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EVALUATION OF VARIOUS METHODS IN THE IDENTIFICATION OF STRAIN HOMOLOGY AMONG PSEUDOMONAS ISOLATES

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ABSTRACT: INTRODUCTION: Pseudomonas spp are generally found in environment and causes nosocomial infection in hospital environment. Nearly 25 species are found to be associated with human infection. The case fatality has been reported in 50% of infections. This study has been taken up to evaluate the suitable diagnostic method to suit the need of tertiary care hospital. **OBJECTIVES:** To evaluate the various methods in identification of strain homology among Pseudomonas Isolated from samples collected from different wards. **METHODS:** samples collected from different wards were subjected for culture, isolation and identification of strain by different methods. **RESULTS:** 183 samples collected from Burn ward, Intensive Medical Care Unit, [IMCU] ENT OP and medicine OP. Pseudomonas isolated 51% in Burn wound ward and contributed 11% in IMCU isolates 28% in ENT OP isolates and 10% from Medicine OP. 24.59% isolated from 21-40 age group and 23.5% was resistant to <6antibiotics and 12.5% was 4 antibiotics less number of similar isolates. **CONCLUSION:** Modified Diene's Mutual Inhibition test and the antibiotic susceptibility test which are the phenotypic tests had detected equal number of related isolates whereas Random amplified polymorphic DNA study (RAPD) which is a genotypic test detects less number of similar isolates. Diene's Mutual Inhibition test is considered to be the most suitable method for identifying strain homology of Pseudomonas aeruginosa in small laboratories especially during epidemics.

KEYWORDS: IMCU, Modified Diene's Mutual Inhibition Test, Random amplified polymorphic DNA study (RAPD).

INTRODUCTION: The genus Pseudomonas is generally found in soil, vegetation and water and also in skin, throat and stool of healthy individuals. More than 140 species of Pseudomonas have been reported. Of which 25 species are associated with human infections. The opportunistic pathogenic species are Pseudomonas aeruginosa, P. putida, P. cepacia P. stutzeri, P. maltophilia and P. Putrefaciens. Pseudomonas is a gram negative aerobic bacillus which accounts for 80% of opportunistic infections in patients with cancer, cystic fibrosis, burns, endocarditis, Pneumonia, urinary tract, central nervous system, wounds, eyes, skin and musculoskeletal infections also encounter pseudomonas infection. The case fatality rate has been reported as 50%. The organism spread via contact with fomites or by ingestion of contaminated water and food.¹ In many parts of the world burn injury is a major problem. Burn injury impairs the normal Skin barrier function, permitting the microbial growth in the burn wounds and contamination is almost common.² Multidrug resistance of Pseudomonas has been reported widely in many countries.^{3,4,5,6,7} P. Aeruginosa was the most frequent organism isolated in many studies. There are many laboratory diagnostics are practiced for the isolation and analysis and of Pseudomonas Speciation. This study was under taken with the objectives to isolate Pseudomonas aeruginosa from various infections like Burns, Chronic Suppurative Otitis Media (CSOM), ventilator associated pneumonia and Catheter associated Urinary tract infections and to find the strain

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similarities of *Pseudomonas* by antibiogram, Diene's Mutual inhibition test and Random amplified polymorphic DNA study and to compare the three methods and to evaluate the easy, cheap, and the most suitable method which can be adopted in small laboratories at times of outbreaks.

MATERIALS AND METHODS: Study Site: This study was carried out in the Department of Microbiology, Madurai Medical College, Madurai and Government Rajaji Hospital, Madurai, Tamilnadu, India.

Institutional Ethics committee certificate was obtained and informed consent was received from the patients from whom samples were collected.

Inclusion Criteria: Samples collected from Abscess of skin, Infections of burn wounds, chronic suppurative Otitis media, Ventilator associated pneumonias, and Catheter associated Urinary tract infections of more than 1week.

Exclusion Criteria: Post-operative and other wound infections, Acute Suppurative Otitis media, Pneumonias not associated with ventilator, Urinary tract infections not associated with catheters and catheter related UTI's less than 1 week, Patient with DM, Malignancy, HIV & other cause of Infectious disease.

Institutional Ethics Committee Certificate: This research proposal was presented before IEC and certificate received from the IEC committee.

