Isolation of Multi-Drug Resistant *Staphylococcus aureus* from the soil samples of Hyderabad

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Abstract

The use and misuse of antibiotics over the past 50 years in man has lead to the emergence of various multidrug resistant microorganisms (MDROs). These organisms are of great concern in discharging health care facilities throughout the world. *Staphylococcus aureus* (*S.aureus*) one of the most important pathogens affecting humans has acquired resistance to various antibiotics. According to the resistance data given by World Health Organization (WHO), Community Acquired Methicillin Resistant *Staphylococcus aureus* (CA-MRSA) has increased significantly all over the world.

In the current study *S.aureus* was isolated from the soil samples of the residential and hospital areas of the Hyderabad city and was evaluated for their sensitivity against twelve antibiotics clinically used against *S.aureus*. Methicillin Resistant *Staphylococcus aureus* (MRSA) were isolated from the soil samples collected from the residential and hospital areas of Hyderabad.

Keywords: Antibiotic sensitivity, MDROs, MRSA, soil samples, Staphylococcus aureus

ISSN: 2278 – 7798

Introduction

Though the antimicrobial resistance or drug resistance is not new, the numbers of resistant organisms are increasing enormously through the length and breadth of geographic locations day-by-day. Diseases and disease agents that were once thought to be controlled by antibiotics are returning in new leagues resistant to these therapies [1], [2]. *Staphylococcus aureus* (*S.aureus*), one of the most important pathogens affecting humans has acquired resistance to various antibiotics and is a leading cause of hospital and community acquired infections, manifesting from minor skin diseases to life-threatening infections [3], [4]. Drug-resistant strains initially appeared in hospitals, where most antibiotics were being used. Penicillin resistant *Staphylococcus aureus* confronted London civilian hospitals very soon after the introduction of penicillin in the 1940. Initially, MRSA was mainly a problem in hospital-acquired infections. Over the past decade, CA-MRSA has increased significantly in a number of countries. Fortunately, many of these CA-MRSA strains have so far retained susceptibility to a number of non-beta-lactam antimicrobials, whereas most health-care associated MRSA infections are caused by difficult-to-treat multi-resistant strains.

The present study is an attempt to isolate and characterize the multi-drug resistance (MDR) *Staphylococcus aureus* from soil surrounding houses and hospitals, which was obtained from various place of Hyderabad. The isolates were investigated for their sensitivity against twelve antibiotics.

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Methodology

Chemicals reagents and media: The antibiotic discs, bacteriological media such as nutrient agar, SIM agar, Simmon's citrate agar, MRVP broth, nutrient broth, tryptophan broth, Mac Conkey's agar, Mannitol agar etc., chemicals required for the preparation of kovac's reagent, Barritt's A and B reagents, stains like crystal violet, methyl red, saffronin, malachite green, ethanol, iodide etc were procured from Himedia (Mumbai, India).

Collection of sample: The soil samples were collected from the residential areas and the hospital areas in the city of Hyderabad. Randomly from different parts of the city twenty five soil samples from the residential areas and twenty five samples from the hospital surroundings were collected for the experimental. The bacteriological analysis of these samples was done by serial dilution and agar plate culture techniques. The obtained pure cultures were characterized based on their morphological and biochemical characteristics as described in *Bergey's Manual* for bacteriology [5].

Serial dilution and agar plating technique: This method is based on the principle that when soil sample containing bacterial colonies are cultured, every living bacterium develops into a visible colony on the nutrient agar plate. One gram of the collected soil sample is suspended in 9ml of saline to obtain a 10^{-1} dilution (10 times dilution). From the above dilution 1ml is transferred to a fresh 9ml saline solution to obtain a 10^{-2} dilution. The process is repeated in order to produce 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} serial dilutions. From the dilutions ranging from 10^{-3} to 10^{-7} 0.1 ml of the suspensions were added to nutrient agar plates (each dilution in 3 replicates) under sterile conditions and incubated at 37^{0}C for 24 hours. The number of bacteria in the testes soil sample can be calculated by the following formula:

ISSN: 2278 – 7798

Organisms per milliliter per gram soil = number of colonies (average of 3 replicates)/ volume plated (0.1) x dilution

The isolated cultures were differentiated by their morphological characteristics and transferred to fresh nutrient agar media to produce in pure form. Golden yellow colonies on the nutrient agar plate were sub cultured and confirmed as *S. aureus* by performing motility, grams staining and biochemical tests [6].

