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CONJUGATIVE PLASMIDS ISOLATED FROM ENTEROPATOGEN *Shigella sonnei* CODE ANTAGONIST SUBSTANCE(S) AND ANTIMICROBIAL RESISTANCE

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Abstract: *Shigella*, an invasive enteropathogen, is responsible for bacillary dysentery or shigellosis, a severe diarrheal disease that mainly affects children in underdeveloped countries. The bacterium expresses several virulence factors, including antagonistic substances and antimicrobial resistance. These substances are often encoded by plasmid genes, which can be transferred by recombination processes such as bacterial conjugation. The present study was carried out with the objective of determining whether resistance to antimicrobials and the antagonist substance(s) synthesized by *Shigella sleepy* SS9 are encoded by plasmid genes. As a developer of plasmid antagonist and receptor expression, samples *S. sonnei* SS12 and *Escherichia coli* ATCC 25922, respectively, were used. For the SS9 and *E. coli conjugation assay*, *E. coli* was evaluated for their antimicrobial susceptibility profiles. After antimicrobial selection, SS9 and *E. coli* were grown separately, cultures adjusted to 0.5 McFarland scale and diluted 1:50. An aliquot of each sample was added to the same tube and the material was incubated. At predetermined time intervals, aliquots were cultured on MacConkey Agar with and without antimicrobial. lac⁺ colonies were evaluated for antagonism expression. Positive colonies were subjected to plasmid extraction and electrophoresis analysis. After culturing a transconjugant sample, the material was centrifuged and the supernatant analyzed by the antagonist assay. Analysis of the growth curve of the transconjugant with the synthesis of bioactive substance(s) was also performed. The results show that the transconjugant sample started to harbor conjugative plasmids with the presence of genes that encode resistance to the antimicrobials ampicillin and trimethoprim, in addition to the production of antagonist substance(s). Proving the presence of

virulence factors, which can be transferred by conjugation, from the enteropathogen *S. sonnei*, which was recognized by the World Health Organization as one of the twelve pathogens that urgently need the development of new drugs.

Keywords: *Shigella sonnei*, antagonist substance, enteropathogens, bacterial conjugation, antimicrobial resistance.

INTRODUCTION

Belonging to the phylum Proteobacteria, class Gamaproteobacteria, order Enterobacteriales, family Enterobacteriaceae, the genus *Shigella* is constituted by Gram negative rods, facultative anaerobes, immobile, not encapsulated and incapable of fermenting lactose [11; 16; 20; 22; 28]. The four species of the genus can be distinguished on the basis of biochemical and serological evidence on *S. sonnei* (1 serotype), *S. flexneri* (18 serotypes), *S. dysenteriae* (15 serotypes) and *S. boydii* (20 serotypes) [10].

Shigella spp. is the etiologic agent of shigellosis, for which humans are the only known reservoir [29]. The bacterium has a worldwide dissemination pattern, with about 188 million infected individuals and approximately 164,000 deaths/year, most of them children [9]. Transmission occurs mainly by the fecal-oral route, via ingestion of contaminated water and food, but transmission via fomites, flies and sexual contact is also observed [29]. From 10 to 100 bacterial cells are required for the development of the disease [7]. It is manifested by the occurrence of fever above 38 °C, abdominal cramps and mucous bloody stools, with the presence of the viable pathogen for a few weeks [29]. More severe forms of presentation include vomiting, anorexia, and formation of abscesses and mucosal ulcerations. In some cases, more than 20 bowel movements per day are reported [1; 6; 13; 15; 16; 20; 26].

Patients with moderate forms of shigellosis can be treated with oral hydration and a balanced diet. However, antibiotic therapy is recommended in order to reduce the duration and severity of the condition, in addition to limiting the spread of the bacteria [12; 17; 21].

Antimicrobial drug resistance, even *multi-drug profile resistant* (MDR) and *extensively drug-resistant* (XDR) has been described for *Shigella* spp. Even worryingly, in the last year, the World Health Organization (WHO) was notified of the occurrence of high cases of Shigellosis caused by *S. sonnei* (XDR) in several countries such as the United Kingdom and Great Britain. This raises concern, due to the low availability of active drugs capable of controlling the spread and severity of the disease [29]. To this end, the WHO included *Shigella* spp. in the list of twelve pathogens that urgently require the proposition of new treatment measures [6].