Informed Consent: Informed consent obtained from the patients/relatives for the collection of samples.

METHODOLOGY: Sample collection: 183 samples were taken from Burns ward, IMCU, ENT OP and General Medicine ward at Govt. Rajaji Hospital were processed and analyzed at the microbiology laboratory. Heterogeneous clinical samples like swabs from burn wound and Pus, discharge from CSOM, endotracheal aspirate from VAP and aspirated urine from catheter and ear infection.

The specimens were also examined macroscopically. The pus was examined for any peculiar odour, colour and consistency. The swabs in the transport media were examined for the purulence, odour and the colour changes. The sputum was examined for purulence, odour, blood tingling and viscosity. The urine was examined for the turbidity or for the presence of deposits or suspended materials. The samples were subjected to Gram staining. The samples showing gram negative bacteria were streaked on Nutrient Agar Plate, Macconkey Agar, and 5% Sheep Blood agar and incubated at 37deg Cover night [18-24 hours]. On the next day, the colonial morphology, pigmentation and the odour were noted. The organisms showing iridescent colonies with mawkish odour on nutrient agar, Beta hemolysis with serrated edges on Blood agar were presumably identified as *Pseudomonas* species.

They were confirmed by bio chemical tests like Oxidase, Indole, Triple sugar iron agar, Citrate, Urease, Catalase, Oxidation- fermentation test and Acetamide test. The motility of the organism was confirmed by Hanging Drop method. The organisms which were showing indole negative, non-fermentation with glucose, lactose and sucrose, Positive reactions for Citrate, Oxidase, Urease, Acetamide and Catalase, Oxidative utilization of glucose in O-F medium and Motility by hanging drop

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were identified as *Pseudomonas aeruginosa*. The antimicrobial sensitivity test was carried out disc diffusion technique by the Kirby Bauer method.⁵

The similarity of *Pseudomonas* strains were identified by Modified Diene's Mutual Inhibition Test^{8,9} in this test, capillary tubes were aseptically sectioned in 1 cm segments in biological safety cabinet. *Pseudomonas aeruginosa* strains were grown separately in 5% sheep blood agar plates and 3 capillary tubes were placed in each plate and incubated at 37deg C for 24-48 hrs. When full growth of organism was noted on the tubes, the tubes were removed from the plates. Fresh 5% blood agar plates were prepared and 3 capillary tubes from 3 different isolates were placed over each fresh plate in such a way that the three tubes form an equilateral triangle on the surface of each fresh plate.

All the plates were incubated in ambient air for 48 hrs. After 48 hrs the plates were examined for intersection of colonial growth from the side tubes with the base tube which had standard strain growth. If there was no clearly visible line of demarcation, it was interpreted as indicating high degree of relatedness (Positive). If there was an observable line of demarcation, it was interpreted as unrelated (Negative test). The isolates which showed high degree of relatedness with each other were taken as similar strains.

The DNA typing of Random Amplified Polymorphic DNA typing was done by at Centre for Research in Medical Entomology [CRME] at Madurai an Indian Council of Medical Research [ICMR].

RESULTS: Out of 183 samples collected burn wound samples were 40.43%, CSOM 31.15%, Ventilator associated Pneumonia 10.93% and Catheter associated UTI 17.49%. [Table: -1]

Sl. No	Sources of organism isolation	No. of isolates	Percentage
1	Burn wound infection	74	40.43
2	CSOM	57	31.15
3	Ventilator associated Pneumonia	20	10.93
4	Catheter associated UTI	32	17.49
Table 1: Sources of isolation organisms			

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Burn wound isolation was higher among all the age groups and highest in 31-40Years [13.11%] CSOM was the next to burn wound and highest in 11-20 age group. Ventilator associated pneumonia and catheter associated UTI was least isolation and not found in the 0-1 age group. [Fig. 1] Among the 183 samples analyzed 16.04% were gram- positive and 83.60% were gram-negative bacilli. Of the gram-negative organisms isolated *Pseudomonas aeruginosa*, *E.coli*, *Klebsiellasp*s, *Proteus sp*s and others were 47.06%, 24.18%, 11.76% and 10.46% and 6.54 % respectively. [Table-2]

Sl. No.	Total Samples collected	Organism isolated		Gram Negative Organisms				others
		Gram Positive bacilli	Gram Negative bacilli	<i>Pseudomonas aeruginosa</i>	<i>E.coli</i>	<i>Klebsiellasp</i> s	<i>Proteus Sp</i> s	
1	183	30 [16.40]*	153 [83.60]	72[47.06]	37 [24.18]	18 [11.76]	16 [10.46]	10 [6.54]

Table 2: Organisms Isolated from samples

*Figures in the parenthesis denote percentage.

