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Recent Prevalence of Clinical Multidrug Resistant *Staphylococcus aureus* in West Bengal

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Abstract:
Introduction*: Antibiotic resistance is increasing day by day like a tsunami. About 100,000 tons' antibiotics are being prepared annually throughout the world. Staphylococcus aureus is one of the pathogenic bacteria which are rapidly evaluating themselves to become resistant for several antibiotics.*
Materials & Method*: 137 clinical bacterial isolates have been collected for study. 21 S. aureus isolates were identified by positive catalase, coagulase and mannitol fermentation tests from 54 gram-positive bacteria. Antibiotics susceptibility test were performed using DAD (Disc agar diffusion) method. Azithromycin, Clarithromycin, Levofloxacin, Rifampicin and Amikacin were the antibiotics which were used in experiments. ATCC25923 was reference strain of S. aureus.*
Results & Discussion*: Out of 21 S. aureus isolated from 54 gram-positive bacterial strain. 15 were resistant for at least one antibiotic agent among 5 agents used in the experiment. That means 15 MDR S. aureus identified among which one XDR was also found which was resistant for all the agents studied in the present work.*
Conclusion and future aim*: Throughout the study, it has been seen that clinical isolates of bacteria have capability to grow resistance for antibiotics, which is the reason we got non-susceptibility against vancomycin too. Therefore, speed of developing new antibiotics is lesser than bacteria are growing resistance. We need to combat this serious issue with some another possible way.*
Keywords: *VRSA, Staphylococcus, amikacin, azithromycin, MDR*

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I. Introduction:

"Antibiotics are manufactured at an estimated scale of about 100,000 tons annually worldwide, and their use had a profound impact on the life of bacteria on earth"(1). With the increase in production and use, the resistance against antibiotic is also increasing every day.While WHO has already declared "combat drug resistance: no action today, no cure tomorrow" in 2011(1). According to a research conducted at Jawaharlal Nehru Medical college, India in 2015 says that the antibiotic susceptibility profile of 1060 bacterial strains give 393 (37.1%) MDR (Multidrug resistant) bacterial strains, 146 (13.8%) XDR (Extensively Drug resistant) strains, and no PDR (Pan drug resistant) bacteria. All (100%) Gram negative bacterial strains were sensitive to colistin whereas all (100%) Gram positive bacterial strains were sensitive to vancomycin(2). On the other hand, resistance for vancomycin was first observed in the form of VRSA (Vancomycin resistant *Staphylococcus aureus*) in 2002 from USA(3)(4). Only four VRSA was obtained from USA till 2007(5). On the same time there were no VRSA isolates from Asia, except for Vancomycin Intermediate *Staphylococcus aureus* (VISA) in Japan and Korea(6) in 1997 and 2000 respectively. In the year 2006 a *van* gene negative VRSA isolate was also observed(5). The present scenario is that 14 VRSA infections have been found in US till 2017(4). It has been seen that 90% *Staphylococcus* strains contains resistance against penicillin(7)(5), and they are getting resistant vigorously against methicillin, aminoglycosides, macrolides and lincosamides(8)(9)(10)(11). This increase in AMR (Antimicrobial resistance) is leading to increased morbidity as well as a huge economic loss for the patients and for the nation(2)(3).*S. aureus* has a fundamental biological property of being able to asymptomatically colonize normal people and approximately 30% humans are carriers for *S. aureus*(4)(5).The recent epidemiology of *S. aureus* reveals that, the bacterium has evolved resistance against penicillin (first antibiotic) to vancomycin (last resort)(6). Apart from all the above incidents, if we see the community acquired antimicrobial resistance in *staphylococcus aureus*. In the early 1980s, the first emergence of CA MRSA (community acquired methicillin resistant *Staphylococcus aureus*) was reported in adults and then later in the same year it was also reported in children(7)(8)(9). They were not truly CA (community acquired), because the patients had a history of being in frequent contact with healthcare workers, so it must be HCA (healthcare associated) instead of CA(10). Now a days, CA-MRSA have been reported from every corner of the world, from US (United States) to Norway(6) and the same is increasingly reported from India as well(11)(12). The MRSA

studied by Joshi et al, in 2013 were also resistant for gentamicin, co-trimoxazole, erythromycin and clindamycin(12).

II. Aims And Objectives

The above worldwide scenario motivated us towards this study in the local proximities. Various surveys conducted in different hospitals of West Bengal, especially in Kolkata. Those surveys concluded that anyhow people are unaware about doses of antibiotics as well as the need of completion of the dose. They are reluctant to complete particular antibiotic dose prescribed by doctor, which further enhances birth of MDR (multidrug resistant) bacterial isolates (especially*S. aureus*). Some of the friendly bacteria present in our body also become resistant towards some antibiotics due to this unawareness and it may cause severe problem through secondary infection. Every gram-positive isolate is a threat to human beings but we focused mainly on *Staphylococcus aureus*, as it causes more severe infection and its infection is quite common in Kolkata and other parts of West Bengal. Our aim is to assess the sensitivity pattern of different antibiotic for *S. aureus* and other gram-positive clinical isolates, that can focus on the local emergence of MDR bacterial infection and aware the concern.

