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Dissemination of Serum Resistant Multiple Antibiotic Resistant *Acinetobacter* sp in Clinical settings - a New Threat

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Abstract

The pathogenic feature including slime production and serum resistant contributes a significant role in virulence of *Acinetobacters* leading to a successive multi-drug resistant organism in the Clinical care. The present study is sought for isolation and identification of Slime producing, Multi drug resistant *Acinetobacter* sp in clinical samples. Out of the total 92 samples analyzed 39 (42.3%) were positive for *Acinetobacter* sp in which 23, 6 and 10 were identified as *A.baumanni* (59%), *A.lwoffi* (15.3%) and *A.hemolyticus* (25.6%) respectively. All the isolates were screened for multiple antibiotic resistant capabilities. The slime production were confirmed in 10 (43.4%), 5(83.3%) and 1(10%) of *A.baumannii*, *A.lwoffi* and *A.hemolyticus***.** Similarly 19 (82.6%), 5(83.3) and 8 (80%) of A.baumannii, *A.lwoffi* and *A.hemolyticus* were resistant to normal human serum bactericidal activity. In conclusion our study claims that all the serum resistant strains were potential slime producers and expresses high MAR index which appears to be a strong correlation between these virulent factors that leads to high pathogenicity of *Acinetobacter* sp in the human environment.

Key words: *Acinetobacter* sp, Slime, MAR index, Serum Resistant

1. Introduction

Multidrug-resistant (MDR) *Acinetobacter* sp has recently been established as a leading nosocomial pathogen in several hospitals[1]**.** *Acinetobacters* are common colonizers of the respiratory tract and skin of hospital patients, particularly in patients located in the intensive care unit setting. Infections may be either nosocomial or community acquired (Fournier and Richet, 2006). *Acinetobacters* comprises many species and all can cause human disease, among them *Acinetobacter baumanni* accounts for about 80% reported infections so far. Nosocomial *A. baumanni* bacteremia associated with other

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clinical diseases with high mortality rate of about75% is also reported [2].

In a earlier studies, 6 out of 7 patients with *Acinetobacter* blood stream infections found *A. baumannii* colonizing their gastrointestinal tract.[3]. One of the most associated factors with reservoirs is biofilm formation capability of *Acinetobacter sp* especially in *A. baumanni* wherein it is responsible in part for the intermittent release of pathogens that leads to outbreaks.One of the mechanisms which facilitates the survival of Gram-negative bacteria in the host is resistance to the bactericidal action of Normal Human Serum (NHS) contributes to the virulence of many gram-negative pathogens [4]. Some strains of *A. baumanni* are responsible for bacteremia with a high morality rate due to their ability to resist the killing action of Normal Human Serum[5]. Serum resistance may be due in part to lipopolysaccharide, but lipopolysaccharide alone was not entirely responsible for serum resistance activity [6]. Hence it is very urged to analyze slime production and their capacity to resist bactericidal activity of normal human serum in MDR *Acinetobacter* sp from clinical samples for their association in clinical infections.

2. Materials and Methods

2.1. Isolation and Identification of *Acinetobacter* sp

Totally 92 clinical samples (Pus, Sputum, Urine, Stool, Bed swabs etc.) were collected from various hospitals in and around Puducherry, India. All the clinical samples were inoculated in Brain heart infusion agar (HiMedia, India) for enriching the bacterial growth and further cultured on Herellea agar and Leeds *Acinetobacter* agar with an incubation time of 24 hrs at 37°C for obtaining selective growth of *Acinetobacter* sp. All the presumptive *Acinetobacter* isolates were identified to species level by using slandered microbiological methods. A control strain of *A.baumanni* MTCC-1425 and *A.lwoffi* MTCC-496 (MTCC, India) were used in parallel.