Only one *Pseudomonas aeruginosa* isolated from the age group 0-1 in female. The age group 21-40 contributes the higher number of *Pseudomonas* isolates. Equal number of isolates obtained from 40 and above age groups both male and female. [Fig. 2]

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Samples collected from burn ward showed more number of [20.22%] *Pseudomonas* isolates, than ENT OP which was only 4.37%, whereas IMCU and Medicine ward samples yielded 4.37% and 3.82% *pseudomonas* isolates respectively.[Fig. 3]

Microbial Susceptibility pattern of *Pseudomonas* showed 17 out of 72 isolates (23.5%) were resistant to more than 6 antibiotics (MDR). Among them, 9(12.5%) were resistant to Carbencillin, Tobramycin, Ciprofloxacin, and Piperacillin – tazobactam which were the antibiotics used in the hospital during the study period against *Pseudomonas* spp. Among them 6 were isolated from Burns ward (67%), 2 were from ENT OP (22%), and 1 was from IMCU (11%). [Table 3][Fig. 4]

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Sl. No.	Name of Ward	Total	%	Resistant to > 6 Antibiotics		Resistant to 4 Antibiotics	
				Total	%	Total	%
1	Burns	37	51 %	9	12.5%	6	8.3%
2	ENT	20	27%	5	7%	2	3%
3	IMCU	8	12%	2	3%	1	0.5%
4	Medicine	7	10%	1	1%	—	—
	Total	72	100%	17	23.5%	9	12.5%

Table 3: Antibiotic Susceptibility Pattern of Pseudomonas aeruginosa n=72

Diene's Mutual Inhibition test was positive in that 9 out of 72 samples (12.5%), showing similarity with each other. Among them, 6 were isolated from burns ward (8.3%) and 3 from ENT OP (4.2%). No positive was detected in IMCU and General Medicine ward. It was found that 12.5% showed strain randomly and 8.3% of them were from burns ward. [Table: 4]

Sl. No	Name of ward	Positive		Negative	
		Total	%	Total	%
1.	Burns	6	8.3	31	43.2
2.	ENTOP	3	4.2	17	23.6
3	IMCU	-	-	11	15.2
4	General Medicine	—	—	4	5.5
	Total	9	12.5	63	87.5

Table 4: Modified Diene's Mutual Inhibition test n=72

In the burn ward samples, isolates 4, 5, 6, 23, 25, 27 and 30 showed strain randomly and 5, 25, 23, 25, 27 isolates showed relative strain similarity. In ENTOP isolates 22, 28 showed similar strain character.

Whereas 6, 22, 28 confirmed with related strain identity. [Table: 5] [Fig-5]

Sl. No.	Ward	Similar Strain	Relative Strain Confirmation
1	Burns	Isolate 4, 5, 6	Isolates 5, 25, 27 (Relatedness)
		Isolates 25, 27	Isolates 5, 23, 30 (Relatedness)
		Isolates 23, 30,	-
2	ENT OP	Isolates 22, 28	Isolates 22, 28, 6 (Relatedness) _
3	IMCU	-	-
4	Medicine	-	-

Table 5: Isolates vs. Diene's positives

In Random amplified polymorphic DNA study, it was found that 7 out of 72 samples were positive (9%). Among them, 5 were isolated from Burns ward (6.9%) and 2 were isolated from ENT OP (2.7%) It was found that 9.7% showed positive RAPD and 6.9% of them were from burns ward. [Table 6]

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Sl. No	Name of ward	Similar Band		Dissimilar Band	
		Total	%	Total	%
1.	Burns	5 Isolates 4, 5, 6, 25, 27	6.9%	32	44.4%
2.	ENTOP	2 Isolate 22, 28	2.7%	18	25%
3.	IMCU	-	-	11	15.3%
4.	Medicine	-	-	4	5.5%
	Total	7	9.7%	65	90.3%