III. Materials And Methods:

Ethical approval: Collection of clinical isolates was approved by institutional ethical committee of University of Calcutta, Kolkata. Patient's consent was also taken before taking bacteria from the concerned institutes.

Study design & sampling: About 137 cultured bacteria were collected from different hospitals and pathological labs of Kolkata and other parts of West Bengal. All the cultures were collected with proper consent of patients as per ethical guidelines. Collected cultures were only from the outdoor patients, hence theresistance grown in these isolates can be said as community acquired AMR (antimicrobial resistance).Clinical isolates were preserved in NA (nutrient agar) slant at 4˚C in refrigerator.

Table 1: Strains collection from different hospitals and labs of Kolkata and its periphery.

Hospital/Lab	City	Number of Strain
SSKM Hospital	Kolkata	24
NRS Medical College and Hospital	Kolkata	25
School of Tropical Medicine	Kolkata	12
Private pathological labs	Kolkata	46
Durgapur Sub-divisional Hospital	Durgapur	8
Katwa Sub-divisional Hospital	Katwa	15
Murshidabad Medical College & Hospital	Berhampore	7

Species identification: All the strains were purified by single colony isolation method in pure Nutrient Agar (NA) media. Then categorized as gram-positive and gram-negative strains by gram staining method(12)shape of the organisms was also confirmed with this test under oil immersion lens of microscope (Magnus MLXi& Magnus MLX-DX). Categorized gram positive isolates were the part of our study, these isolates were characterized by various biochemical tests, such as catalase test(13), coagulase test(14), Indole test, Methyl Red (MR), Vogues Proskauer (VP), Oxidase, Citrate, Urease, TSI (Tri-Sugar Iron) tests. Catalase and coagulase positive isolates were identified as *Staphylococcus aureus* and these isolates were mannitol fermenting too(15)(16). ATCC25923 was taken as reference strain to identify *S. aureus* isolates.

Antibiotic Susceptibility testing: 54-gram positive isolates were treated through Disk Agar Diffusion (DAD) method(17)(18) toassess their susceptibility pattern. Antibiotic disc was prepared in departmental laboratory using Azithromycin (Azithral 500), Clarithromycin (Clarigard 250), Levofloxacin (Loxof 500), Rifampicin (R-Cin 600), Amikacin (Mikacin INJ 500mg) antibiotics tablets and injections available in market of Kolkata and outside Kolkata. Disc content was maintained as per CLSI (Clinical and Laboratory Standards Institute) guidelines(19) these are: Azithromycin - 15µg, Clarithromycin - 15µg, Levofloxacin - 5µg, Rifampicin - 5µg and Amikacin - 30µg per disc. All sensitive reference strain ATCC25923 was used as quality control strain.

Choice of antibiotics: Azithromycin, Clarithromycin and levofloxacin was taken for the present study because these were commonly prescribed by the medical practitioners and are commonand first line antibiotics to be used to treat such bacterial infection. Rifampicin and amikacin were taken as, these are the antibiotics used or prescribed very rarely. And we tried to establish any difference in susceptibility in *S. aureus* for such kind of antibiotics.

DAD: Disc agar diffusion test is used to test susceptibility pattern of bacteria against different antibiotics. It requires muller hinton agar and broth (himedia). 2-3 freshly cultured colony were suspended in 5ml MHB (muller-hinton broth) and kept overnight. On the next day the freshly prepared culture was again inoculated in fresh MHB and adjusted the turbidity of 0.5 MacFarland standard after incubation for 3-4 hours at 37˚C. then again inoculated the MHA (muller-hinton agar) plate by spreading the inoculum uniformly on the media with the help of sterile cotton swab. After 15-20 minutes of incubation at room temperature, antibiotic discs were

placed accordingly and the results were taken on the next day after incubating the plates at 37˚C for overnight. Diameter of zone of inhibition was measured according to CLSI guidelines and interpreted later(13).

MDR identification: There are some standardized terminologies defined by CDC (Center for Disease Control & prevention) and ECDC (European Center for Disease Control & Prevention). These terminologies include MDR (Multidrug resistant). Any bacteria can be said as MDR when the same will show resistance for at least one agent in three or more antimicrobial categories or groups(14)(1).

Statistical analysis: t-test (one sample) in 'SPSS Statistics 20' was used to examine any significant relationship between sensitivity and resistivity of strain towards different antimicrobial agents.