Motility: The motility of the native isolates was detected using hanging drop technique, a drop of bacterial suspension, preferably in mid-logarithmic phase, was placed in the centre of a cover slip coated with grease. Glass slide having a central depression was placed over the cover slip. The slide was then inverted without disrupting the drop and examined using 40x optical microscope (Labomed, Vision 2000, India) for motility of the bacteria [6].

Gram staining of bacteria: The gram-negative bacterial cell wall is thin, complex, multilayered structure and contains relatively a high lipid contents, in addition to protein and mucopeptides. The higher amount of lipids is readily dissolved by alcohol, resulting in the formation of large pores in the cell wall which do not close appreciably on dehydration of cell wall proteins, thus facilitating the leakage of crystal violet-iodine (CV-I) complex and resulting in the decolorization of the bacterium which later takes the counter stain and appears pink. In contrast, the gram positive cell walls are thick and chemically simple, composed mainly of protein and cross-linked mucopeptides. When it was treated with alcohol, it causes dehydration and closure of cell wall pores, thereby not allowing the loss of (CV-1) complex and cells remain purple [9]. Heat fix a thin smear of culture on glass slides, cover the smear one by one with crystal violet (60 seconds), gram's iodine (60 seconds), 95% ethanol (20 seconds) and safranin (40 seconds). Air dried the slides after washing with distilled water and observed under microscope [6]. Gram positive bacteria appear in violet color and the Gram negative bacteria appear in pink color.

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Biochemical tests:

According to the *Bergey's manual of systematic bacteriology* the biochemical characterization of *Staphylococcus aureus* was done by the tests shown in the Table I [7].

Antibiotic sensitivity test [8]:

The identified *S. aureus* cultures (each 20µl) was poured over the basal plates containing 25ml of nutrient agar media in nine sterile petriplates and spread using L-shaped glass rod. The antibiotic discs used commercially for Gram's positive bacteria were placed in the plate. The effect of the each of the organism was tested in triplicate. The plates were incubated at 37^0C for 24 hr. and there after the zones of inhibition were recorded by measuring the diameter of zone of inhibition by the following formula:

Zone of inhibition (mm) = D-d

Where,

D= diameter of zone of inhibition

d= diameter of the antibiotic disc (6 mm)

The twelve antibiotics used in the experimental are Pencillin G (P), Amoxicillin (AMX), Carbenicillin (CB), Methicilin (MET), Azithromycin (AZM), Clindamycin (CD), Roxithromycin (RO), Lincomycin (L), Vancomycin (VA), Rifampicin (RIF), Teicoplanin (TEI) and Linezolid (L).

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Results and Discussion

Among the Gram-positive pathogens, *S. aureus* continues to cause skin and soft tissue infections in the community and invasive infections in the hospitalized patients. In a recent Europe-wide survey, the most common organisms in skin and soft tissue infections were *S. aureus* (71% cases) with 22.5 per cent being MRSA [9]. The proportion of infections with MRSA varied among countries ranging from 0.4 per cent in Sweden to 48.4 per cent in Belgium. In the United States in a span of 10 years, there was an increase in the overall incidence of *S. aureus* and CA-MRSA infections [10].

In the current study 50 soil samples were collected randomly in the city of Hyderabad. Out of these 50 samples, 25 each were collected from the residential and hospital areas. *S. aureus* was isolated from four RS samples and five HS samples. These isolates were identified as *S. aureus* by performing biochemical tests.

The identified organisms were tested for their sensitivity against 12 antibiotics clinically used against gram positive bacteria. Among the four *S. aureus* isolated RS1 is resistant to P and AMX; RS2 and RS4 are resistant to P; RS3 resistant to P, AMX and MET. Among the five isolates of the hospital area HS1 is resistant to P, AZM and LZ; HS2 is resistant to P, MET, CD and LZ; HS3 is sensitive to AZM, RO, VA and RIF; HS4 is sensitive to RO, VA and RIF; HS5 is sensitive to the antibiotics RO and VA only.

Conclusion

Soil is the outermost layer of the earth composed of complex minerals and organic matter. The microbial profile in a particular portion of the soil is determined by the interaction of sunlight, rainfall, temperature, moisture, soil pH, vegetation and redox potential. Pathogens may

ISSN: 2278 – 7798

be indigenous or enter by the animal deposits, manure application, sewage, floods or contaminated water.

