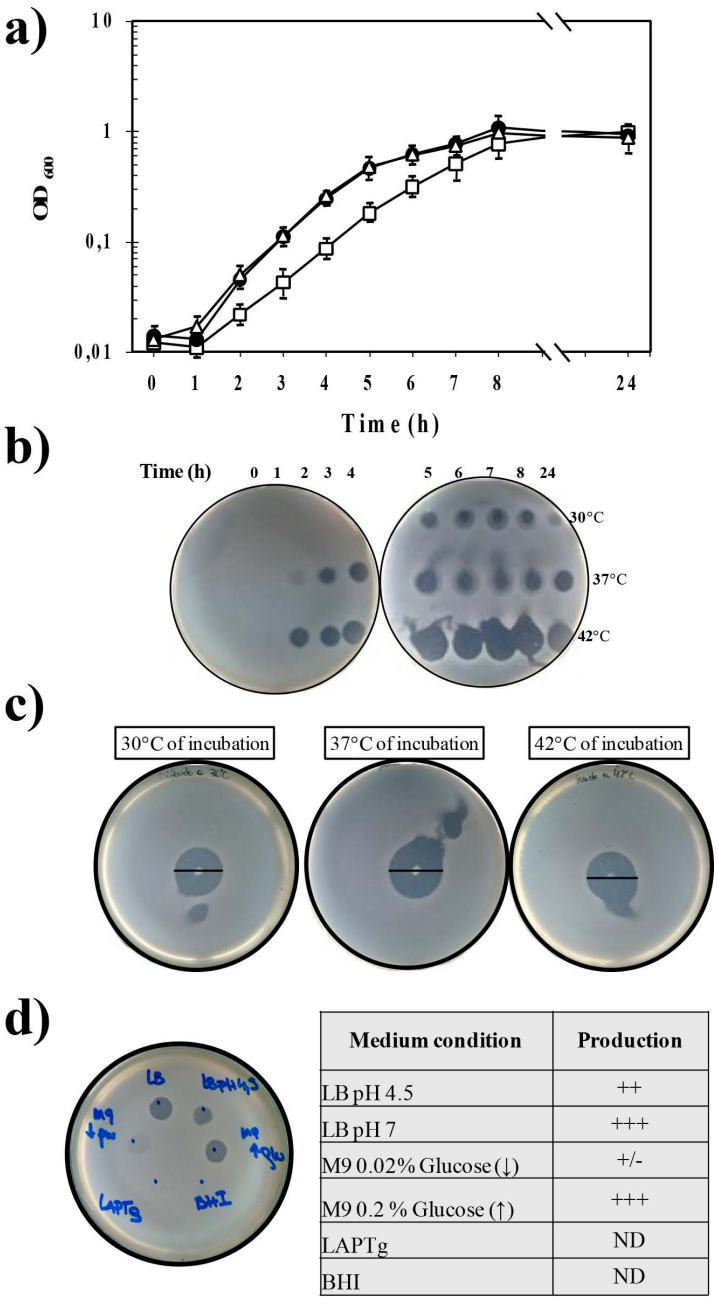


Figure 1. ***Shigella*** **clinical isolates producing antimicrobial peptide of the Northwest Argentina Region (NWA). a) Representative plate of deferred production test, carried out to determine those clinical isolates (CI) able to produce antimicrobial peptide. 1 to 6 CI obtained in the Santiago del Estero; 7 to 17 CI obtained in the Tucumán and 18 to 20 CI obtained in the Catamarca. b) Classification and number of the** ***Shigella*** **producer CI, by specie and serovar.**

Determination of factors involved in the antimicrobial compound production from CI172.