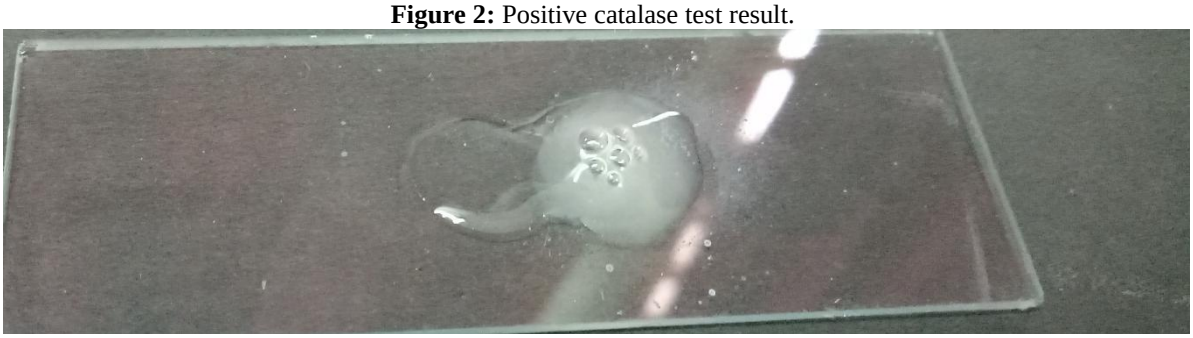
IV. Results

Gram staining: As per gram staining(12) method, it has been evaluated that, after random sample collection and purified by single colony isolation a total of 137 strains gives 54 (39.41%) gram positive and 83 (60.59%) gram-negative bacteria.

***Staphylococcus aureus* and its occupancy**: Among all the 54 gram-positive bacterial strains 21*Staphylococcus aureus* has been identified with catalase, coagulase and mannitol fermentation test. Rest of the gram-positive clinical isolates includes *Streptococcus* sp., *Lactobacillus*, *Corynbacterium*, etc.

Figure 1: Positive and negative control for catalase test.

Figure 2: Positive catalase test result.



Sensitivity pattern of total gram-positive bacteria: Among all the gram-positive isolates 53.70%, 51.90%, 31.50%, 29.60 &9.30% were resistant for Azithromycin, Clarithromycin, Levofloxacin, Rifampicin & Amikacin respectively. Whereas 5.60%, 5.60%, 1.90%, 1.90% &11.10% were intermediateto Azithromycin, Clarithromycin, Levofloxacin, Rifampicin & Amikacin respectively.

Antibiotics susceptibility test result of *S. aureus*: After treating all 21*S. aureus* isolates with Azithromycin, Clarithromycin, Levofloxacin, Rifampicin & Amikacin. They showed 57.40%, 52.40%, 38.10%, 23.81% &4.80% resistance and 4.80%, 9.50%, 4.80%, 0% &4.80% intermediate respectively.

Table 2: Percentgage of resistant and intermediate against different antibiotics.

Percent resistant for antibiotics		**Antibiotics**				
		Azithromycin	Clarithromycin	Levofloxacin	Rifampicin	Amikacin
All gram-positive isolates	Resistant	53.70	51.85	31.5	29.60	9.30
	Intermediate	5.6	5.55	1.85	1.85	11.10
S. aureus	Resistant	57.40	52.40	38.09	23.80	4.80
	Intermediate	4.76	9.5	4.76	0	4.76

Prevalence of MDR: Out of 21 *Staphylococcus aureus* collected and isolated from the above said sources, 15 were resistant for at-least one agent (antibiotics) out of 5 agents, among four different groups of antibiotics were used for the study. One clinical *S. aureus* was found to have resistance for all the five agents. Whereas 6 *S. aureus*isolates were found to be susceptible for all the agents used in the study.

From this study we can see that 15 MDR *Staphylococcus aureus* is being emerged from 21 strains, it is a huge percentage of emergences i.e., 71.43%. The one which showed resistance for all the antibiotics tested can be said as XDR (Extensively drug resistant), as it is unable to show its susceptibility of any agent tested in the present study, it may show susceptibility, if it gets treated with some other agents.

Table 3: Prevalence of MDR and XDR in the present study.

	Antibiotic numbers	All gram-positive isolates	*Staphylococcus aureus*	MDR/XDR
Sensitive	Zero	19	6	
Resistant	One	7	4	MDR
	Two	9	3	
	Three	10	6	
	Four	6	1	
	Five	3	1	MDR+XDR
Total		54	21	

Statistical analysis: One sample t-test (SPSS Statistics 20) was used to analyze the data. Intermediate strains were excluded from the analysis.

Table 4: t-test statistics.