Antagonist substances, among these antimicrobial peptides and proteins, can be encoded by plasmid or chromosomal genes. They are considered virulence components produced by bacteria because they help in the conquest of a habitat and in the competition for nutrients, since they act against other microorganisms. However, due to the molecular bioactivity of these substances, they also arouse great interest in the development of new drugs and may contribute to the development of new antimicrobial compounds, which may, in the future, be an alternative in the treatment of some diseases, including Shigellosis [23; 25].

Based on the increasing profile of resistance to multiple drugs, the ability of *S. sonnei* to produce substances with antagonistic action to another isolate of the same species and the possibility that these genes of resistance and production of antagonist substances are encoded by conjugative plasmids. The objective of this work was to investigate the

presence of conjugative plasmids present in an isolate of *S. sonnei*, capable of contributing to the virulence of the pathogen, by coding the production of antagonist substance(s) and antimicrobial resistance profile.

MATERIALS AND METHODS

BACTERIAL SAMPLES

Two samples of *S. sonnei*, (SS9 and SS12) were used in the study as producer(s) of antagonist(s) and antagonism-revealing substance(s), respectively. They were obtained from fecal samples from children with shigellosis treated at Hospital Infantil João Paulo II, Belo Horizonte, Brazil [24]. To choose the receptor sample used in the conjugation assay, the overlayer diffusion test was performed, using a panel of three samples, *E. coli* K12 Row, *E. coli* ATCC 25922 and *S. flexneri* ATCC 12022. All samples are part of the library of the Laboratory of Oral and Anaerobic Microbiology and have been kept in Brucella broth (BBL, Sparks, MD, USA) added with 10% glycerol (v/v) at -80°C.

OVERCOAT DIFFUSION TEST

Before use, all strains were cultured for three consecutive days on Tryptic Soy Agar (TSA; Difco, Sparks, MD, USA) at 37 °C, for 24 h, under aerobic conditions. The samples were inoculated separately in Tryptic Soy Broth (TSB; Difco), and incubated under the same conditions. The concentration of each culture was adjusted to read from 0.08 to 0.10 using λ of 625 nm [4]. Sample aliquots were analyzed as producing and revealing antagonist action (QUA. 1). For the assay, 10 μ L of the culture of the possible producer sample was deposited on the surface of TSA and the plate incubated in the same condition. Viable cells were eliminated by exposing steam to chloroform for 30 min followed by an open plate in a sterile environment to eliminate chloroform vapor. The plate was coated with 3.5 mL of semi-solid TSA (TSB + 0.7% agar) containing 10 μ L of the possible developer sample and incubated as described above. Antagonist activity was observed by the presence or absence of an inhibition halo around the producing sample [3, 8; 18; 19].

Producer of antagonistic substance	Revealing the expression of antagonism				
	<i>S. sonnei</i> SS9 ^b	<i>S. sonnei</i> SS12	<i>S. flexneri</i> ATCC 12022	<i>E. coli</i> K12 Row	<i>E. coli</i> ATCC 25922
<i>E. coli</i> K12 Row ^a	T ^c	T	NT	NT	NT
<i>E. coli</i> ATCC 25922	T	T	NT	NT	NT
<i>S. flexneri</i> ATCC 12022	T	T	NT	NT	NT
<i>S. sonnei</i> SS9	NT ^d	CP ^{and}	T	T	T

^a samples evaluated as producing antagonist substance; ^b samples tested as revealing the expression of antagonism; ^c test performed; ^d not tested; ^{and} positive control.

Table 1: Evaluation of antagonist activity and cellular susceptibility to the antagonist substance for selection of a recipient sample for conjugation test.

ANTIBIOGRAM

Samples *S. sonnei* SS9 and *E. coli* ATCC 25922 were used in the antibiogram assay by the disk diffusion method. *E. coli* ATCC 25922 is recommended by CLSI 2018 as a control sample for antibiogram assays involving members of the *Enterobacteriaceae* family. In the present study, it was also used as a candidate for the recipient in the conjugation process. For the assay, a panel of 19 antimicrobial drugs was used, from the following classes: penicillins, first to fourth generation cephalosporins, carbapenems, aminoglycosides, quinolones, monobactams, amphenicols and sulfonamides. Discs were selected based on antimicrobial resistance conferred by plasmids.

For the test, 50 ml of *Mueller Hinton Agar* (Difco)/150x30 mm plate were dispensed and, after solidification of the medium, samples *S. sonnei* SS9 and *E. coli* ATCC 25922, both grown in *Mueller- Hinton Broth* (Difco), adjusted in a spectrophotometer with absorbance of 625 nm, reading from 0.08 to 0.10, were seeded with the aid of a *swab*. Antimicrobial disks were deposited on the surface of the medium and the material was incubated at 37 °C for 16 h under aerobic conditions. The reading was performed by macroscopic observation of the presence of inhibition halo and measurement, with a caliper, of each halo [4; 5].