The cities are thoroughly populated with human population. The *S. aureus* being a commensal of human skin is expected to reach the soil in various forms. Hence, the soil in the residential areas of the city are analyzed for the presence of *S. aureus* .The expectation of isolating drug resistant *S. aureus* from the hospital zone is high due to the disposal of hospital waste by the in-patients, out-patients and the hospital authorities. The prevalence of these MDROs in the soil may have a greater ability to infect susceptible organisms. According to a survey conducted by the Indian Network for Surveillance of Antimicrobial Resistance (INSAR) group during the year 2008 and 2009, the percentage of occurrences of MRSA in India is 42 and 40 respectively in the samples collected from the patient wounds [11].

In the current study the percentage of occurrence of *S. aureus* around the residential and the hospital areas in the city of Hyderabad is 16 and 20 percentage respectively. The *S. aureus* isolated from the residential areas were found to be highly sensitive to most of the antibiotics compared to the *S. aureus* isolated from the hospital area which have exhibited a wide range of resistance to the test antibiotics. All the HS isolates were found to be sensitive to Ro and VA. The regular surveillance of MRSA is essential for selecting an appropriate antibiotic, for limiting the use of powerful antibiotics as initial treatment and also to help in postponing the development of resistant and life-threatening staphylococcal infections.

Acknowledgement

I express my sincere thanks to SERO-UGC, Hyderabad for funding the minor research project and encouraging budding scientist.

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I also thank the Principal and the Department of Microbiology, Indira Priyadarshini Government Degree College, Hyderabad for providing the necessary infrastructure to carry out the work successfully.

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Table I: Biochemical Characterization of *Staphylococcus aureus.*

Biochemical test	Reaction
Catalase	+
Oxidase	-
Indole production	-
Methyl red	+
Voges-Proskauer	-

ISSN: 2278 – 7798

Citrate utilization	-
Glucose fermentation	+
Mannitol fermentation	+
Lactose fermentation	+
Sucrose fermentation	+
Blood hemolysis	+
Coagulase	+

Table II: Results of morphological and biochemical tests for *Staphylococcus aureus*.

Note: Mo- motility test, I- indole test, M- methylred, VP- Voges Proskauer's , C-citrate, O-oxidase, Ct- catalase, H- hemolysis, Co- coagulase, Mn- mannitol, G- glucose, S- sucrose, L-lactose

Isolates no.	**Nutrient Agar Media**	**Mo**	**Gram's staining**	**I**	**M**	**VP**	**C**	**O**	**Ct**	**H**	**Co**	**Mn**	**G**	**S**	**L**
RS1	Small round golden yellow color colonies	-	+ cocci in clusters.	-	+	-	-	-	+	+	+	+	+	+	+
RS2	Small round golden yellow color	-	+ cocci in	-	+	-	-	-	+	+	+	+	+	+	+

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	colonies		clusters.												
RS3	Small round golden yellow color colonies	-	+ cocci in clusters.	-	+	-	-	-	+	+	+	+	+	+	+
RS4	Small round golden yellow color colonies	-	+ cocci in clusters.	-	+	-	-	-	+	+	+	-	+	+	+
HS1	Small round golden yellow color colonies	-	+ cocci in clusters.	-	+	-	-	-	+	+	+	-	+	+	+
HS2	Small round golden yellow color colonies	-	+ cocci in clusters.	-	+	-	-	-	+	+	+	+	+	+	+
HS3	Small round golden yellow color colonies	-	+ cocci in clusters.	-	+	-	-	-	+	+	+	+	+	+	+
HS4	Small round golden yellow color colonies	-	+ cocci in clusters.	-	+	-	-	-	+	+	+	+	+	+	+
HS5	Small round golden yellow color colonies	-	+ cocci in clusters.	-	+	-	-	-	+	+	+	+	+	+	+

Table III: Antibiotic sensitivity of the residential area soil sample: Diameter of zone of inhibition (mm) of the isolates (RS1, RS2, RS3 and RS4) against the twelve test antibiotics comparing with the standard zone of inhibitions to be produced and the control (disc with distilled water); '+' indicates positive result and '-' indicates negative result.

