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ORIGINAL ARTICLE

Shigella flexneri strains produce bacteriocins active against members of the human microbial intestinal flora

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ABSTRACT. The principal aim of this work was to detect the bacteriocinogenic capacity of *S. flexneri* strains on members of the human intestinal flora. A total of 49 bacteriocinogenic *S. flexneri* strains were isolated from individuals of both sexes and different ages. The bacteriocins were detected by means of the drop method using *E. coli* and *B. fragilis* as target strains. The serotypes of the *S. flexneri* were determined. The producer capacity of bacteriocins was analysed in 10 different colonies of the same cellular clone and also the arbitrary units were determined. The highest number of bacteriocinogenic *S. flexneri* strains were obtained from diarrhoeal individuals from 0-10 years old and the *S. flexneri* serotype 2a was the most abundant. It was demonstrated that *E. coli* and *B. fragilis* isolated from the normal intestinal flora of healthy individuals were susceptible to the bacteriocinogenic *S. flexneri* strains. By means of the determination of arbitrary units per ml of the bacteriocin, it was demonstrated that colonies from a single colony isolate of a same clone of bacteriocinogenic *S. flexneri* produce different quantities of bacteriocin.

Key words: *Shigella flexneri*, serotypes, diarrhoea, bacteriocins.

RESUMEN. El principal objetivo de este trabajo fue el detectar la capacidad bacteriocinogénica de *S. flexneri* sobre miembros de la flora intestinal humana. Un total de 49 cepas de *S. flexneri* bacteriocinogénicas fueron aisladas de individuos de diferentes sexos y edades. El mayor número de estas cepas fue obtenido de individuos con diarrea de entre 0-10 años y el serotipo 2a de *S. flexneri* fue el más abundante. Se demostró que *E. coli* y *B. fragilis* aislados de la flora intestinal de individuos sanos fueron susceptibles a las bacteriocinas de *S. flexneri*. Mediante la determinación de unidades arbitrarias por ml de bacteriocina, se demostró que colonias derivadas de un mismo clon celular de *S. flexneri* bacteriocinogénica sintetizaron diferentes cantidades de bacteriocina.

Palabras clave: *Shigella flexneri*, serotipos, diarrea y bacteriocinas.

INTRODUCTION

The bacteriocins are proteins produced by several bacterial species with lethal activity over a wide range of Gram positive and negative bacteria. Bacteriocinogenic strains have an immune mechanism of self-protection against its own bacteriocin.[6] The genetic determinants of most of the bacteriocins are located in plasmids.[5] These bacterial products exert their bactericidal activity through the adsorption to specific receptors located in the external surface of susceptible bacteria. This process is followed by metabolic and morphological changes leading to the death of the target bacteria.[7]

In general, many of the described bacteriocins present ecological properties related mainly with the natural nutritional competence with other related bacterial genus.[10] Many bacteriocins of the *Enterobacteriaceae* family are known; the colicins produced by different strains of *E. coli* are the most studied, and so far the best characterized.[2] It has been reported that some strains of the genus *Shigella*, like *S. sonnei* and *S. boydii*, are able to produce bacteriocins.[8,11] The main objective of this paper is to detect bacteriocins from *S. flexneri* strains isolated from diarrhoeic faeces from individuals of both sexes and to relate the ages of the patients with the presence of the bacteriocinogenic strains. The other purpose of this research is to determine the susceptibility to these antibacterial substances, of microorganisms in the normal intestinal bacterial flora, as *E. coli* and *B. fragilis*, isolated from healthy individuals.