We used the biochemical and genetic criteria determined for each bacteriocin to classify the compound produced by CI172, referred as *Sh*pCI172. On these bases, we determined the bacterial growth time at which *Sh*pCI172 begins to be produced. Since this strain was obtained from a patient suffering acute diarrhea with febrile symptoms, we first determined the production levels of the antimicrobial agent



Medium condition	Production
LB pH 4.5	++
LB pH 7	+++
M9 0.02% Glucose (↓)	+/-
M9 0.2 % Glucose (↑)	+++
LAPTg	ND
BHI	ND

Figure 2. Characterization of the *Sh*pCI172 production by the CI172 strain. a) Growth phase curve of CI172 strain determined by OD_{600} during the growth in LB medium at 30 (white squares), 37 (black circles) or 42°C (white triangles). All data correspond to mean values and standard deviation of three independent experiments, carried out by duplicate. b) Presence of *Sh*pCI172 in the supernatant of the CI172 strain growing in LB medium at above mentioned temperatures, determined each hour by the spot in plates sensitivity test, using *E. coli* AB1133 strain as indicator (most representative image). c) The effect of the temperature on the production levels was determined in solid medium. A colony of the CI172 strain was deposited on the plate with toothpick and incubated for 24 h at 30, 37 or 42°C, and then was covered with a soft agar stock containing 12 µl of an overnight culture of AB1133 as the indicator strain. The clear halo around the colony indicates growth inhibition of the AB1133 strain by the presence of *Sh*pCI172, while the diameter of the inhibition halo indicates a semi-quantitative amount of the antimicrobial synthesized. d) The effect of the LB, M9 LAPTg and BHI media components on the *Sh*pCI172 production levels was determined from the supernatant of the CI172 strain growing in liquid media. The semi-quantitative amount of *Sh*pCI172, in each analyzed condition, was determined by spot in plate sensitivity test using AB1133 as indicator strain. The left table represents the comparative levels of *Sh*pCI172 in each condition determined by spot antimicrobial activity again AB1133 as indicator: +++ maximal activity, ++ intermediate activity, +/- low activity, ND not detected activity. All data correspond to mean values of three independent experiments.

according to the bacterial growth rate at 30, 37 and 42°C. As shown in the Figure 2, the *Sh*pCI172 begins to be detected during the early exponential growth phase at 37 and 42°C. The maximum *Sh*pCI172 levels were reached at OD=0.5 (5 h approximately) and maintained until the stationary growth phase (24 h) (Figure 2). In these assays, we did not observe differences in the rate of growth of the strain at both mentioned temperatures (37 and 42°C) (Figure 2a). However, at 30°C the growth was slower and the production of *Sh*pCI172 was observed only after 5 h of incubation at a lower level than those observed at 37 or 42°C (Figure 2a and b).

We also investigated the effect of the temperature on *Sh*pCI172 production when the

