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Isolation of *Serratia Marcescens* from the Soil and *In vitro* Prodigiosin Production as Source of Antibiotic, Active against Oxacillin-Resistant *Staphylococcus aureus*

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Authors' contributions

This work was carried out in collaboration among all authors. The work was a research project of an author INA under the supervision of author BCA with the assistance of author IWO. All the authors read and approved the final manuscript.

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ABSTRACT

Background: Wide range of microorganisms produced secondary metabolites as microbial activities in extended habitats.
Aims: The aim of this research is the extraction of the red colored pigment, prodigiosin from Serratia marcesens isolated from the soil and evaluate its antibacterial activity against different strains of oxacillin/methicillin-resistant *Staphylococcus S. aureus.*
Study Design: Two isolates, namely, RMN1 and RMN2, belonging to the Genus, *Serratia* from two soil samples collected from two strategic locations in University of Abuja, Nigeria were isolated. The isolates were morphologically distinct on the basis of spore colour, aerial and substrate mycelium formation and production of diffusible pigment. Isolates were Gram stained, observed under a microscope and were seen to be Gram negative. Biochemical tests revealed that the two isolates were catalase and citrate positive, and were oxidase negative. One of the two isolates was

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observed to have significant antibiotic producing potential, and the antibacterial activity of the produced antibiotics (red pigment extracted from cultural supernatants of the isolates grown on Peptone glycerol agar) was assessed using the agar-well diffusion method and streaking agar method.

Results: The results indicated that the pigment extracted *in vitro* had varying antibacterial activity at different concentrations of 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml against four strains of the test organism (*Staphylococcus aureus*) titled S1 to S4, the extracted pigment was more effective at concentration 500 mg/ml against the antibiotic resistant *S. aureus* with the MIC at 125 mg/ml.

Conclusion: The results indicated that the soil of this region could be a good source of *prodigiosin* having antibacterial activity and thus enable the use of micro-organisms as biological control agents.

Keywords: Isolation; Serratia; in vitro; prodigiosin; antibacterial.

1. INTRODUCTION

Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century [1]. *Staphylococcus aureus*, a virulent pathogen that is responsible for a wide range of infections including pimples, pneumonia, osteomyelitis, endocarditis and bacteraemia, has developed resistance against most classes of antibiotics against Gram-positive pathogens [2]. For more than two decades, clinicians and public health officials have faced hospital acquired methicillin-resistant *S. aureus* (HA-MRSA), which also bears resistance to many antibiotics, being one of the most common multidrug-resistant (MDR) pathogens. During much of this time, vancomycin has been the therapeutic answer to MRSA infections, but that paradigm has changed. Vancomycin-resistant strains have emerged clinically [3,4]. Vancomycin-resistant S. *aureus* (VRSA) challenges clinicians, not only because of vancomycin and methicillin-resistance, but also because of resistance to many other antibiotics, including aminoglycosides, macrolides, and fluoroquinolones. Fortunately, newer therapeutic agents, daptomycin, linezolid, and a streptogramin combination (quinupristin/ dalfopristin) have entered the clinical arena in the past few years [5]. However, certain undesirable side effects and the spread of pathogens with this new antimicrobial drug resistance emphasize the need for the development of other newer antimicrobial agents with activity against such Gram-positive bacteria [5,6,7,8].

Microorganisms are known to produce different pigments like carotenoids, melanins, flavins, quinones, prodigiosins and more specifically monascins, violacein or indigo [9]. Therefore, the aim of this research is the extraction of the red colored pigment, prodigiosin from *Serratia marcesens* isolated from the soil and evaluate its antibacterial activity against different strains of oxacillin/methicillin-resistant *S. aureus*.

2. MATERIALS AND METHODS

2.1 Soil Sample Collection

The soil sample was collected from a termite hill at two strategic locations in University of Abuja, Nigeria; Namely: Termite hill beside the Girls hostel dump site permanent site University of Abuja and Faculty of Agriculture farmland. The samples were collected with sterilized containers and sterilized hand trowel using sterile gloves and brought to the laboratory of Microbiology of the University of Abuja.

