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Research Article

SCREENING OF ACTINOMYCETES WITH ACTIVITY AGAINST CLINICAL ISOLATES OF GRAM POSITIVE COCCI WITH MULTIRESISTANT PROFILE

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ABSTRACT

Actinomycetes are Gram-positive bacteria widely distributed in the environment and known for the diverse biologically active molecules they produce. This study assesses the activity of compounds produced by 40 actinomycete isolates against *Enterococcus* spp. *Staphylococcus aureus* and *Staphylococcus epidermidis* clinical isoalates. The susceptibility profile of the samples was assessed using the agar disk diffusion method. Antimicrobial activity of actinomycetes was evaluated according to the double layer method. Here, two isolates (50 and 8S) exhibited activity against 90% of clinical *Staphylococcus* spp. and *Enterococcus* spp. These isolates were grown in starch casein broth at 30°C for 7 days with constant shaking. After, the culture obtained was filtered to obtain a crude extract, whose antibiotic activity was assessed using the well diffusion technique. In this assay, isolate 50 presented higher activities than that exhibited by isolate 8S. The isolate 50 has been used in different assays looking for production of metabolite optimization.

Keywords: Antibiotic;Resistance;*Staphylococcus* spp; *Enterococcus* spp.; Actinomycetes

1. INTRODUCTION

The knowledge of microorganism resistance to chemical and physical agents dates back to the time when antibiotics were first developed. Resistance to antibiotics may emerge as a result of genetic changes (mutations) or the acquisition of resistance genes mediated by horizontal gene transfer (HGT) [1, 2]. Some organisms have a typical phenotype that presents susceptibility to antibiotics [3], which was acquired before the introduction of antibiotics in therapy.

In the past 30 years, resistance to antibiotics has increased worldwide to a significant extent, which has prompted the World Health Organization [4] to declare antibiotic resistance a public health issue in 1999. Ever since, a wide array of measures to control resistance has been put in place, including the collection of information on resistance profiles observed in different countries and regions [5].

Acknowledging this crisis in public health, several nations, international agencies and other organizations across the globe have taken measures to fight antimicrobial resistance to antibiotics based on specific strategies conceived to work in relevant sectors. Several resolutions issued by the World Health Assembly (WHA) have warned about the need to adopt specific steps addressing antimicrobial resistance, and the WHO published a strategy to check the phenomenon in 2001. On World Health Day 2011, WHO urged countries to adopt a six-item policy, which were: (A) commit to a comprehensive, financial national plan with accountability and civil society engagement, (B) strengthen surveillance and laboratory capacity, (C) ensure uninterrupted access to essential medicines of assured quality, (D) regulate and promote rational use of medicines in animal husbandry and to ensure proper patient care, (E) enhance infection prevention and control, and (F) foster innovations and research and development of new tools [4].

Gram positive bacteria today pose a challenge in anti-infection therapeutics, causing great concerns among medical doctors, microbiologists and the scientific community. This is due to the resistance that microorganisms present against antimicrobial drugs used in clinical treatment protocols. Moreover, the paucity of new antibiotics introduced in markets makes it urgent to conceive measures to prevent health crisis at global level.

Actinomycetes are Gram positive bacteria known for their capacity to produce antibiotics and other important secondary metabolites such as antitumor, anti-inflammatory, antifungal, anthelmintic and herbicide agents. Among these, antibiotics are the most important from the economic and therapeutic standpoint. Approximately two thirds of antibiotics produced today are isolated from actinomycetes. Considering the potential of actinomycetes in the production of bioactive molecules and the need for new antibiotic compounds, this study assessed the antibiotic activity of actinomycete isolates against clinical *Enterococcus* spp, *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates.

2. MATERIAL AND METHODS

2.1. Actinomycete isolates

Forty actinomycete isolates were used in this study. These isolates were obtained from a compost and landfarming plant and belong to the bacterium collection of the Laboratory of Environment Microbiology, Department of Microbiology, Immunology and Parasitology, ICBS, Federal University of Rio Grande do Sul (UFRGS). Microorganisms were isolated and identified using morphological and biochemical assays [6, 7].

2.2. Gram positive cocci isolates

The 15 *Enterococcus* spp. isolates used belonged to the bacterium collection of the Laboratory of Environment Microbiology, Department of Microbiology, Immunology and Parasitology, ICBS, UFRGS. The isolates were obtained from clinical samples collected in Hospital Santa Casa de Misericórdia, Porto Alegre, Brazil, by Dr. Pedro Alves d'Azevedo (UFCSPA). The 10 *Staphylococcus* spp. isolates were obtained from clinical samples collected in Hospital de Clínicas de Porto Alegre, and belong to the bacterium collection of the Laboratory of Molecular Biology, School of Pharmaceutics, UFRGS, and were kindly supplied by Dr. Ana Lucia Antunes (Table 1).