CONJUGATION

SS9 was used in the assay to verify the ability to transfer, by conjugation, plasmids that harbor genes encoding antagonist substance(s) and antimicrobial resistance. *E. coli* ATCC 25922 was evaluated as a recipient of such plasmids.

Samples in studies were inoculated in 2 mL of Penassay broth (Difco) and incubated for 24 h, without agitation, in an aerobic atmosphere at 37 °C. The cultures were adjusted in a spectrophotometer with absorbance of 625

nm, for reading from 0.08 to 0.10, and diluted 1:50 v/v, with a final volume of 3 mL, in the same broth. The tubes were incubated under the same conditions as above, for 3 h, under agitation at 300 rpm. The cultures were diluted, in the same broth, in the proportion 1:10 v/v. A 1 mL volume of cultures from the producer and recipient samples were then mixed in a sterile test tube. The samples were incubated in an aerobic atmosphere at 37 °C. At time intervals of 0, 15 and 30 min, 1, 3, 5 and 24 h, 100 µL aliquots were seeded on MacConkey Agar (Difco) plus ampicillin (Sigma-Aldrich, São Paulo, SP, Brazil) 32 µg / ml. The same samples were diluted 1:10 v/v in Penassay broth and 100 µL were seeded in medium without antibiotics. As a control, 100 µL of the producer and recipient samples were inoculated in the same medium with and without ampicillin. The plates were incubated in an aerobic atmosphere at 37 °C for 24 h. Reading was performed by counting lactose positive colonies (Lac⁺) [27].

CONFIRMATION OF CONJUGATION AND ANTAGONIST EXPRESSION RESULTS

To ensure the absence of *Shigella colonies* in the samples recovered in the conjugation assay, the lac⁺ colonies were streaked on MacConkey Agar and incubated in aerobiosis at 37 °C for 24 h. The *E. coli* colonies obtained were cultured in TSA under the same conditions described above. Clones of the studied samples were obtained using the replica plating technique [14]. The overlay diffusion assay was performed with the transconjugant *E. coli samples from the replica plate*.

PLASMID EXTRACTION AND ELECTROPHORESIS

plasmid extraction assay was performed on *S. sonnei* SS9, *E. coli* ATCC 25922 and *E. coli* transconjugant, using the *kit 96 Well*

Plasmid Kit (Geneaid, New Taipei City, Taiwan). The products obtained and the molecular mass standard GeneRuler™ DNA Ladder Mix # SM0331/2/3 (Fermentas, Life Sciences, Germany) were added to DNA dye GelRed™ (Biotium, Houston, TX, USA) and electrophoresed in 0.7% (w/v) agarose gel (Kasvi, Curitiba, PR, Brazil) prepared with TBE buffer (Tris/Borate/EDTA). The electrophoretic run was performed in a vat containing TBE buffer, under a current of 75 V, for approximately 2 h and 15 min. After the run, the plasmid profile was visualized in an ultraviolet light transilluminator.

GROWTH CURVE AND TRANSCONJUGANT ANTAGONIST ACTIVITY TEST

Based on the antagonist activity tests, by the overlayer diffusion test, and electrophoretic profile in 0.7% agarose gel, the transconjugant sample 9 was chosen for the evaluation of the antagonist substance synthesis in relation to the culture time. The chosen sample was cultivated in TSA, at 37°C, for 24 h, under aerobic conditions. Colonies obtained were transferred to TSB broth and incubated under the same conditions for 12 h. The culture was adjusted in a spectrophotometer with absorbance of 625 nm, reading from 0.08 to 0.10, fractionated in pre-identified tubes and incubated. Then, the sample from tube 1 was subjected to optical density reading, an aliquot of the culture was centrifuged and the supernatant evaluated by the overlayer diffusion test and titration [18]. Another aliquot was diluted in 0.85% saline, 100 µL of the 10⁻⁹ dilution was plated, in duplicate, in TSA medium and incubated. The same steps were performed for the other tubes, respecting the time interval, 20 min between each tube, for 9 h. The reading of the growth curve was performed by counting the number of colonies against time and the

synthesis of antagonist compound(s) was determined during the bacterial growth curve.