Note: Pencillin G (P), Amoxicillin (AMX), Carbenicillin (CB), Methicilin (MET), Azithromycin (AZM), Clindamycin (CD), Roxithromycin (RO), Lincomycin (L), Vancomycin (VA), Rifampicin (RIF), Teicoplanin (TEI) and Linezolid (L).

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Antibiotics	Sensitivity	RS1	RS2	RS3	RS4
P	29	0	0	0	0
AMX	20	**19**	30	**15**	20
CB	23	30	30	30	25
MET	14	15	24	**0**	26
AZM	12	20	23	26	28
CD	21	23	28	22	27
RO	15	18	20	17	28
L	21	21	24	24	26
VA	12	20	26	26	25
RIF	26	26	22	30	27
TEI	21	22	23	30	22
LZ	32	32	32	32	32
CONTROL	0	0	0	0	0

Table IV: Antibiotic sensitivity of the hospital area soil sample: Diameter of zone of inhibition (mm) of the isolates (HS1, HS2, HS3, HS4 and HS5) against the twelve test antibiotics comparing with the standard zone of inhibitions to be produced and the control (disc with distilled water).

Note: Pencillin G (P), Amoxicillin (AMX), Carbenicillin (CB), Methicilin (MET), Azithromycin (AZM), Clindamycin (CD), Roxithromycin (RO), Lincomycin (L), Vancomycin (VA), Rifampicin (RIF), Teicoplanin (TEI) and Linezolid (L).

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Antibiotics	Concentration	Standard	HS1	HS2	HS3	HS4	HS5
P	10 units	29	0	0	0	0	0
AMX	10 µg	20	26	25	0	0	0
CB	100 µg	23	26	25	0	0	0
MET	5 µg	14	26	0	0	0	0
AZM	15 µg	12	0	25	21	0	0
CD	2 µg	21	25	0	0	0	0
RO	15 µg	15	20	22	19	25	17
L	2 µg	21	24	23	0	13	12
VA	30 µg	12	20	19	23	22	16
RIF	5 µg	26	26	26	26	26	12
TEI	30 µg	21	23	26	0	17	17
LZ	30 µg	32	24	0	0	0	0
CONTROL		0	0	0	0	0	0