2.2. Determination of Multiple Antibiotic Resistances

Determination of Mulitple Antibiotic Resistance were done by using Kirby Bauer disc diffusion method in Mueller Hinton Agar (MHA). The following antibiotic discs (Hi Media) were used in this study: Gentacmicin (10ug), Cephadroxil (30µg), Ampicillin (10µg), Kanamycin (30), (30µg), Carbenicillin 100µg), Neomycin (30µg), Cefuroxime (30µg), Penicillin G(10U), Tobramycin (30µg), Cefdinir (5µg), Piperacillin/ tazobactam(100/10µg), Amikacin (30µg), Ceftriaxone (30µg) Cloxacillin (10µg), Netillin (30µg), Cefpirome (30µg), Cephotaxime (10µg), Ciprofloxacin (5µg), Meropenem (10µg), Nitrofurantoin (300µg), Nalidixicacid (30µg), Levofloxacin(10µg), Chloramphenicol (30µg), Tetracyline (30µg), Norfloxacin (10µg), Streptomycin (10µg), Rifampicin (5µg), Clindamycin (2µg), Azithromycin (15µg), Metronidazole (5µg), Polymyxin B (300U) and Trimethoprim (5µg).

2.3. MAR index of *Acinetobacter* sp

The MAR index to a single isolate is defined as a/b, where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the number of antibiotics to which the isolate was exposed. MAR index value higher than 0.2 is considered to have originated from high-risk sources of contamination [6].

2.4. Screening of slime production

BHA plates were prepared containing 0.8g/l Congo red [7]. *Acinetobacter* isolates were inoculated onto the surface of the medium and the plates were incubated at 37ºC for 24h. Slime producing bacteria appeared as black colonies, whereas non - slime producers remained non pigmented.

2.5. Serum susceptibility Test

Group 'O' blood was obtained by vein puncture from healthy individuals with no history of infection; Pooled sera were separated and used immediately or stored at 7°C Fresh or thawed normal human serum (NHS) was used unaltered. *Acinetobacter strains* were challenged against 65% NHS in a micro colorimetric assay. The strains tested were transferred to micro dilution well containing 100µL of peptone 1% (v/v) and glucose (1% w/v) broth (PGB). After overnight incubation at 37°C, 20µL of each PGB culture were transferred to the 200µL of fresh PGB and incubated at 37°C for 2 hrs. Log phase bacteria were then inoculated (20µL, 10^7 bacteria) into 100µL PGB containing 65% NHS and 0.5% of 1.5µL of stock solution of bromothymol blue (Final concentration 0.0075 %) serum resistance was assayed by visible color change from green (inhibition) to yellow (growth) of the PGB containing NHS. Control consisted of PGB with 65% heat- inactivated serum 56 °C for 4hrs [8].

3. Result and Discussion

Of the total 92 samples analyzed, 39 (42.3%) were found to harbor *Acinetobacter* sp. (Fig.1a and Fig 1b). Fig.2 represents the distribution of *Acinetobacter* sp. in clinical samples. All the positive isolates were identified by standard biochemical methods and also by 16S rRNA-PCR method by compared with reference the strains of *A. baumanni* MTCC-1425 and *A.lwoffi* MTCC-496.

Out of the 39 *Acinetobacter* isolates 23, 6 and 10 were identified as *A.baumanni* (59%), *A.lwoffi* (15.3%) and *A.hemolyticus* (25.6%) respectively. Our findings were higher than our earlier findings 25% of incidence [9] and

10.8% incidence in Pune, Maharastra, for *A.baumanni* [10]. The present incident report was lesser than 87% incidence and higher than 0.93% and 0.93 % for *A.baumanni*, *A.lwoffi* and *A.hemolyticus* respectively in Transilvania [11]. The similar pattern of higher incidence for *A.baumanni* (79%), lesser incidence for *A.lwoffi* (9.7%) and *A.hemolyticus* (1.3%) were also reported in Chennai [12].

3.1.Multiple antibiotic Resistances

All the 39 isolates of *Acinetobacter* sp when subjected for multiple antibiotic resistances all the isolates (*A.baumanni*, *A.lwoffi* and *A.hemolyticus*) were completely resistant to Penicillin G and Tetracycline (100%). High degrees of resistance were exhibited by *A.baumanni*, *A.lwoffi* and *A.hemolyticus* towards Rifampicin (95.6%, 100% & 80%) and Cloxacillin (87%, 100% and 80%) respectively.