2.3. Susceptibility profile of *Enterococcus* spp. and *Staphylococcus* spp.

The susceptibility profile of *Enterococcus* spp. and *Staphylococcus* spp. samples was determined using the agar disk diffusion method following the recommendations by the Clinical and Laboratory Standards Institute (CLSI 2009). The antimicrobial agents used for the assay with the *Enterococcus* spp. were: nalidixic acid (30 μg), ampicillin (10 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), erythromycin (15 μg), streptomycin (10 μg), nitrofurantoin (300 μg), norfloxacin (10 μg) and vancomycin (30 μg). Susceptibility of *Staphylococcus* spp. was assessed using the antibiotics amoxicillin/ clavulanic acid (20/10μg), ampicillin (10μg), ciprofloxacin (5 μg), clindamycin (2μg), chloramphenicol (30μg), erythromycin (15μg), oxacillin (1 μg) and rifampicin (5 μg).

2.4. Antimicrobial activity of *Streptomyces* isolates

2.4.1. Double layer assay

Antimicrobial activity of the 40 *Streptomyces* spp. isolates against Gram positive coccus samples was first determined using the double-layer method. *Streptomyces* spp. isolates were inoculated using the spot inoculation method onto plates with in starch casein agar (SCA) prepared with 10g starch, 0.12g casein, 2.0g NaCl, 2.0g KNO_3, 2.0g K_2HPO_4, 0.05g $MgSO_4$, 0.01g $FeSO_4$, 0.02g $CaCO_3$, and 6g bacteriological agar). Incubation took place for 14 days at 30°C. Each plate was inoculated with four different isolates of *Streptomyces,* and three plates were prepared for each test microorganism. The *Enterococcus* and *Staphylococcus* strains to be tested were grown on trypticase soy broth (TSB) until the concentration of 10^8 cells/mL was reached. One milliliter (1 mL) of each bacterial culture was mixed with 9 mL of melted Mueller-Hinton agar and poured over the layer with *Streptomyces* grown on the plate. After the inoculation, the plates were incubated for 24-48 h at 37°C and the halo formation was evaluated.

2.4.2. Submerged growth culture

To produce the extract, isolates that presentedantimicrobial activity against *Enterococcus* spp. and *Staphylococcus* spp. in the double-layer assay were grown in submerged culture. Isolates were grown in 250mL flasks containing 50mL starch casein broth (SCB) (10g starch, 0.12g casein, 2.0g NaCl, 2.0g KNO_3, 2.0g K_2HPO_4, 0.05g $MgSO_4$, 0.01g $FeSO_4$, 0.02g $CaCO_3$) for 7 days at 30°C under agitation. Pre-inoculum was prepared in SCB in 50mL, for each *Streptomyces* spp and the cultures were grown for 48 h at 30°C with agitation at 100 rpm. After, 5mL of the cell growth culture was transferred to a new flask containing 50mL of the same culture medium. The culture was grown for 7 days under the same temperature and shaking conditions as used to prepare the pre-inoculum. On the 7^{th} day, 1mL was collected from the cell growth medium and centrifuged at 13,400 rpm for 10min to obtain crude extracts free of cells. Production assays were carried out in duplicate.

2.4.3. Antimicrobial assay using the supernatant well diffusion method

The well diffusion method consists of seeding using a swab soaked in a tested bacterium culture at 10^8 cells/mL (0.5 McFarland scale) on a Petri dish containing 20mL Müeller Hinton agar. After seeding, wells were made on agar using cylinders measuring 7 mm in diameter at identical distances across one another. Then, 100μL of the crude extract of each isolate was added to each well. The Petry dish

was kept at 4°C for 16h to promote the diffusion of the extract in the culture medium and then incubated at 37°C for 48h. The technique allowed determining activity of extracts based on the measurement of antibiosis index formed by extracts produced by isolates against the microorganism investigated. The antibiosis index was determined based on the ratio mean inhibition halos formed by the crude extracts to well diameter [8].

2.5. Statistical analysis

Data were analyzed using the STATISTICA software 7.1 (StatSoft Inc 2005) and the Tukey test ($\alpha = 0.05$).

3. RESULTS

3.1. Susceptibility profile of *Enterococcus* spp. and *Staphylococcus* spp.

The phenotypic profile of *Enterococcus* spp. isolates observed using the disk diffusion method showed that they were resistant to at least 55.55% of the antimicrobial agents used (Table 1). All *Enteroccocus* isolates exhibited resistance to ciprofloxacin and nalidixic acid. Phenotypically, 93% of the samples were resistant against erythromycin, norfloxacin, vancomycin and streptomycin. In turn, all samples were sensitive to penicillin and nitrofurantoin.