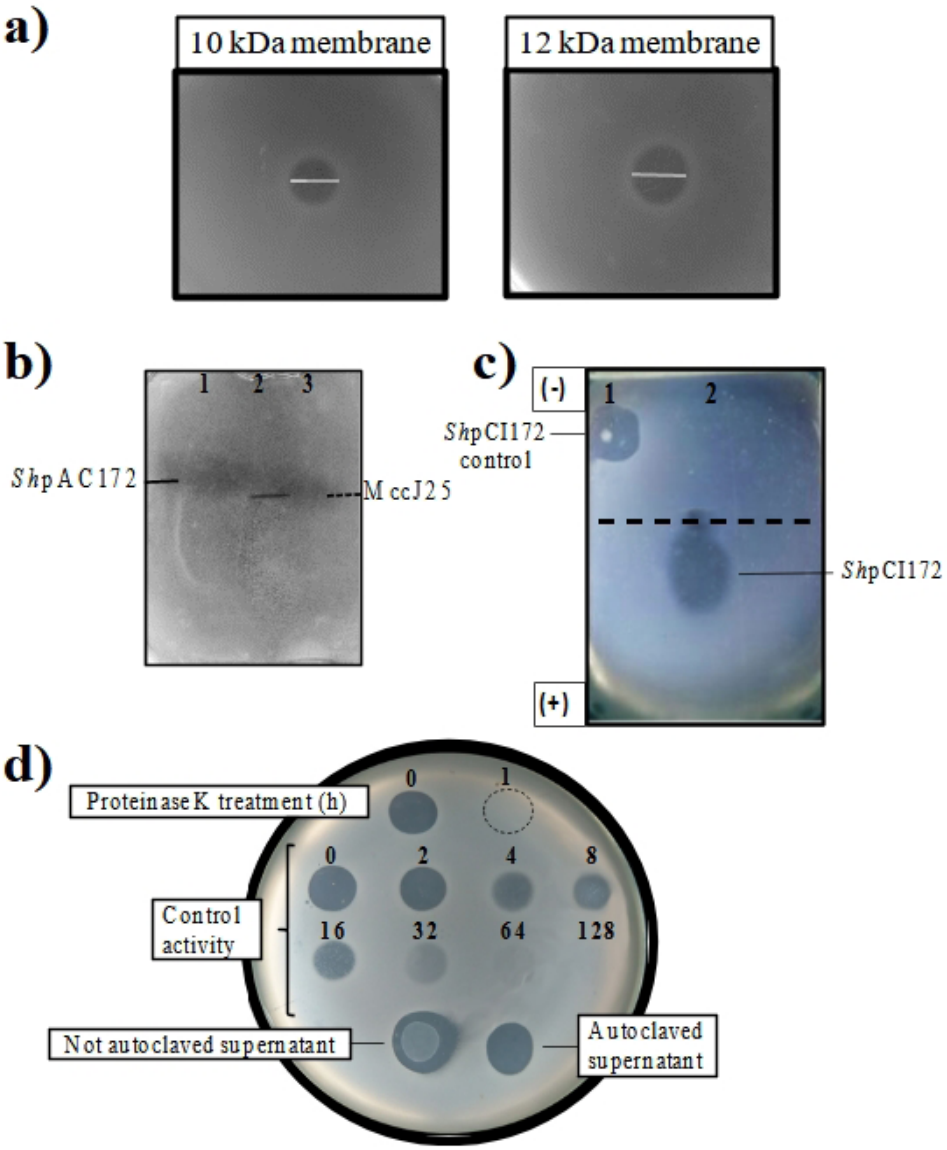


Figure 3. Characterization of the *Sh*pCI172 bacteriocin. a) Determination of the molecular size by its capability to pass through the membrane pore of dialysis. The less mass of 12 kDa size of *Sh*pCI172 was estimated by its ability to cross the pores of 10 and 12 KDa of cellulose membranes placed on LB medium plates containing a lawn of the indicator strain. This characteristic was observed by the antibiotic activity, producing a growth inhibition halo. b) Electrophoretic analysis in polyacrylamide gels determined that *Sh*pCI172 has a higher molecular weight than MccJ25. The electrophoretic run was performed under denaturing conditions using a 6% polyacrylamide gel, revealed by antibiotic activity as detailed in Materials and Methods. The appearance of the AB1133 strain growth inhibition halo revealed the position of both microcins in the gel. 1- MccJ25 (2.1 kDa); 2- Insulin B-chain (coomasie blue stained); 3- *Sh*pCI172. c) *Sh*pCI172 charge determination by electrophoretic analysis. The negative charge at pH 7 of *Sh*pCI172 was determined in a 1% agarose gel and detected by antibiotic activity test again AB1133 strain. 1) *Sh*pCI172, added after the electrophoresis run, as a positive activity control; 2) *Sh*pCI172 assayed. The dashed-line represents the seed point. d) Proteases and temperature sensitivity to *Sh*pCI172 microcin. The effects of both treatments were determined by the remaining antimicrobial activity of *Sh*pCI172 related to non-treatment *Sh*pCI172, again AB1133 as indicator strain by spot in plate. Control activity: remaining antibiotic activity of AB1133 sensitive strain produced by serial double-dilution of *Sh*pCI172 treated with proteinase K. The absence of growth inhibition halo of the indicator strain suggests that *Sh*pCI172 is sensitivity to the treatment.

bacteria grow in solid LB medium at 30, 37 or 42°C. The results demonstrated that temperature does not affect the production levels of bacteriocin in solid medium since the appearance of a growth inhibition halo of equal size was observed after the incubation with the indicator strain at all analyzed temperatures (Figure 2c).

The effect of culture medium composition on the *Sh*pCI172 production was also tested. As shown in the Figure 2d, we did not find differences between cells that were grown in LB pH 7 or in M9 supplemented with physiological amount of glucose (0.2%). However, in M9 containing low glucose concentrations or in acidic LB medium the *Sh*pCI172 activity decreased dramatically. Moreover, the *Sh*pCI172 production was not detected in LAPTg or in BHI medium (Figure 2d).