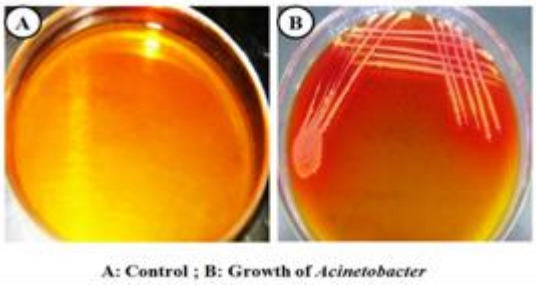

Fig 1a. Growth of Acinetobacter isolates in leeds *Actinobacter*

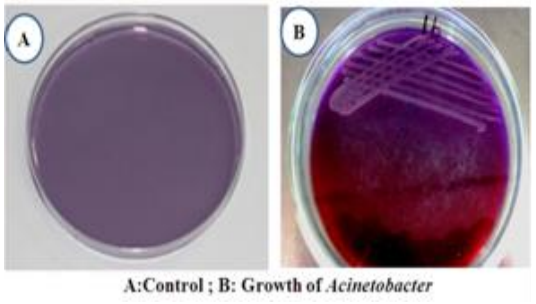

Fig.1. Growth of *Acinetobacter* isolates in Herellea agar

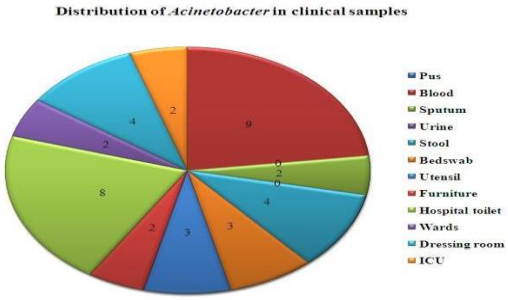

Fig. 2. Distribution of *Acinetobacter* in clinical samples

Ampicillin and Cefaclor were also found to be resistant to all the three species (74%, 100%, 80% and 82.6%, 100, 60% each). Resistance to Cephadroxil (69.5%, 66.6%,& 80%), Cefuroxime(47.8%, 100% & 90%), Kanamycin (43.4%, 66.6% & 50%), Cefpirome (34.7%, 83.3% & 70), Chloramphenicol (60.8%, 66.6%, & 60%), Clindamycin (62.2%, 66.6%, & 70%) and Polymyxin B(74%, 50% & 70%) were also observed for *A.baumanni*, *A.lwoffi* and *A.hemolyticus* during the study(Table.1a).

In our findings apart from *A.hemolyticus* the other two species *A.baumanni* and *A.lwoffi* were found resistant to Carbenicillin (43.4% & 50%), Amikacin (30.4% & 83.3%), Ceftriaxone (26.08 % & 50%), Nitrofurantoin (78.2% & 66.6%), Nalidixic acid(65.2% & 83.3%), Streptomycin(56.5% & 83.3%) and Metronidazole (82.6% & 83.3%) respectively. Moderate levels of resistance to Azithromycin (43.4%), Netillin (34.7%) and Neomycin(34.7%) was recorded for *A.baumanni* alone.