Table 1. Phenotypical antimicrobial resistance profile of clinical Enterococcus spp. isolates

Enterococcus spp	NAL[a]	AMP[b]	CIP[c]	CLO[d]	ERI[e]	NIT[f]	NORF[g]	STREP[h]	VAN[i]
488*	R	S	I	I	I	S	I	R	S
1950*	R	S	R	R	S	S	R	R	R
2389*	R	S	R	S	R	S	R	R	R
2074	R	S	R	R	R	S	R	R	R
2688	R	S	R	R	R	S	R	I	R
2714	R	S	R	R	R	S	R	R	R
2499	R	S	R	R	R	S	R	R	R
2390	R	S	R	R	R	S	R	R	R
1854	R	S	R	R	R	S	R	S	R
1884	R	S	R	R	R	S	R	R	R
1885	R	S	R	S	R	R	R	R	R
1950	R	S	R	R	S	S	R	R	R
1953	R	S	R	R	R	S	R	R	R
2072	R	S	R	R	R	S	R	R	R
2319	R	S	R	R	R	S	R	R	R

[a]*Nalidixic acid;* [b]*Ampicillin;* [c]*Ciprofloxacin;* [d]*Chloramphenicol;* [e]*Erythromycin;* [f]*Nitrofurantoin;* [g]*Norfloxacin;* [h]*Streptomycin,* [i]*Vancomycin. R= resistent; I= intermediate; S= sensitive.* Samples selected for the evaluation of antimicrobial activity of actinomycete isolates.*

Table 2 Phenotypical antimicrobial resistance profile of clinical Staphylococcal spp. isolates

	Samples	AM+ÁC[a]	AMP[b]	CIP[c]	CLO[d]	ERI[e]	OXA[f]	RIF[g]	CLIN[h]
S. aureus	21	R	R	R	S	R	R	R	R
	28	R	R	R	S	R	R	I	R
	53*	R	R	R	R	R	R	S	R
	103*	S	R	R	R	R	R	R	R
	209	R	R	R	S	R	R	S	R
S. epidermidis	177	S	R	S	I	R	R	R	R
	157*	S	R	I	R	R	R	R	R
	221*	S	R	R	S	R	R	R	R
	229	S	R	S	S	S	S	S	S
	51*	R	R	S	R	R	R	R	R

Clavulanic acid + Amoxicillin; [b]*Ampicillin;* [c]*Ciprofloxacin;* [d]*Chloramphenicol;* [e]*Erythromycin;* [f] *Oxacillin;* [g]*Rifampicin,* [h]*Clindamycin. R= resistent; I= intermediate; S= sensitive. * Samples selected for the evaluation of antimicrobial activity of actinomycete isolates.*

Staphylococcus spp. isolates exhibited resistance to at least 75% of the antimicrobials used, 100% were resistant to amplicillin and 90% were resistant to erythromycin, oxacillin and clindamycin (Table 2). These isolates presented high percent of resistance to oxacillin: 100% of *S. aureus* and 80% of *S. epidermidis* isolates.

3.2. Evaluation of the antimicrobial activity of Actinomycete isolates

Enterococcus spp. and *Staphylococcus* spp. isolates used in the double-layer assays were randomly selected, since there were no differences in susceptibility profiles between species in each genus. Therefore, these assays were carried out using the isolates *E. faecium* (488, 1300), *E. faecalis* (2389, 1950), *S. epidermidis* (221, 157, 51) and *S. aureus* (53, 21,103).

In the double-layer assay, 57.3% of actinomycetes exhibited antimicrobial activity against the clinical isolates used. Actinomycete isolates 50 and 8S were active against 90% of the clinical samples of *Staphylococcus* spp and *Enterococcus* spp. Of the Gram positive microorganisms used, samples 1950 (*E. faecalis*), 488 (*E. faecium*) and 1300 (*E. faecium*) were inhibited only by actinomycete isolates 50 and 8S. The more sensitive samples, that were inhibited by a larger number of actinomycete isolates were, respectively, 103 (*S. aureus*), 157 (*S. epidermidis*) and 2389 (*E. faecium*). No antibiotic activity of isolates 50 and 8S was observed against the sample *S. epidermidis* 51.

3.3. Activity of the crude extract by the well-diffusion method

Table 3. Antibiotic activity profile of actinomycete isolates 8S and 50 against clinical Gram positive cocci by the well-diffusion technique. Cell growth in SCB for 7 days at 30°C with agitation of 150 rpm.