Determination of ShpCI172 general properties

In accordance with colicins and microcins description, we determine the approximate molecular mass of *Sh*pCI172. To this end, we analyzed the capacity of *Sh*pCI172 to diffuse through pores of different membranes (cut offs of 10 and 12 kDa). The results demonstrated that *Sh*pCI172 was able to diffuse through pores of all the membranes assayed, since a growth inhibition halo of the sensitive strain was observed after overnight incubation at 37°C (Figure 3a). These results indicated that the bacteriocin produced by CI172 strain is a small antimicrobial peptide.

To confirm the molecular mass of *Sh*pCI172, we performed an electrophoretic assay using a denaturing polyacrylamide gel (12%), which was developed using coomassie blue, and its inhibitor activity. We observed that the coomassie blue staining did not reveal a protein band. However, when the gel was developed based on activity, we noted the appearance of an AB1133 growth inhibition halo corresponding to *Sh*pCI172, at approximately 3 kDa related to migration of controls, insulin B-chain and MccJ25 peptides (Figure 3b).

The charge of *Sh*pCI172 was analyzed determining its migration by electrophoresis assay in agarose gel and antimicrobial activity against the AB1133 strain. We observed that *Sh*pCI172 has a negative charge (pH 8), since the inhibition growth of indicator strain displaced from the seed point toward the cathode by (+ pole) (Figure 3c).

On these bases, we could classify this bacteriocin as an antimicrobial peptide belonging to the ribosomal synthesized class of microcins.

Characterization of *Sh*pCI172 as a new microcin

First, we determined the effect of proteolytic enzymes on *Sh*pCI172 activity. We observed that the *Sh*pCI172 antibiotic activity was strongly affected by proteinase K, since no growth inhibition halo was observed after the treatment, compared to the control (Figure 3d).

Then we evaluated the thermostability of *Sh*pCI172. The results demonstrated that *Sh*pCI172 was extremely thermostable, since its activity remained unchanged after boiling, producing a similar growth inhibition halo of the AB1133 strain than the supernatant without heat treatment (Figure 3d).

Since the different microcins already characterized have been defined by cross-immunity criteria between producer strain members of the same group, we analyzed whether the CI172 strain displays immunity to any of these microcin groups. As shown in Figure 4a, the CI172 strain showed sensitivity to all five different microcins tested, while the producer strains of MccE492, MccB17 microcin and colicin V were immune to *Sh*pCI172 but those producing MccH47 or MccJ25 were not (Figure 4b).

Chromatographic analysis and mass spectrometry of *Sh*pCI172

To discard the possibility that more than one compound with antimicrobial activity are present in the CI172 cell-free supernatant, we carried