2.2 Sample Preparation

The soil samples were dried in a dry air oven for 30 mins; this was done to kill off some of the competitor bacteria of the genus *Serratia.*

2.3 Antibiotic Sensitivity Assay

The antibiotic sensitivity assay of four clinical strains of oxacillin/methicillin-resistant *Staphylococcus aureus* (coded SA1 to SA4), isolated from four different clinical samples was conducted through Kirby-Bauer disk diffusion method. Twenty-four hours fresh culture of pathogenic bacteria was inoculated into 5 mL nutrient broth and then incubated at 37^{o}C for 24 to 48 hrs, then turbidity of indicator for the clinical isolates were adjusted with McFarland solution adding 0.89% saline solution in culture tube of indicator pathogenic strain. Uniform lawns were prepared on Muller-Hinton agar media for each

pathogen using sterile cotton swabs. Antibiotic discs were gently placed on the prepared lawn at equal distance using sterile forceps. Incubated for 24 to 48 hrs at 37ºC, after incubation, zone of inhibition was measured for determination of sensitivity towards a commercially produced antibiotic such as oxacillin.

2.4 Plating

Ten-fold of serial dilution was carried out, the serial dilution was done for each soil sample collected in a separate test tubes that has already been labeled according to their serial dilution factors and soil samples. Dilution factors 10^{-4}, $10^{-5,}$ $10^{-6,}$ 10^{-7} was plated starting with 10^{-4}. About 0.1 mL (100 µl) of soil solution was placed onto the labeled plates and it was immediately spread with flamed glass spreader. This same procedure was carried out for other dilution factors 10^{-5}, 10^{-6} and 10^{-7}. The plates were labeled and incubated in the incubator at 37ºC for 24 hrs.

2.5 Identification of Growth

The red pigment producing bacterial isolate was plated on Nutrient Agar and allowed to grow for 1 day (24 hrs) and studied for morphological, physiological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology [10]. The bacterial strain was studied for different cell morphological parameters such as colony evaluation, colony configuration, colony margin, colony surface, colony texture, colony opacity and colony pigmentation. Gram's reaction, spore formation and motility tests of the bacterial cells were performed using standard methods in order to determine the physiology of the isolated red pigment producing bacteria.

2.6 Gram Staining

After 24 hrs of growth, Gram-staining test was carried out to determine if the isolate is a Gram-positive or negative organism and also to determine the shape of growth.

Gram-staining procedure was carried out before microscopy, based on previously described protocol.

2.7 Motility Test

Motility testing was carried out to determine the motility of the soil isolate. A clean, scratch free slide was labeled with name of the test soil isolate; 20 µL of the fresh culture was placed in the middle of the slide. A clean cover slide was lowered over the drop as though it were hinged at one side in order to avoid bubbles. The prepared slide was then examined under the microscope first under 4x followed by 100x magnifications.

2.8 Biochemical Identification

The following Biochemical tests were carried out for further identification of the soil isolate according to the methods of Cheesebrough et al. [11].

2.8.1 Oxidase test

A piece of filter paper was placed on a clean petri dish and 3 drops of freshly prepared oxidase reagent 10 g/L tetramethyl-p-phenyl-enediaminedihydrochloride was also placed on the filter paper in the petri dish. A sterile glass rod was used to remove a colony of the test isolate from the culture plate and was smeared on the filter paper.

2.8.2 Catalase test

A clean glass slide was divided into two sections using a marker. One section was labeled with the name of the test soil isolate and the other was labeled as control. A drop of normal saline was placed on each area. A sterile inoculating loop was used to pick a small culture from the petri dish and two colonies were emulsified on each drop making a smooth suspension. A Pasteur pipette was used to place a drop of hydrogen peroxide on the smear. Hydrogen peroxide was not added to the drop that served as control.

2.8.3 Citrate utilization test

This was used to test the soil isolate's ability to utilize citrate as its sole source of carbon and energy. The media was prepared by dissolving 4.4g of Simmon's citrate agar in 15 ml of deionized water and heated gently with stirring to dissolve properly. 5ml each was dispensed into two clean bijou bottles and covered tightly. This was then autoclaved at 121ºC under 15 psi pressure for 15 minutes. The bijou bottles were then allowed to cool in a slant position. One of the slants was labeled with the test soil isolate and the other slant was labeled as control.

A Bunsen burner was used to sterilize an inoculating loop by flaming. The cap of the slant labeled with test soil plate was removed and the mouth of the bijou bottle was flamed properly.

The sterile inoculating loop was used to was used to pick an inoculum from the culture plate of the unknown soil isolate. The inoculum was immediately transferred into the fresh sterile medium as labeled and was streaked back and forth. The mouth of the bijou bottle was flamed once again, and the cap was replaced. The inoculated slant was then kept in an incubator at 37ºC for 5 days. The inoculating loop was re-flamed to sterilize before keeping. The control slant was not inoculated.