MATERIAL AND METHODS

In Talca (Chile), 116 strains of *S. flexneri* were isolated from individuals with diarrhoea, ranging from 0 to 50 years old. All these bacteria were identified by a biochemical test and serological typing, and were numbered arbitrarily.[3] The serotypification of the bacteriocinogenic *S. flexneri* strains was performed with monovalent antisera (Seiken) according to the instructions provided by the manufacturer. The following bacterial strains were analysed as target of the lethal activity of the bacteriocinogenic *S. flexneri*: 40 *E. coli* and 40 *B. fragilis* abacteriocinogenic strains were obtained from faecal cultures of healthy people. All the target

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strains were identified according to Bergey's Manual of Systematic Bacteriology.[4] None of the target strains showed any bacteriocinogenic reverse interaction with bacteriocinogenic *S. flexneri* strains.

S. flexneri strains were cultured in 20 ml of BHI broth (Merck) at 37ºC for 36 h, with constant stirring. Later on, the culture was centrifuged at 10,000 x g for 20 min. The supernatants were studied to detect bacteriocins. *E. coli* target strains were grown in BHI broth until the first stage of exponential growth (O. D.: 0,4 at 660 nm) and immediately sown in lawns in Petri dishes containing Mueller Hinton agar (Merck). The dishes were dried for 10 min at 37ºC and then 5 μl of the supernatants were spot in lawn. The dishes were incubated at 37ºC for 5 h and then the inhibitory zones were observed. The same procedure was applied with *B. fragilis*; however, these bacteria were grown in 5% human blood agar containing kanamycin (1%) and vitamin K (4%). After the bacteriocin was applied, the dishes were kept in a strict anaerobic system (Bio-Merieux) for 48 h at 37°C and then the inhibitory zones were observed. The same assays described above were performed with *E. coli* and *B. fragilis* using the bacteriocinogenic *S. flexneri* as target strains.

In order to confirm their proteic nature, the bacteriocins were treated with proteolytic enzymes such as proteinase K, protease type IV and pepsin (Sigma), according to the instructions of the manufacturer.

The production capacity of bacteriocins was analyzed in different colonies from the same clone. Ten bacteriocinogenic *S. flexneri* strains were grown in 50 ml of BHI broth (Merck), incubated overnight at 37ºC and then a 10^{-6} dilution in sterile distilled water was prepared. Fifty μl of each dilution were sown in lawn over Mueller-Hinton agar and incubated at 37ºC for 24 h in order to obtain isolated colonies. Later on, the dishes were covered with a thin layer of 5 ml of fresh BHI medium containing 0.7% agar (soft agar), mixed with 2 ml of a log phase culture of the indicator *E. coli* strain and the dishes were incubated at 37ºC for 5 h in order to observe the inhibitory zones. From these colonies, ten bacteriocinogenic *S. flexneri* strains were selected that showed different sizes of inhibitory zones, including some with or without any inhibitory zone,. These colonies were separately grown in 5 ml of BHI broth, incubated overnight at 37º C and then the cultures were centrifuged at 6,000 x g for 10 minutes. One ml of the each of the supernatants was serially diluted in sterile distilled water (in two-fold decrements) and 5 μl of each dilution was assayed to detect bacteriocin activity by the method described before. One arbitrary unit ml^{-1} (AU/ml) of bacteriocin was defined as 5 μl of the highest dilution of supernatant yielding a definite zone of growth inhibition in a lawn of susceptible *E. coli* cells. The AU was calculated as follows: 1000/5 x (reciprocal of highest positive dilution).[1]

Ten colonies of each one of the bacteriocinogenic *S. flexneri* clones without inhibitory zones were treated with 0.5 μg/ml of mitomycin C (Sigma) in order to investigate the bacteriocin induction