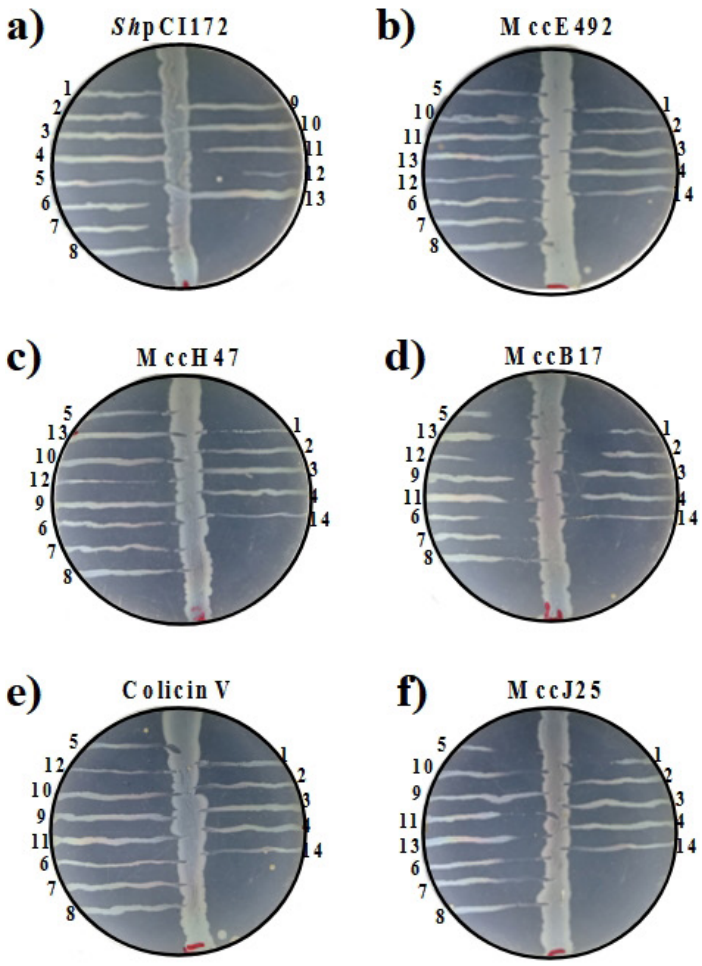


Figure 4. Cross-immunity assay of different strain against *Sh*pCI172. This test allowed us to rule out that *Sh*pCI172 does not belong to a known microcins group and its antimicrobial action spectrum. a) A liquid culture of MccE492, MccH47, MccB17, Colicin V and MccJ25 microcin-producing strains was deposited in the center of M9 plate as indicated in material and methods. After overnight incubation the AC172 strains was deposited parallel to the central streak of the producing strain. The absence of bacterial growth until the central streak indicates sensitivity. b) The AC172 strain was deposited in the center of M9 plate as indicated in material and methods. After overnight incubation the following strains were deposited parallel to the central streak:1) *Shigella flexneri* collection strain; 2) *Shigella sonnei* collection strain; 3) *Salmonella* Typhimurium collection strain; 4) *Salmonella* Enteritidis collection strain; 5) CI310 clinical isolates strain; 6) DH5α, *E. coli* strain; 7) AB1133, *E. coli* strain; 8) MC4100, *E. coli* strain; 9) RYC492, *K. pneumonia*-MccE492 producer strain; 10) BM21, *E. coli*-MccB17 producer strain; 11) H47, *E. coli*-MccH47 producer strain; 12) MC4100, *E. coli*-MccJ25 producer strain; 13) V517, *E. coli*-Colicin V producer strain; 14) CI172, *Shigella flexneri Sh*pCI172-producer strain. The absence of bacterial growth until the central streak indicates sensitivity.

out a RP-HPLC assay. The fractions obtained from this assay, showing a peak at OD_{280} were concentrated and their antimicrobial activity was analyzed. The results showed that the fraction IV with 34 min retention-time inhibited the AB1133 growth (Figure 5a and b). This active fraction was subjected to mass spectroscopy (HPLC-MS/MS) to determine the molecular mass and composition of the antimicrobial agent. The MS results revealed that the active compound was purified to homogeneity, since the range of the mass spectrum between ~1450 *m/z* and ~1750 *m/z* displayed a majority species with 1592.8 *m/z* (Figure 5c). Amplification of this zone revealed the presence of second specie of 1593.35 *m/z*, which differs from 1592.8 *m/z* in the signals corresponding to C^{12} and C^{13} in $\Delta m/z$ ~0.51 (Figure 5d). This difference corresponds to a double-charged species ($[M+2H]^{+2}$), indicating that it is derived from a molecule of ~3184 Da. The results obtained in this assay suggests that in the cell-free supernatant only one antimicrobial agent is present, whose molecular weight is similar to that previously determined by electrophoresis.

Antimicrobial activity of the *Sh*pCI172

Here, we investigated the antimicrobial spectrum of *Sh*pCI172, using cross-streaking test. Our results demonstrated that *Sh*pCI172 displays a narrow antimicrobial spectrum, restricted to gram-negative bacteria species, since we only detected inhibition of DH5α, AB133 and MC4100 *E. coli* K-12 strains, as well those producing MccH47 and MccJ25 (also *E. coli* strains), *Shigella sonnei* (ATCC) and CI310 clinical isolate corresponding