2.9 *In vitro* Prodigiosin Production

Peptone glycerol agar medium was used for the biosynthesis of the pigment at 27ºC for 24 hours. The pigment was multiplied by widespread streaking using sterile swab sticks on freshly prepared medium in 30 petri plates and was left for 5 days to enable more yield of pigment. The pigmented growth was harvested in absolute ethanol and was allowed to stand for 24hours. This was then centrifuged at 5000 rpm for 15 minutes. The supernatant was decanted into a beaker leaving behind the sediments consisting of dead cells of *S. marcescens* and crumps of media. The beaker containing the supernatant was placed in water bath at 60ºC to evaporate ethanol leaving behind dry flakes of pigment. The dry pigment was used as the antimicrobial.

2.10 Antimicrobial Activity of Extracted Red Pigment on Clinical Isolates (Oxacillin/methicillin-Resistant *S. aureus*)

This was done according to the methods described by Mallikharjuna et al. [12]. About 20 mL of sterile Muller-Hinton agar was poured into a set of petri dishes under aseptic conditions and was allowed to solidify. Then each plate was inoculated with 0.2 mL of pure cultures of the test organism and was evenly spread with a bent glass rod to ensure proper seeding of the organisms on plates. After allowing the sensitivity agar surface to dry, a sterile cork borer (4 mm in diameter) was used to punch four holes along the sides, a control plate was equally provided and a hole was made at the center. Exactly 0.2 mL of the 500 mg/mL, 250 mg/mL, 125 mg/mL and 62.5 mg/mL, while the 5^{th} well also contained 0.2 mL of Chloramphenicol was introduced into the hole at the center to serve as control. This was done in duplicates. The plates were allowed to stand for 30 minutes for proper diffusion and incubated at 37ºC for 24 hours. After the incubation period has elapsed, the plates were observed for zones of inhibition which was recorded to the nearest size in millimeter using a transparent ruler. The results were expressed in terms of the diameter of the inhibition zone < 9 mm, inactive, 9-12 mm, partially active, 13-18 mm active; >18 mm, very active [13].

2.11 Determination of Minimum Inhibitory Concentration (MIC) of the Extracted Red Pigment

The MIC of the red pigment that showed activity against the organism was determined according to the macro broth dilution technique as described by Junior and Zanil [13]. Two drops of standardized suspension of the test organisms were inoculated separately into a series of sterile test tubes containing 2ml of nutrient broth each then 4 drops of different concentration of the red pigment were separately added to the tubes. The concentrations used are 500 mg/mL, 250 mg/mL, 125 mg/mL and 62.5 mg/mL. The tubes were then properly corked and incubated at 37ºC for 24 hours. The MIC was read as the least concentration that inhibited the growth of the test organism.

3. RESULTS

3.1 *Staphylococcus aureus* Antibiotic Resistance Pattern

The antibiotic resistance pattern of *Staphylococcus aureus* to commercially produced methicillin/oxacilin antibiotics is shown in Table 1.

3.2 Physical Characteristics of Soil Sample Collected

The physical characteristics of soil samples collected from two different locations at university of Abuja permanent site the soil collected from a termite hill titled RMN1 is a clay soil and soil sample collected from a farm land titled RMN2 is a humus soil is shown in Table 2.

3.3 Morphological Characteristics of Soil Isolate

The morphological characteristics and Gram staining result of isolates gotten from the soil sample is shown in Table 3. An isolate from RMNI soil sample was picked for the research because it had a good growth with aerial mycelium and as shown on culture plate 1.

Table 1. Antibiotic sensitivity and resistance pattern of four strains of *S. aureus* against Oxacillin

Strains of *S. aureus*	Oxacilin sensitivity and resistant pattern			
	Resistance	%	Susceptibility	%
SA1	8	62	5	32
SA2	12	92	1	8
SA3	9	69	4	31
SA4	7	54	6	46

Key: SA= Staphylococcus aureus strains
No zone of inhibition = 0; Resistance % = No of resistant isolate/No of isolate x 100/1
Susceptibility % = No of susceptible isolate/No of isolate x 100/1