TITRATION OF ANTAGONIST SUBSTANCE

The tubes obtained from the growth curve and antagonist substance production assay were subjected to serial dilutions in ultrapure water and 10 µL of each dilution were deposited on a layer of TSA. After exposure to chloroform vapor and evaporation of residual chloroform for 30 min each, overlay diffusion tests were performed. The titer, defined as the reciprocal of the highest dilution that results in a clear zone of inhibition of the developer sample, was determined. Then, the antibacterial activity was calculated and expressed in arbitrary units (UA).mL⁻¹ using the formula “UA = titer x 1000 µL/v(µL) [2; 8; 18; 19].

RESULT

RECIPIENT SAMPLE SELECTION FOR THE CONJUGATION ASSAY

Based on the overlayer diffusion assay, for the evaluation of antagonist action, *E. coli* ATCC 25922 was chosen as the recipient for the conjugation test. The sample was not active against *S. sonnei* SS9 and *S. sonnei* SS12 and was also not inhibited by *S. sonnei* SS9 (TAB. 1). The choice of the recipient sample is an important step in the search for conjugative plasmids that may encode the production of antagonist substance(s), and antimicrobial resistance to drugs.

ANTIBIOGRAM

Phenotypic analysis, by antibiogram, evidenced the resistance of *S. sonnei* SS9 to the antimicrobial's trimethoprim and ampicillin, while *E. coli* ATCC 25922 was susceptible to them.

Producer of antagonistic substance	Revealing the expression of antagonism				
	<i>S. sonnei</i> SS9 ^b	<i>S. sonnei</i> SS12	<i>S. flexneri</i> ATCC 12022	<i>E. coli</i> K12 Row	<i>E. coli</i> ATCC 25922
<i>E. coli</i> K12 Row ^a	- ^c	-	NT	NT	NT
<i>E. coli</i> ATCC 25922	-	-	NT	NT	NT
<i>S. flexneri</i> ATCC 12022	-	+++ ^{and}	NT	NT	NT
<i>S. sonnei</i> SS9	NT ^f	+++	+ ^d	+++	-

^a samples evaluated as producing antagonist substance; ^b samples tested as revealing the expression of antagonism; ^c absence of antagonist activity; ^d small and cloudy inhibition halo; ^e large and clear inhibition halo; ^f not tested.

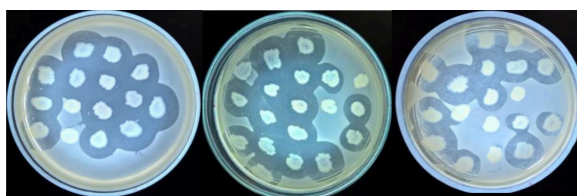
Table 1: Evaluation of antagonist activity and cellular susceptibility to the antagonist substance for selection of a recipient sample for conjugation test.

CONJUGATION

E. coli colonies, which are lactose positive, were considered. Conjugation between *S. sonnei* SS9 and *E. coli* ATCC 25922 with transfer of the plasmid encoding ampicillin resistance was observed from 15 min. *E. coli* isolates Selected transconjugants also showed resistance to trimethoprim. In the medium without antimicrobial, the number of lac⁺ and lac⁻ colonies was uncountable, which made the use of this assay unfeasible.

TRANSCONJUGANT CELLS

The overlayer diffusion test for evaluation of antagonist expression by samples of *E. coli* ATCC 25922 transconjugants recovered from the medium with ampicillin showed a positive result for some samples (FIG. 1). The data demonstrate the horizontal transfer, by conjugation, of plasmids that confer the phenotypic characteristics of antibiotic resistance and antagonist activity against SS12.



Inhibition halos produced by *E. coli* ATCC 25922 transconjugant samples recovered from ampicillin medium, against the developer sample SS12.

Figure 1. Antagonist activity of transconjugant samples.