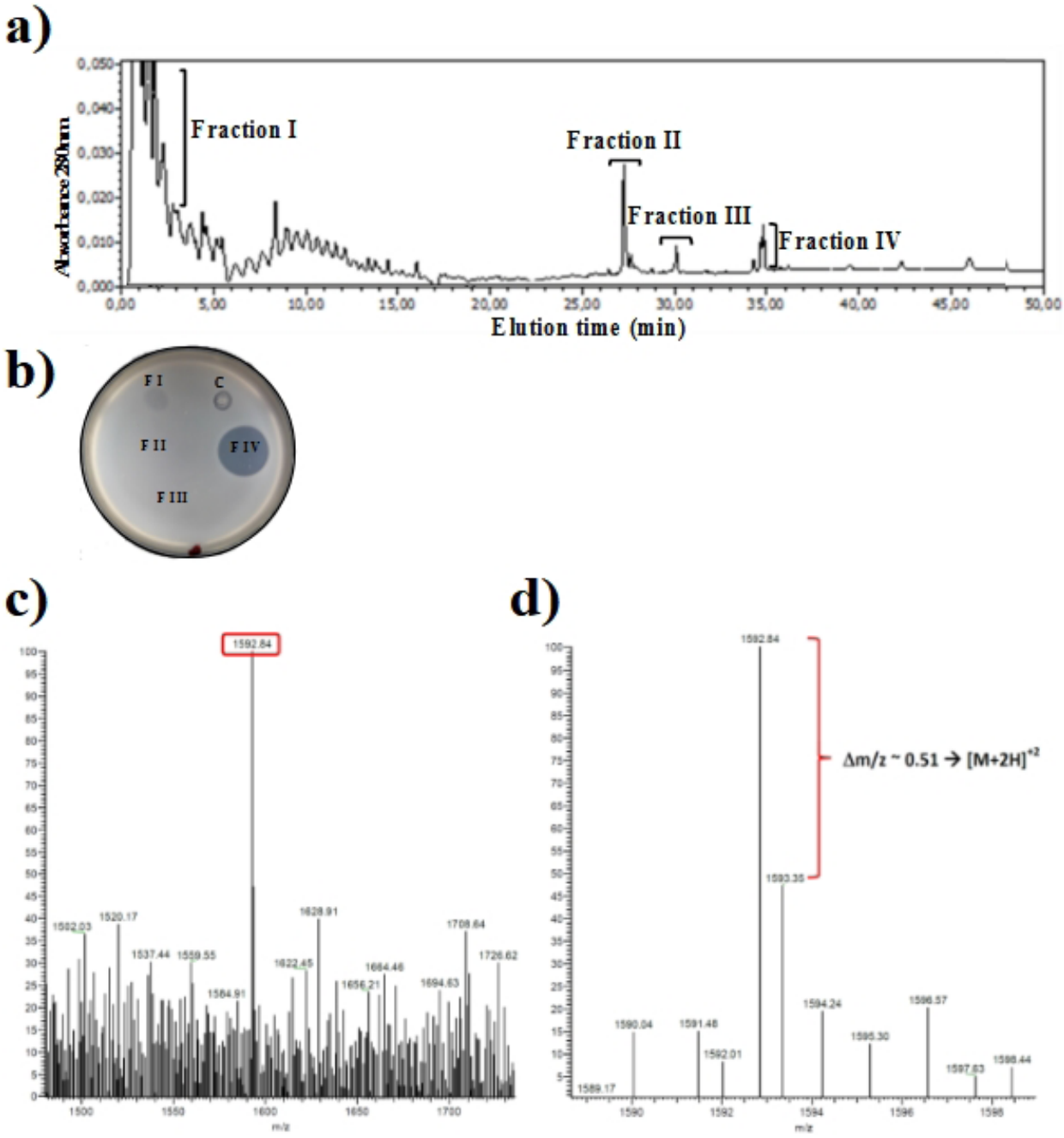


Figure 5. High performance chromatography (HPLC) and HPLC-MS analysis of *Sh*pCI172. a) The cell-free supernatant of CI172 culture, previously ammonium sulfate precipitated, was used to inject into a C18 column. The elution carried out by linear gradient of TFA/acetonitrile was followed measured OD_{280nm} every one minute. b) Antimicrobial activity displayed by the HPLC-peak of higher OD_{280} intensity, against the sensitive AB133 strain. FI, concentrated sample corresponding to the fraction eluted during the first three minutes; FII, concentrated sample corresponding to the fraction eluted during minutes 27-28, FIII; concentrated sample corresponding to the fraction eluted during minutes 29-30; FIV, concentrated sample corresponding to the fraction eluted during minutes 34; C, *Sh*pCI172 production from AC172 strain used as control. The presence of growth inhibition halo indicated the presence of *Sh*pCI172 in the fraction. c) The fraction with antimicrobial activity was then subjected to mass spectrometry, resulting in a peak with identified mass-over-charges of 1590.84 *m*/*z*. d) Amplification of the mass spectrum range ~1450 and ~1750 *m*/*z*, indicating the presence of second specie of 1593.35 *m*/*z*.

to *Shigella flexneri* (Figure 4a). The sensitivity to *Sh*pCI172 of Gram-positive bacteria was determined by spot test, using species-specific growth medium. Here, we determined that *Sh*pCI172 inhibits the growth of some Gram-negatives but not Add: Gram-positive bacteria species (Table II).