Table 2. Characteristics of soil samples collected

Sample places	Sample type and characteristics	Sample designation
Termite hill besides girls hostel dump UNIBUJA	Clay	RMN1
Faculty of Agric farm land UNIBUJA	Humus	RMN2

Key RMN1 = soil sample one; RMN2 = soil sample two

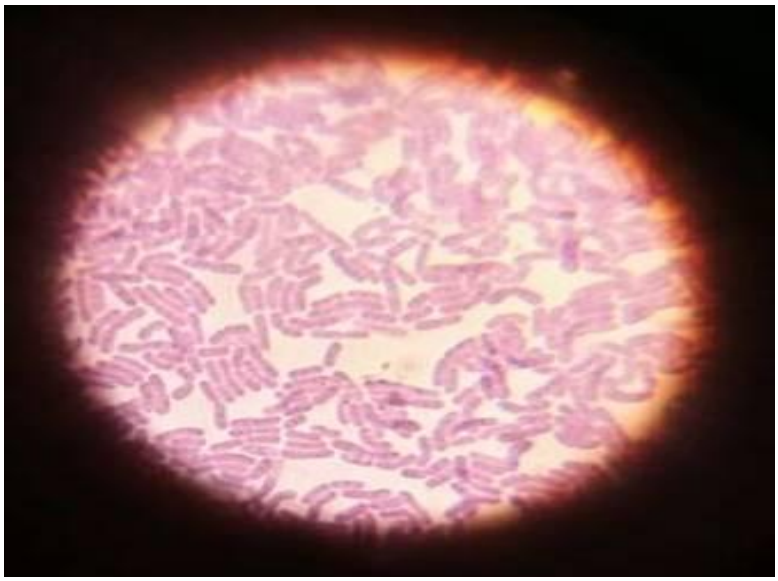

Plate 1. Microscopic view of soil isolate *Serratia marcescens* after Gram-staining x100

3.4 Biochemical Characterization of RMN1 Isolate

The result of the biochemical screening of *Serratia marcescens* isolated from RMNI soil is shown in Table 4.

3.5 Antimicrobial Assay

The antimicrobial activity of Prodigiosin, Red pigment extract on the test organism represented by the mean zones of inhibition produced on each concentration level is shown in Table 5.

3.6 Minimum Inhibitory Concentration (MIC) of the Red Pigment Produced by *S. marcescens*

The Minimum Inhibition Zone of Red pigment on the test organism is shown in Table 6.

4. DISCUSSION

Due to the lack of education and proper training and health regulations for sale of antibiotics without prescription, antibiotic resistance increases in an alarming rate especially oxacillin-

Table 3. Cultural Characteristics of soil Isolates

Serratia marcescens	**Growth**	**Aerial mycelium**	**Substrate mycelium**	**Elevation**	**Opacity**	**Consistency**	**Microscopic features**	**Soluble pigment**
RMNI	Good	Red	Dark Red	Convex	Opaque	Smooth	Gram Negative Pink Rod shaped Motile	Red
RMN2	Poor	Not determined	Light brown	Not determined	Not determined	Rough	Gram negative	Not determined

Key: RMN 1 = soil Sample one; RMN 2 = soil sample two

Table 4. Biochemical characterization of *Serratia marcescens* from RMN1

Test	Result	Colour change	Gas production
Catalase	+	No colour change	Production of bubbles
Citrate	+	Green Blue	
Oxidase	-	No colour change	

Key + = Positive; - = Negative; RMN 1 = soil sample one

Table 5. Antimicrobial activity of red pigment extract

S.aureus strain	Prodigiosin red pigment extracts at different concentrations			
	500 mg/mL	250 mg/mL	125 mg/mL	62.5 mg/mL
SA1	18.0 ± 0.61	16.0 ± 0.36	14.2 ± 0.62	0
SA2	16.1 ± 0.36	14.1 ± 0.2	10.8 ± 0.37	0
SA3	13.1 ± 0.35	11.2 ± 0.35	9.3 ± 0.35	0
SA4	12.7 ± 0.28	10.1 ± 0.2	8.2 ± 0.61	0

Key SA = Staphylococcus aureus strains; 0 = No inhibition

Table 6. Minimum inhibitory Concentration (MIC) of the Red pigment Produced by *S. marcescens*

S. aureus strains	Prodigiosin pigment mg/mL				Control (Chloramphenicol) mg/mL			
	500	250	125	62.5	500	250	125	62.5
SA1	-	-	-	+	-	-	-	-
SA2	-	-	-	+	-	-	-	-
SA3	-	-	-	+	-	-	-	-
SA4	-	-	-	+	-	-	-	-

resistant Staphylococcus aureus and Gram negative multi drugs resistant pathogenic bacteria (MDR). It is clear from the results gotten from this study that there are open option for large scale screening of soil for isolation of *Serracia marcescens* producing bioactive metabolites against resistant pathogens to develop novel antibiotics for control of resistant pathogenic bacteria [14].