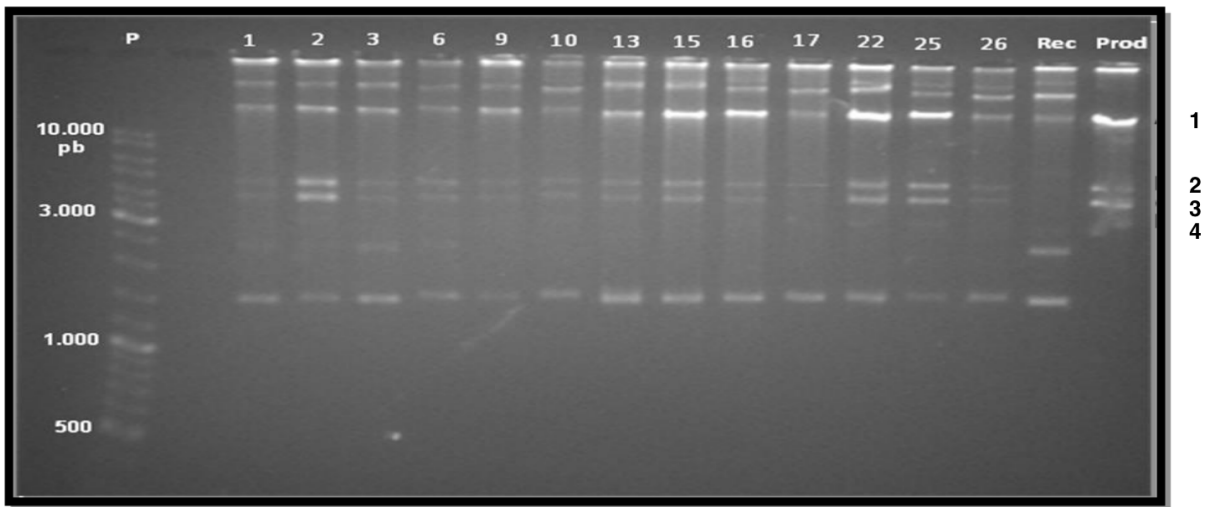
ELECTROPHORESIS

Plasmid profile of the samples is shown in FIG. 2. The *S. sonnei* SS9 sample showed four bands with different molecular masses, namely: greater than 10,000 bp and approximately 5,000 bp, 4,000 bp and 3,000 bp. The receiving sample showed a different profile from the producing sample, with the exception of a band with a molecular mass greater than 10,000 bp, two bands greater than 10,000 bp and two with about 2,500 bp and 1,500 bp are also observed, respectively. SS9

bands 2 and 3 were seen in all transconjugant samples, while band 4 was it was seen in only a few samples. The electrophoretic profile proves the efficiency of transfer by conjugation of plasmids.

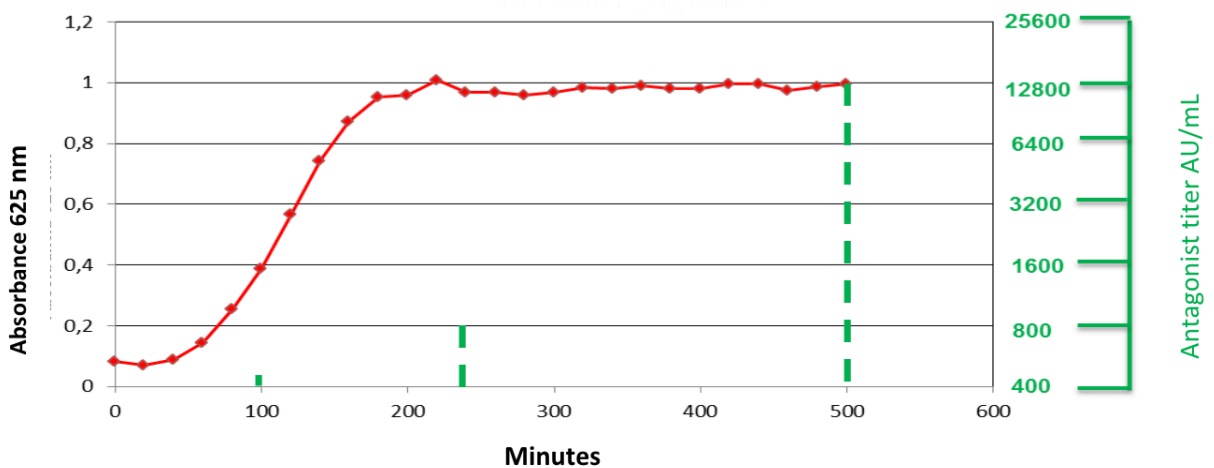
Growth curve and transconjugant antagonist activity test the transconjugant sample 9, evaluated in relation to the bacterial growth curve and the production of antagonist substances, presented the beginning of the exponential and stationary phases at 60 and

180 min, respectively. The presence of the antagonist substance(s) in the extracellular medium started, during the exponential phase, at 1h 40 min, with a titer of 400 AU/mL with the presence of unclear inhibition halos. At 4 h the antagonist titer was 800 AU/mL with halos still unclear. The best result of the inhibitory action was observed during the stationary phase, from 8:20 am, with 400 AU/mL in clear halos and 12,800 AU/mL with less clear halos (FIG. 3 and 4).