Table 6: Random Amplified Polymorphic DNA study

The phenotypic characters of the pseudomonas identified by antibiotic Susceptibility test and Diene's Mutual Inhibition test were compared with the genotypic characters identified by RAPD and it was found that 7 isolates (9%) showed similar banding pattern in RAPD, 9 isolates (12.5%) showed relatedness with each other by Diene's. Further, it was found out that the nine isolates (12.5%) were resistant to Carbencillin, Tobramycin, Piperacillin-Tazobactam, and Ciprofloxacin. [Table 7][Fig-6]

Sl. No.	Antibiogram		Modified Diene's Mutual Inhibition Test		RAPD	
	Total	%	Total	%	Total	%
1.	9	12.5%	9	12.5%	7	9%

Table 7: Comparison of Three Methodologies

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DISCUSSION: In this study, it was found that 52% of samples were collected in the age group 21-40 years, (mean age 30.5 years) especially in the burns ward. Similar study by Ahmed¹ et al showed that 49.3 % of their study population was in the mean age group 33.3 years. Which is in accordance with our study. Faridaaketal¹⁰ in their study showed that 59.2% of their study population was from burns ward. The cause for the increased incidence of infection in burns ward may be attributed to the loss of natural cutaneous barrier to infection, coagulated proteins and other microbial infections in the burn wound and combined avascularity of the wound which might lead to microbial infection. In this study, 84% bacteria isolated were Gram negative. Akay leyetal¹¹ in their study also demonstrated that 77.9% organisms in their study were Gram negative. The normal flora colonizing the skin influences the infection. Although the Gram positive organisms colonize skin more abundantly, their influence will be there only during the first phase. The Gram negative organisms are the predominant inhabitants of the deeper wound because they have greater invasive potential than Gram positives. In this study, it was found that 51% pseudomonas was present in burns wards. Mehta manjulaet al¹² showed 51% pseudomonas in burns infection which correlates with this study. Pseudomonas aeruginosa from the patient's endogenous intestinal flora and/or an environmental source is the most common cause of burn wound infection by this organism. In this study, 79% pseudomonas was isolated from pus. Anupurpashambaet al¹³ in their study showed 69% pseudomonas were isolated from pus.

In this study it was observed that 23.5% pseudomonas was resistant to more than 6 antibiotics and 8.3% were resistant to carbenicillin, tobramycin, piperacillin-tazobactam, and ciprofloxacin. Obritschet al¹⁴ demonstrated 24.3% multi drug resistance and Jung et al¹⁴ demonstrated 24% multi drug resistance. Multiple drug resistance may be due to inappropriate therapy, delay in starting appropriate therapy, prolonged hospital stay or due to derepression of the chromosomal Amp-C beta-lactamases which reduce the susceptibility to beta lactum antibiotics.

In this study, by Diene's mutual inhibition test, 8.3% isolates showed relatedness with each other and all the isolates were from burns ward. Munsonet al⁹ in his study showed 9.2% related strains in Pseudomonas by Diene's phenomenon but the isolates were from varied infections. But Munson used central venous catheters instead of capillary tubes, which were used in this study. As both the techniques give the same result, substituting central venous catheter by capillary tube is justified.

In this study, 6.9% isolates showed similar bands by Random Amplified Polymorphic DNA Study. All the isolates were from burns ward. Menonet al¹⁵ demonstrated 20% isolates with similar bands by RAPD but the isolates were from ophthalmic cases only. Maureen Campbell et al¹⁶ demonstrated 94% isolates showing similar bands in cystic fibrosis cases. The variations in the isolation rates in the three studies might be due to the variation in the specimens.

CONCLUSION: Thus it was found that Modified Diene's Mutual Inhibition test and the antibiotic susceptibility test which are the phenotypic tests had detected equal number of related isolates whereas RAPD which is a genotypic test detects less number of similar isolates. Among the two phenotypic tests, Diene's Mutual inhibition test is easy to perform, and interpreted easily and it is also less expensive whereas Antibiotic susceptibility test is costlier and interpretation is difficult. Genotypic tests are expensive, need sophisticated equipments, and time consuming. Hence Diene's Mutual Inhibition test is considered to be the most suitable method for identifying strain homology of Pseudomonas aeruginosa in small laboratories especially during epidemics.

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LIMITATIONS: This is a single centered study.

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