RESULTS

All the proteolytic enzymes analysed, produced inactivation of all the bacteriocins synthesized by the studied *S. flexneri* strains. Furthermore, the cross-reaction between all the bacteriocinogenic *S. flexneri* gave negative results. Table 1 shows no differences between the numbers of *S. flexneri* strains obtained from individuals of both sexes. Table 2 shows that the serotype 2a was the most abundant in the all the *S. flexneri* analysed. It is also observed that only four serotypes of *S. flexneri* were detected in the bacteriocinogenic groups in comparison to six serotypes in the non-bacteriocinogenics. Table 3 shows that the highest numbers of bacteriocinogenic strains were isolated from individuals ranging from 0 to 10 years. On the other hand, all the bacteriocinogenic *S. flexneri* strains presented lethal activity over the 36 of the 40 *E. coli* strains and 34 of the 40 *B. fragilis* analysed, being both species isolated from faecal cultures of healthy individuals (Table 4). It was also observed that *S. flexneri* colonies derived from the same clone present differences in bacteriocin production, when analysed according to the diameter of the inhibitory zone or the absence of it (Fig. 1a). A direct relationship was also demonstrated between AU and the diameter of the inhibitory zones for all the studied colonies of *S. flexneri* (Fig. 1b). The production of the bacteriocin was not induced by mitomycin C in any of the *S. flexneri* colonies without inhibitory halo (data not shown).

DISCUSSION

It was demonstrated that all the studied antibacterial products were sensitive to the studied proteolytic enzymes. These results confirm the proteic nature of these antibacterial products. In addition, according to the results of the

Table 1. Number of bacteriocinogenic *S. flexneri* strains isolated from individuals of both sexes.

Individuals	Number of *S. flexneri*	*S. flexneri*	
		Bacteriocinogenic	Non bacteriocinogenic
Male	59	26	33
Female	57	23	34
Total	116	49	67

Table 2. Numbers of bacteriocinogenic and non-bacteriocinogenic *S. flexneri* serotypes according to age of patients.

Range of ages (years)	*S. flexneri* serotypes: Bacteriocinogenic 2a	3a	4b	5b	Non-bacteriocinogenic 1b	2a	3b	4b	5b	6
0 - 10	14	–	2	4	8	2	1	4	3	4
11 – 20	7	1	–	2	2	1	–	2	2	1
21 – 30	5	1	2	1	1	10	2	6	–	–
31 – 40	3	–	1	1	–	6	2	–	2	2
41 – 50	3	1	1	–	–	2	1	–	2	1
Total	32	3	6	8	11	21	6	12	9	8

Table 3. Numbers of bacteriocinogenic and non-bacteriocinogenic *S. flexneri* according to age.

Range of ages (years)	*S. flexneri* strains: Bacteriocinogenic N°	%	Non-bacteriocinogenic N°	%
0 - 10	20	17.2	14	12.0
11 - 20	10	8.6	28	24.1
21 - 30	9	7.7	16	13.7
31 - 40	5	4.3	4	3.4
41 - 50	5	4.3	5	4.3
Total	49	42.1	67	57.5

Table 4. Sensitivity of *E. coli* and *B. fragilis* isolated from faeces of healthy individuals to bacteriocins produced by *S. flexneri* strains.[1]

Target strains (n°)	Bacteriocin activity: Sensitive	Resistant
E. coli (40)	36	4
B. fragilis (40)	34	6

[1]The 49 *S. flexneri* strains were analyzed against all the target strains.