Clinical Gram-positive	50	8S
21	3.42*	2.92*
53	3.28	1.42
103	3.78	2.92
157	3.21	1.57
221	2.21	0
488	3.85	1.71
1300	3.21	1.57
1950	3.35	2.28
2389	3.35	2.28
S. aureus ATCC 27664	4.00	3.42
SEE (Fiocruz)		
ATCC *Enterococcus faecium*	3.21	2.14

*Antibiosis index (AI)= mean inhibition halo/well diameter. Means followed by * in columnsdo not differ according to the Tukey test (α=0.05).*

Due to the fact that isolates 8S and 50 exhibited antibiotic activity against 83.33% and 91.66% of the clinical samples selected, they were chosen for the assay using submerged culture and the antimicrobial activity was observed using the well-diffusion method. Isolates 8S and 50 were grown in submerged SCB culture for 7 days at 30°C. Actinomycete isolate 50 inhibited all the clinical samples used, producing antibiosis indices between 2.21 (sample 221) and 3.78 (samples 21, 103 and 488). The crude extract produced by isolate 8S did not exhibit inhibitory activity against *S. epidermidis* samples 221 and 51 (Table 3).

4. DISCUSSION

Much of the progress recently seen in medicine such as chemotherapy, organ transplant and other procedures and treatments depend on the use of drugs that are active against infections caused by microorganisms. The predictable consequences of antimicrobial resistance include increased morbidity and mortality, longer disease spans and greater higher risks of complications. However, the phenomenon also takes its toll in economic terms, affecting productivity of workers, more costly diagnosis and treatment protocols (more consultations, more complex medical infrastructure, tracing of infection outbreaks, and more expensive drugs). In Europe, estimates reveal that over 25,000 deaths a year are caused by hospital acquired infections [4].

Due to the increased resistance to multiple drugs in staphylococci, mainly in strains resistant to vancomycin, new antimicrobial agents are required as an alternative to treat infections caused by these microorganisms. In this sense, the remarkable property of producing bioactive metabolites, such as antibiotics, exhibited by actinomycetes may become a pathway to fight resistant infectious microorganisms.

In the present study, *Enterococcus* spp. isolates were resistant to 55.55% of the antimicrobial agents used. Also, 100% of these isolates were resistant to vancomycin. Of the *Staphylococcus* spp. isolates, both *S. aureus* and *S. epidermidis* 75% were resistant to the antimicrobial agents, of which 100% were resistant to oxacillin. The double-layer assay showed that 57.3% of the actinomycetes tested were active against the isolates selected based on the susceptibility profile analyzed in the antibiogram. This is an interesting result concerning the quest for new drugs to treat multiresistant pathogens that emerge today, mainly methicillin-resistant *Staphylococcus aureus* (MRSA), methicilin-resistant *S. epidermidis* (MRSE), and vancomycin-resistant *Enterococcus* spp (VRE).

The higher number of patients infected and colonized by VRE is a worldwide problem. In a study carried out in Europe, Latin America and North America

between 1998 and 2004, a two-fold increase was observed in the rate of patients colonized by VRE in North America, though less consistent increases were recorded in Europe and Latin America [9]. Falci & Dalarosa [10] reported a significant increase in both in colonization and infection in several hospitals in Porto Alegre, Brazil, which characterizes an outbreak.

Since resistance to methicillin in *S. aureus* was first reported in the 1960's Barber [11], the increase in resistance to this antibiotic by this microorganism is reason for concern. The National Nosocomial Infection Surveillance (NNIS) reported that the prevalence of resistance to methicillin in *S. aureus* isolated in hospital environments rose from 2.1% in 1875 to 35% in 1991 [12] *Staphylococcus aureus* and *S. epidermidis* resistance to methicillin is codified in mobile genetic elements (MGE), that is, in the staphylococcal cassette chromosome *mec* (SCCmec), which codifies a penicillin-binding protein, BPB2a, which in turns exhibits lower affinity for methicillin in comparison with the affinity for other BPBs [13]

The results obtained in the present study confirm the bioactive potential of actinomycete strains. Actinomycetes produce most of the roughly 10,000 known antibiotics, and 75 to 80% of antibiotics prescribed today are derived from the genus *Streptomyces* [14]. Streptomycetes have become interesting sources of antibiotic compounds. Studies carried out by Cho et al. [15] have shown that the isolate *Streptomyces* CS392 produced highly active compounds against resistant Gram-positive bacteria, such as MRSA, vancomycin-resistant *Staphylococcus aureus* (VRSA) and VRE. Higgingbotham & Murphy [16] characterized a *Streptomyces* isolate from soil that presents bioactive molecules against MRSA.

The results of the present study demonstrate that isolate 50 exerted inhibitory actions against multiresistant clinical isolates, and can be characterized as a producer of bioactive compounds that may be used in future studies. Further investigations should be carried out to characterize and purify compounds, and carry out a molecular analysis by sequencing isolate 50.

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