DISCUSSION

In this work, we report for the first time the isolation of a *Shigella flexneri* 2 strain able to produce an antimicrobial compound. This was an unexpected result, since even when the production of bacteriocins by the *Shigella* genus is widely described, all of them were attributed to the species of *Shigella sonnei* and *Shigella boydii*, and were classified as colicins (Calcuttawala et al. 2015, Sousa et al. 2010, Smarda & Smajs 1998, Sousa et al. 2013). We observed that the largest number of analyzed isolates pathogenic strains belonged to *Shigella sonnei* (62% in our study), while the remaining 38% corresponds to *Shigella flexneri* serovars. These data suggest that the appearance of these new *Shigella flexneri* producer-strains may be a recent event. This observation is clearly supported in Santiago del Estero, since no producer strains were detected in the previous periods. However, these producer strains have been isolated since 2015 in Tucumán and Catamarca provinces. This study demonstrates that there is a large exchange of pathogenic strains between the provinces of our region, allowing these strains to acquire new selective advantages to establish the gastroenteritis diseases in new hosts and environments.

Table II. Antimicrobial spectrum of *Sh*pCI172 action.

ATCC Strains	ShpCI172 Sensitivity[a]
Gram-negative	
Salmonella Typhimurium	R
Shigella flexneri	S
Shigella sonnei	S
Escherichia coli DH5α	S
Escherichia coli MC4100	S
Escherichia coli AB1133	S
Klebsiella pneumonia	R
Vibrio alginolyticus	R
Vibrio parahaemolyticus	R
Gram-positive	
Listeria monocytogenes	R
Clostridium botulinum	R
Clostridium perfringens	R
Enterococcus faecalis	R
Staphylococcus aureus	R

[a]**The microcin effect was detemined by spot sensitiviy in plate. S: sensitive, R: resistant.**

Therefore, in this work we studied the antimicrobial compound produced by the *Shigella flexneri* 2 CI172 strain. We demonstrated here that high temperatures and early exponential growth phase induced the synthesis and/or export of this antimicrobial to the culture medium. *Sh*pCI172 was sensitive to degradation by proteinase K, indicating that this compound is of a protein nature. *Sh*pCI172 was resistant to the autoclaving treatment, showing activity after heating. The *Sh*pCI172 activity was not affected by the medium pH. *Sh*pCI172 showed also a negative charge at pH 8. These antimicrobial compound characteristics suggested that it is a low molecular weight bacteriocin that we named *Sh*pCI172, which positions it as the first antimicrobial peptide described as a member of the microcins family produced by a *Shigella flexneri* serovar 2 strain (Asensio & Perez-Diaz 1976). Furthermore, we confirmed the small molecular mass of *Sh*pCI172 by HPLC-MS of approximately 3,183.68 Da; that *Sh*pCI172 is unable to be stained with coomassie blue, and that it is compound more effective against strains of their own class (Cotter et al. 2013, Salomon &

Farias 1992). Together, these results allowed us to confirm that *Sh*pCI172 is a new member of the microcin family, since CI172 did not display cross immunity with other microcin and showed specific characteristics. It is important to note that this is the first report where the production of this kind of antimicrobial agents by a *Shigella flexneri* 2 strain is described.

According to previously reported, we suggest that the CI172 strain uses the bacteriocin production as a survival mechanism mainly developed in certain hostile environments (Riley & Gordon 1999, Martina Sassone-Corsi 2016, Asensio & Perez-Diaz 1976). We concluded that the finding of this new antimicrobial compound is an important contribution in the biotechnology field, since displays good characteristics for its application in the development of new antibiotics and/or food preservatives agents.

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