Table 1a. Percentages of *Acinetobacter* sp showing Multiple drug resistances

Antibiotic used	*A.baumanni* (n=23)	*A.lwoffi* (n=6)	*A.hemolyticus* (n=10)
Cephadroxil	69.5	66.6	80
Ampicillin	74	100	80
Kanamycin	43.4	66.6	50
Cefaclor	82.6	100	60
Carbenicillin	43.4	50	40
Neomycin	34.7	33.3	40
Cefuroxime	47.8	100	90
Penicillin	100	100	100
Cefdinir	43.4	83.3	70
Amikacin	30.4	83.3	10
Ceftriaxone	26.08	50	20
Cloxacillin	87	100	80
Netillin	34.7	16.6	20
Cefpirome	34.7	83.3	70
Cephotaxime	---	50	30
Ciprofloxacin	39.1	16.6	--
Nitrofurantoin	78.2	66.6	40
Nalidixicacid	65.2	83.3	20
Chloramphenicol	60.8	66.6	60
Tetracyline	100	100	100
Streptomycin	56.5	83.3	30
Rifampicin	95.6	100	80
Clindamycin	62.2	66.6	70
Azithromycin	43.4	16.6	20
Metronidazole	82.6	83.3	80
Polymyxin B	74	50	70
Trimethoprim	69.5	100	80

This kind of high level resistance against Tetracycline(94%), Chloramphenicol (83%) and Carbenicillin (83%), Nalidixic acid(75%), Rifampicin, Ampicillin (80%) and Nitrofurantoin (70%) were reported in earlier studies [13,14]. Resistance to Cefaclor (97.4), Cefuroxime (98.2%), Amikacin(68.3%), Ceftriaxone(93.3%), Cephotaxime(93.2%) were also reported [15]. From our present findings it was clearly observed that all the *Acinetobacter* species developed Multiple drug resistance activity posing a serious threat to hospitalized patients. A strict attention to maintain and control of the environment

and of the antimicrobial use, appears the measures most likely to control the spread of this organism in hospitals.

Regular monitoring of the antibiogram of hospital pathogen is also recommended to keep physician updated on the proper empirical treatment of such rapidly evolving resistant pathogens.

When considering the susceptibilities all the isolates (*A.baumanni*, *A.lwoffi* and *A.hemolyticus*) were completely susceptible to Piperacillin/ tazobactam (100% each) and expressed high level of sensitivity towards Tobramycin (87%, 100% and 80%), Gentamicin (78.2%, 83.3% and 90%) Neomycin (65.2%, 66.6% and 60%), Ciprofloxacin(60.8%,83.3% and 100%), Levofloxacin (91.3%,100% and 90%), Netillin (65.2%, 83.3% and 80%),Ciprofloxacin(60.8%, 83.3% and 100%), Meropenem(95.6%, 100% and 90%) and Norfloxacin(78.2%, 100% and 90%).

A.baumanni and A.hemolyticus were highly susceptible to Amikacin (69.5% and 90%), Ceftriaxone (74% and 80%) and Cephotaxime (100% &\and 70%). Azithromycin found susceptible at the level of 83.3% and 80% to *A.lwoffi* and *A.hemolyticus* respectively. Apart from other two species *A.hemolyticus* expressed its sensitivity towards Gentamicin(90%), Kanamycin(50%), Carbenicillin and Nitrofurantoin (60% each), Nalidixic acid and Metronidazole (80% each) and Streptomycin (70%) at its maximum level.(Table.1b).

Susceptibility of *Acinetobacter* sp to Gentamicin (99.15%), Kanamycin (61.86%), Neomycin (66.95%), Piperacillin (79.66%), Amikacin (94.07%), Cephotaxime (96.61%) Ceftriaxone (66.1%), Ciprofloxacin(97.46%), Meropenem (82.3%) Levofloxacin (83.05%), Norfloxacin (93.22%), Streptomycin (54.24%) and Tobramycin (88.98%) were reported in earlier studies[16].

Though all the *Acinetobacter* species (*A.baumanni*, *A.lwoffi* and *A.hemolyticus*) exhibited susceptibility to certain drugs the resistance to routinely used drugs were higher than the susceptible level and also the susceptibility were decreased to some extent when compared with earlier reports can be observed from the present findings.