In the last few decades several unexplored habitats in the world for isolation of bioactive compounds producing microorganisms were extensively screened [14]. A study conducted in Kolkata area of Merck Indian, reported that a strain of the isolate obtained from the soil sample collected from this area produced a secondary metabolite which was active against antibiotic resistant bacteria [15]. Selected *Serratia* isolate was characterized morphologically and biochemically and designated as *Serratia* (RMN1) spp. which was potent producer of bioactive metabolites confirmed in secondary screening. RMN1 isolate had potent activities against 4 *S. aureus* strains at varying concentrations. Highest activity was observed against *S. aureus* strain at concentration of 500 mg/ml and is similar to results obtained in a research by Jairo et al. [16] in which 500 mg/ml of prodigiosin extracted from *S. marcescens* isolated from a local soil in Kolhapur India proved potent against methicillin-resistant *S. aureus*. Research investigations carried out in this study also correlates with another study conducted in Merck Indian in which *S. marcescens* was isolated from a soil sample collected from Kulkata area of Merck Indian. Isolated *Serretia* had antibacterial activity against 4 indicator bacterial strains including multi drugs resistant bacteria [15]. According to Parani and Saha [17], the prodigiosin was a potent inhibitor against Gram positive bacteria like *Staphylococcus aureus* and *Bacillus cereus* and fungal pathogens like *Candida albicans, C. parapsilosis* and *Cryptococcus* sp at 62.5 mg/mL which was also the MIC. This is in contrast with this research in which MIC is at 125mg/mL. In this research, soil samples were collected from diverse range of habitats in University of Abuja Permanent site, the extracted prodigiosin was active against tested bacteria. It is obvious from the result gotten from this research that the soil micro flora exhibit potential pool of diverse *Serratia* active against antibiotic resistant *S. aureus*. Tests indicator bacteria was Gram positive bacteria culture of fresh isolates of human pathogenic strains isolated from hospitalized patient's samples, identified

morphologically which were resistant to oxacillin antibiotic tested through disc diffusion assay. Prodigiosin extract RMN1 results indicate high activity against oxacilin resistant *S. aureus* at 500 mg/mL concentration.

In brief outcomes, *S. aureus* strains isolated from hospitalized patient exhibited high frequency of resistance to oxacillin, which showed worse situation in Nigeria. To control antibiotic resistance or at least to slow down resistance pattern against available antibiotics there is need for proper regulations at government level, to implement health regulatory laws, to create awareness in the public regarding the use and misuse of antibiotics through print and electronic media and to encourage researcher for the accumulation of antibiotic resistance data time by time and also to find new antimicrobials as a controlling efforts. The isolate potent *Serratia marcescens* strain from University of Abuja Permanent site was effective against oxacillin-resistant *S. aureus* strains. If such trends of research findings continue then there will be an add up to novel type's antimicrobials in the pipe line of pharmaceutical industries at local level, which will be inexpensive, potent and will be able to export such type of drugs to other countries for the benefit of humanity.

5. CONCLUSION

This study shows that a total of (4) oxacilin resistant *Staphylococcus aureus* strains were gotten from hospital patients, and investigated for antibiotic sensitivity assay, Antibiogram showed that percentage resistance of *S. aureus* to oxacillin included those with moderate resistance SA4 (54%) and those with high resistance SA1 (62%), SA2 (92%), SA3 (69%).

A total of (2) *Serratia marcescens* sp was isolated from soil samples but only one out of the two showed a good growth and it was identified morphologically and biochemically. A Primary screening was carried out on the prodigiosin extract for antibiotic producing abilities. Further screening process was carried out which involves the secondary screening. In the secondary screening the prodigiosin extract at different concentration had significant activities against tested bacteria, at the end of the whole screening; primary and secondary screening, and also result obtained from the statistical analysis of the effectiveness of the different concentration level of the prodigiosin extract concentration 500mg/mL was discovered to be more active against the oxacilin resistance *S. aureus.*

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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