	Sensitive	Resistant	N (number of test isolates)	t-value	p-value	Significance
Azithromycin	8	12	20	.472	.642	Not significant
Clarithromycin	8	11	19	1.193	.248	Not significant
Levofloxacin	12	8	20	2.187	.041	Significant
Rifampicin	16	5	21	3.711	.001	Significant
Amikacin	19	1	20	9.980	.000	Significant

V. Discussion

Resistance to antimicrobial agents in pathogenic bacteria is emerging very rapidly and is becoming a serious threat to mankind. Gram positive and gram-negative both type of bacteria are getting affected by this emergence against rise of antimicrobial agents(20). In 2011, WHO declared "Combat drug resistance: no action today, no cure tomorrow"(21). Now-a-days, it's a problem to us that bacteria are developing resistance at a much faster rate than we can develop new antimicrobial agents to kill them(22).

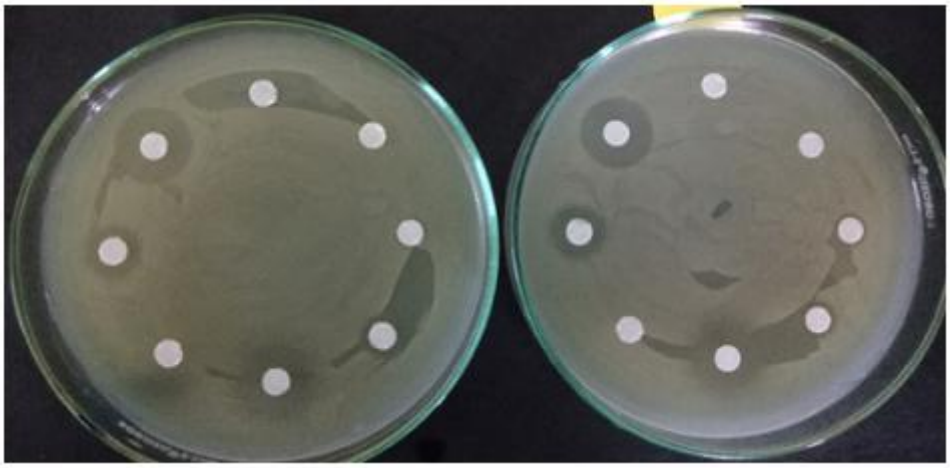
Definition for multidrug-resistant bacteria varies by country(23). An international panel of experts developed the definitions in a simple way, these are as follows: Multidrug-resistant (MDR) means acquired non-susceptibility to at least one agent in three or more antimicrobial categories (antibiotic group, such as penicillin, aminoglycoside, etc.) and extensively drug-resistant (XDR) is defined as non-susceptibility to at least one agent in all, but two or fewer antimicrobial categories, and pandrug-resistant as non-susceptibility to all agents in all available antimicrobial categories(20)(23).

According to the above definitions and studies & tests we performed 15*Staphylococcus aureus* were resistant for at least an agent among all five antibiotic agents. This identifies them as MDR *S. aureus*. Whereas, 1, 1, 6, 3, 4*S. aureus* were non-susceptible against 5, 4, 3, 2& 1 antimicrobial agents respectively (Table 3).
The one XDR found in the study has following resistance pattern for the used antibiotic agents.

Table 5: ZOI (zone of inhibition) of XDR *S. aureus* against different antibiotics.

		Group of antibiotics	**Macrolide**	**Macrolide**	**Fluoroquinolone**	**Rifamycin**	**Aminoglycoside**
Sl. No.	Strain No.	Antibiotic	Azithromycin	Clarithromycin	Levofloxacin	Rifampicin	Amikacin
1	128Ai	ZOI (mm)	10 (R)	6 (R)	8 (R)	8 (R)	9 (R)

Figure 3: DAD of 128Ai.



From the above statistics (Table 4), we can easily say that levofloxacin, rifampicin and amikacin are actively able to treat *S. aureus* infection, as the strains are mostly sensitive than resistant. Whereas, in case of azithromycin and clarithromycin (macrolides), there is no significant difference between sensitive and resistant isolates. It can be said as there are equal chances of having azithromycin and clarithromycin resistant and sensitive strains. Which may lead to failure of treatment.

VI. Conclusion

The present study was undertaken to get a clear picture of the current status of resistance against antibiotics, especially in Kolkata and surrounding. This is not only a major treatment center for the people of west Bengal but also a greater periphery of this eastern region of India, Bangladesh and other nearby countries as well. However, this study opens up further area of vigorous working, not only on this sector but also can be used as a serious awareness program.

VII. Limitations & Future aim

This study deals with very few number of isolates collected, we will try to do the same kind of study with more number of isolates further. As per biochemical analysis is concerned for the identification of strains, it is potent and authentic. But we will try to do genetical identification in our further elongation of the study. XDR found, need to check for other antibiotic agents (like vancomycin, methicillin, etc.) for the susceptibility of the same.

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