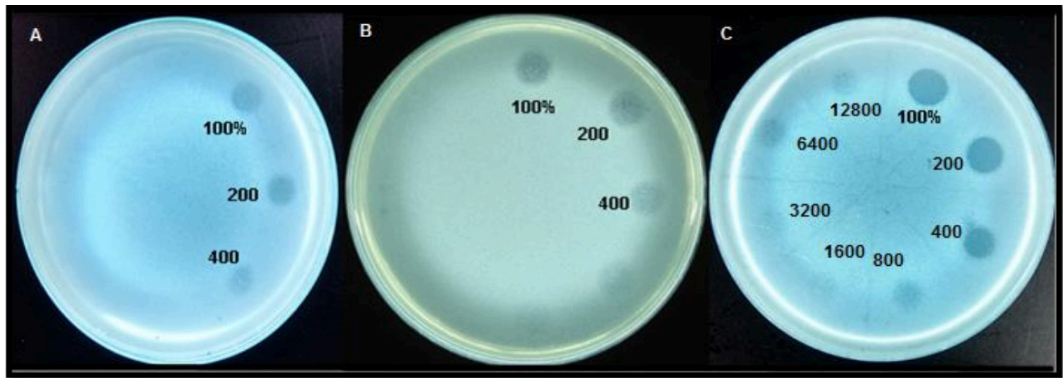
P, molecular mass standard (Gene Ruler™ DNA Ladder Mix # SM0331/2/3); 1 to 26, transconjugants; Rec, recipient sample, *E. coli* ATCC 25922; Producer producer, *S. sonnei* SS9.

Figure 2. Plasmid profile of producer, developer and transconjugant samples.



transconjugant sample 9, *E. coli* ATCC 25922 conjugated to plasmid (s) of *S. sonnei* SS9 producing antagonist substances.

Figure 3. Bacterial growth curve and production of antagonist substance(s).



Activity and antagonist titer of transconjugant 9 against the SS12 developer sample. **A**, beginning of antagonist activity and titer of 400 AU/mL of the sample recovered in the lag phase, with 1h 40min of culture; **B**, activity and antagonist titer of 800 AU/mL of the sample obtained after 4h of cultivation; **C**, Activity and antagonist titer of 12,800 AU/mL of the sample recovered at 8h and 20min of cultivation.

Figure 4. Production of antagonist substance(s) synthesized by transconjugant 9 during the bacterial growth curve.

DISCUSSION AND CONCLUSION

Recent reports demonstrate worldwide concern about the emergence of *Shigella* spp. (MDR) and (XDR), including *S. sonnei* [29]. The *S. sonnei* SS9 sample is resistant to the antimicrobials trimethopril and ampicillin, being the first drug of choice by the Brazilian Ministry of Health in the fight against shigellosis. Resistance genes can be transferred, by conjugation, to other bacteria, including those of a different genus from the original sample. After the conjugation assay, the selected transconjugant sample showed the phenotypic characteristic of resistance to the same drugs. This plasmid transfer can also be observed by the electrophoretic profile from the donor *S. sonnei*, *E. coli* ATCC 25922 and *E. coli* transconjugant. Plasmid transfer encoding ampicillin resistance was observed as early as 15 minutes. Watanabe & Fukasawa (1960) also reported the transfer of antimicrobial resistance over the same time period. Similar results were also reported by Yah (2010), who observed the transfer of a conjugative plasmid encoding antimicrobial resistance, including to ampicillin, between *Shigella* and *E. coli*.

As it was seen, the profile of antibiotic resistance is increasing, which demonstrates the need to develop other compounds that can act against these microorganisms. In addition to the synthesis of molecules that confer resistance, SS9 also produces substance(s) with antagonistic capacity against another isolate of the same species, SS12. After the conjugation assay between *S. sonnei* SS9 and *E. coli* ATCC25922 it was demonstrated that the genes encoding the substance(s) are contained in conjugative plasmid(s). The growth curve and production of antagonist substance showed that the transconjugant *E. coli* sample 9 initiated the synthesis of this molecule during the exponential phase and the best antagonist titer against the clinical isolate SS12 was observed during the stationary phase. The antagonist substance gives the sample that produces it a virulence factor by acting against other bacteria and thus contributing to the installation and permanence in a new habitat [18]. Based on its antagonistic capacity against the etiological agent of shigellosis, this molecule can, in the future, arouse interest as a model for drug production.

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