Table.1b. Percentages of *Acinetobacter* sp showing Susceptibility

Antibiotic used	*A.baumanni* (n=23)	*A.lwoffi* (n=6)	*A.hemolyticus* (n=10)
Gentacmicin	78.2	83.3	90
Kanamycin	56.5	3.3	50
Carbenicillin	56.5	50	60
Neomycin	65.2	66.6	60
Tobramycin	87	100	80
Piperacillin/ tazobactam	100	100	100
Amikacin	69.5	83.3	90
Ceftriaxone	74	83.3	80
Netillin	65.2	83.3	80
Cefpirome	65.2	16.6	30
Cephotaxime	100	83.3	70
Ciprofloxacin	60.8	83.3	100
Meropenem	95.6	100	90
Nitrofurantoin	78.2	33.3	60
Nalidixicacid	34.7	16.6	80
Levofloxacin	91.3	100	100
Norfloxacin	78.2	100	90
Streptomycin	43.4	16.6	70
Azithromycin	56.5	83.3	80
Metronidazole	17.3	16.6	80

3.2. Multiple Antibiotic Resistant index

Multiple antibiotic resistances were calculated for all the *Acinetobacter* isolates and its percentage was established. A graph illustrating the MAR index of all the isolates were shown in Fig. 3. The MAR index calculated in the present study were ranged in between 0.27 and 0.81. All the *Acinetobacter* isolates (*A.baumanni*, *A.lwoffi* and *A.hemolyticus*) expressed MAR index value more than 0.2. In particular *A.baumanni* expressed 0.2 and 0.81 as its least and highest MAR indices level. Similarly in *A.hemolyticus* and *A.lwoffi* the lowest MAR value were 0.30 and 0.45 the highest were 0.75 and 0.60 respectively. The result of present study coincides with the earlier report of *Acinetobacter* species with highest MAR index value [17].The resistance patterns detected in *Acinetobacter* could reflect the antibiotic frequent usage, misuse and lack of regulations on the over the counter sale in some parts of the World [18]. The present study suggested that due to the increasing resistance of *Acinetobacters*, the usage of antibiotics should be judged by making an attempt to distinguish colonization from infections and treatment may be continued to the clinically confirmed *Acinetobacter* infections and not merely colonization

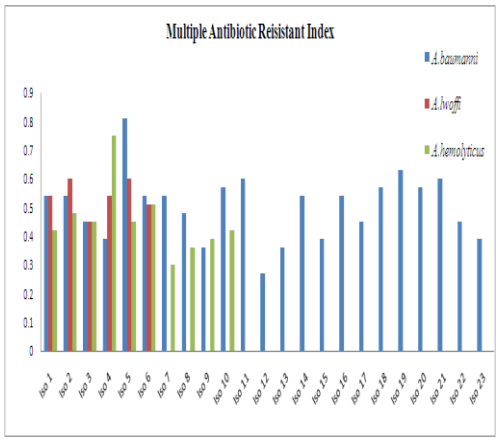

Fig.3. Multiple Antibiotic Resistant Index of *Acinetobacter* sp

3.3. Slime Production

Bacteria producing slime layers are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy [19]. The slime production test was positive for 10 (43.4%), 5(83.3%) and 1(10%) of the total isolates of A.baumannii, A.lwoffi and A.hemolyticus respectively (Fig.4 and Table.2). The percentage of slime production was higher in A.lwoffi than A.baumanni and A.lwoffi. Slime can reduce the immune response and opsonophagocytosis, thereby interfering with host defense mechanisms. The increased biocide resistance and multidrug resistance in A. baumannii associated with the ability to form stronger biofilms. In part, the resistance may be increasing due to low penetration of antimicrobials into biofilms [20].