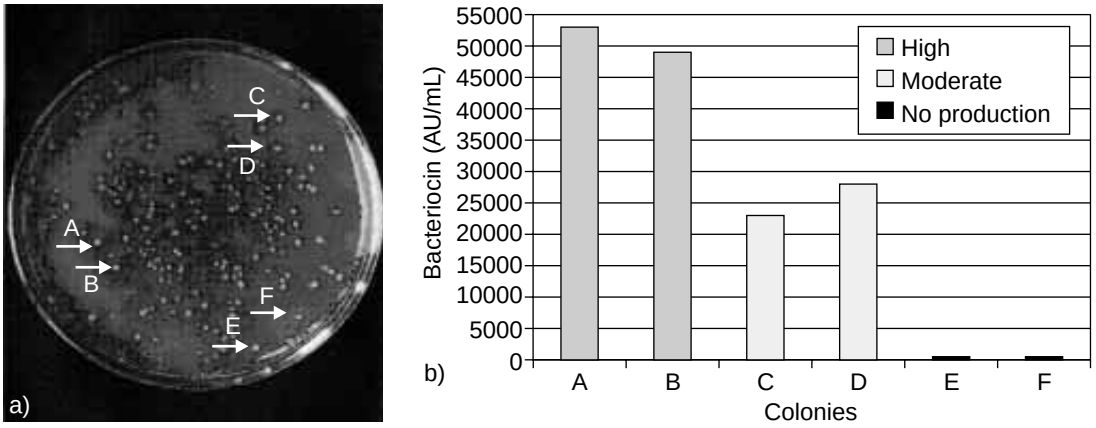

Figure 1. *a) Differences in the diameter of the inhibitory zones observed with several colonies of* S. flexneri 11 *from the same clone. b) Bacteriocin arbitrary units / ml (AU/ml) obtained from colonies of S. flexneri 11 that produce different diameters of inhibition zones. High production: colonies A and B; moderate production: colonies C and D; no production: colonies E and F.*

cross-reaction experiment it can be postulated that all the *S. flexneri* strains produce the same bacteriocin; it can be argued that all the strains contain the immunity gene which activated together with the respective bacteriocin gene. It was interesting to note that the sex is a non important factor in the infection process with these bacteriocinogenic bacterial species. The age of the patient was also not a determining factor for the infection process with the bacteriocinogenic *S. flexneri*, since the age range of the infected individuals varied between 0 to 50 years old. However, the greatest number of the bacteriocinogenic *S. flexneri* were isolated mainly from the first group (0 to 10 years old). This group is always the more affected with bacterial diarrhoea infections in underdeveloped countries.[9]

It was initially thought that all these strains could belong to a unique serotype. However, it was demonstrated that

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the bacteriocinogenic *S. flexneri* strains are distributed between four different serotypes, although the predominant one was 2a, which is the most frequent in Chile.[9] In the non-bacteriocinogenic *S. flexneri*, it was demonstrated the presence of six different serotypes, but also the 2a was the most common one.

It was observed that a high number of *E. coli* strains (36) as well as *B. fragilis* (34) isolated from faecal cultures from different healthy individuals were susceptible to all the studied bacteriocins. Moreover, it is also interesting to note that none of the 49 studied bacteriocins was active on the resistant target strains. These results can indicate a natural susceptibility or resistance in the members of the normal intestinal flora.

Actually, it is known that *S. flexneri* exert its pathogenic capacity through a complex process of invasiveness which has been studied in depth,[13] but still there are some aspects of the process that need to be investigated. Anyway, in accordance with the results obtained in this work, it is suggested that the bacteriocins may play a role in the first stages of the infection process. Thus, these bacteriocins could possibly displace susceptible bacteria of the normal intestinal flora from the epithelial cells and could quickly initiate the invasive stage in the virulence process. For example, strains of *E. coli* responsible for generalized infections and death in livestock and humans usually produce colicin V, that enhances virulence. The loss of this bacteriocin reduce the virulence in both animal and human experiments.[12] It could be argued that a similar effect could be obtained with bacteriocinogenic *S. flexneri* strains.

Furthermore, it was observed that the different colonies of the same clone of *S. flexneri*, varied in their capacity to produce inhibitory zones and a direct relation was demonstrated between the biggest inhibitory zones and the highest arbitrary units produced by the bacteriocinogenic *S. flexneri* strains. Considering these results, it could be argued that the infection with the hyperproductive bacteriocinogenic *S. flexneri* strains and the presence of a normal bacterial flora susceptible to these antimicrobial products, would induce a diarrhoeic infection.

To provide further evidence it will be useful to investigate the association between the genes that participate in the invasiveness process of the infectious agent and the expression of the bacteriocin gene, in order to get a better understanding of the pathogenic capacity of *S. flexneri*

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