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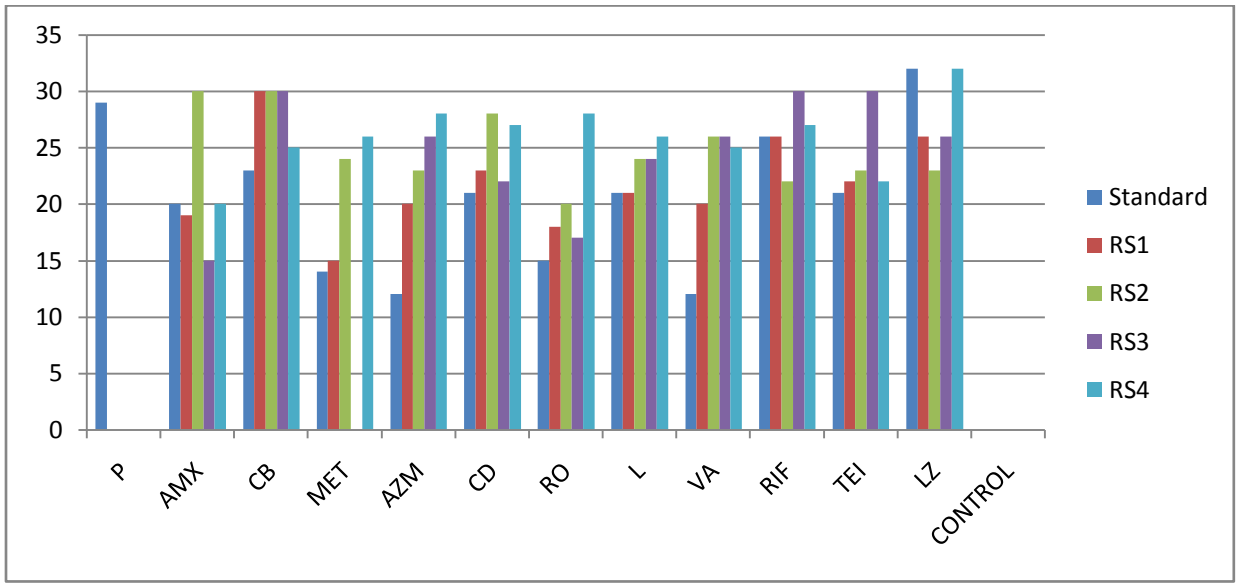
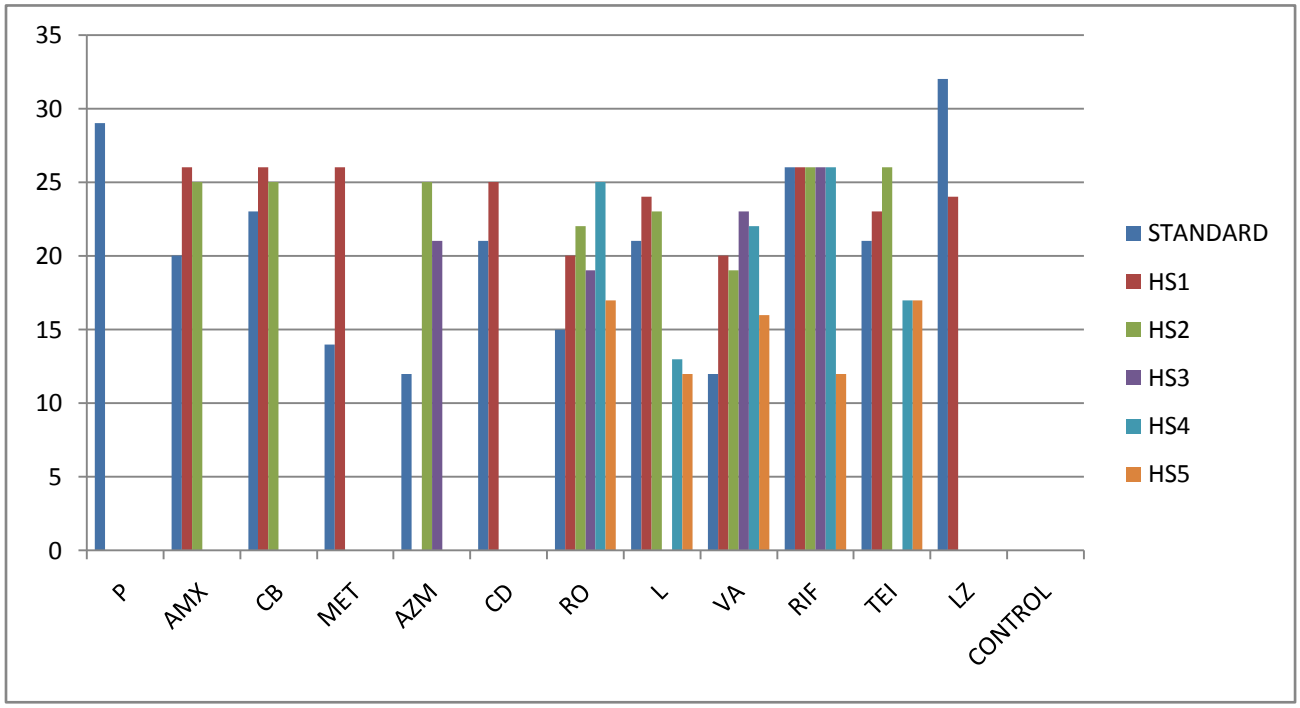


Graph 1: Graphical representation of the residential area soil sample isolates against the twelve test antibiotics comparing with the standard zone of inhibitions to be produced and the control (disc with distilled water).

Note: Pencillin G (P), Amoxicillin (AMX), Carbenicillin (CB), Methicilin (MET), Azithromycin (AZM), Clindamycin (CD), Roxithromycin (RO), Lincomycin (L), Vancomycin (VA), Rifampicin (RIF), Teicoplanin (TEI) and Linezolid (L).

ISSN: 2278 – 7798



Graph 2: Graphical representation of the hospital area soil sample isolates against the twelve test antibiotics comparing with the standard zone of inhibitions to be produced and the control (disc with distilled water).

Note: Pencillin G (P), Amoxicillin (AMX), Carbenicillin (CB), Methicilin (MET), Azithromycin (AZM), Clindamycin (CD), Roxithromycin (RO), Lincomycin (L), Vancomycin (VA), Rifampicin (RIF), Teicoplanin (TEI) and Linezolid (L).

ISSN: 2278 – 7798