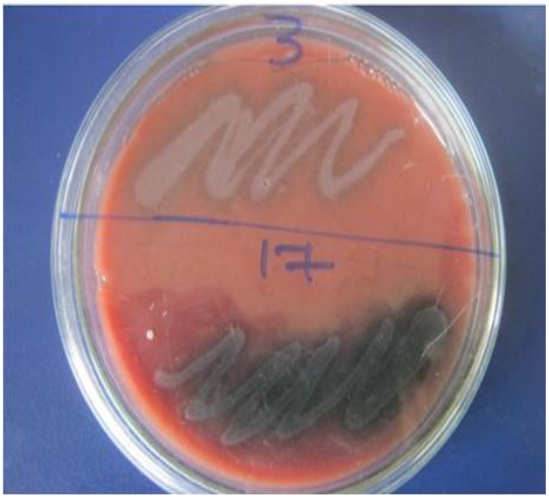

Fig 4: Slime production of *Acinetobacter sp*

Table.2: Slime Production in *Acinetobacter* sp

Isolate No	Slime Production		
	A.baumanni (n=23)	**A.lwoffi (n=6)**	**A.hemolyticus (n=10)**
1.	+	-	+
2.	-	+	-
3.	+	+	-
4.	-	+	-
5.	+	+	-
6.	-	+	-
7.	+		-
8.	-		-
9.	-		-
10.	+		-
11.	+		
12.	-		
13.	-		
14.	-		
15.	-		
16.	-		
17.	-		
18.	+		
19.	+		
20.	+		
21.	+		
22.	-		
23.	-		

3.4. Serum susceptibility

The capacity to resist bactericidal activity of normal human serum (NHS) contributes to the virulence of many gram-negative pathogens. Resistant to the bactericidal activity of normal human serum(NHS) were noticed in 19 (82.6%), 5(83.3) and 8 (80%) of the isolates of *A.baumannii*, *A.lwoffi* and *A.hemolyticus* and the remaining 4(17.3%),

1(16.6%)and 2(20%) of the isolates of the isolates were found to be susceptible to the bactericidal activity of normal human serum(Fig. 5 and Table.3). The results were compared with NHS positive *E. coli* as control strain. The resistant to bactericidal activity of NHS in *A.baumanni* and *A.lwoffi* were reported in previous studies.[21,5].The findings were higher than the previous report of serum resistant activity reported in *A.baumanni* (50%)[5]. Resistance to the bactericidal activity of NHS are most probably connected with the cell surface components of bacteria, including LPS. Apart from LPS other bacterial molecules such as outer membrane proteins are also associated with the serum resistance[21].

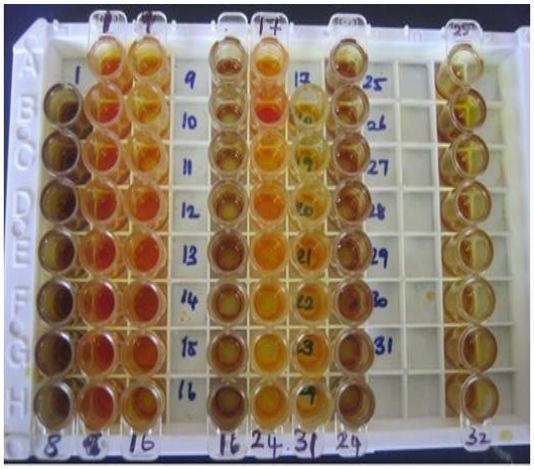

Fig.5. Serum susceptibility of *Acinetobacter sp*

Table .3 Serums Resistant of Acinetobacter sp

Isolate No	Serum Resistant		
	A.baumanni **(n=23)**	***A.lwoffi*** **(n=6)**	***A.hemolyticus*** **(n=10)**
1.	+	+	+
2.	+	+	+
3.	+	-	+
4.	-	+	-
5.	+	+	+
6.	+	+	+
7.	+		+
8.	-		-
9.	+		+
10.	+		+
11.	+		
12.	+		
13.	+		
14.	-		
15.	-		
16.	+		
17.	+		
18.	+		
19.	+		
20.	+		
21.	+		
22.	+		
23.	+		

4. Conclusion

On the basis of above study confirmed the dwelling of highly potential pathogenic mutli drug resistant *Acinetobacters* in the clinical settings. It is also confirmed that all the multi drug resistant *Acinetobacter* isolates were effective slime producers and resist the bactericidal activity of normal human serum expressed increased levels of MAR index values which is further a new threat to the human society. Our study supported this claim and concluded the similar pattern perhaps serum resistance may aid the bacterium in forming slimes due to its increased ability to survive in the presence of the host immune response, or the secreted substance used to form slimes lead to serum resistance.

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