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Original Article

Isolation, identification, enumeration and antibiotic profiling of microbes from soil contaminated with hospital waste dumbing

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ABSTRACT

Hospital waste is a potential health hazards to the health care workers and public acquired infection, transfusion transmitted diseases. Increasing land and water pollution lead to increasing possibility of contracting many diseases. Study has compelled the authorities to think seriously about hospital wastes and the disease transmitted through their improper disposal. Soil samples were collected from two different hospitals in Salem city. A total of six isolated were identified and purified from the samples, further screened for individual antibiotics at their respective concentrations and all the six isolates were found to be strong resistant against antibiotics selected in the study. In this present study, *Pseudomonas* sp only possessed a positive virulence characteristic such as haemolysis, protease, coagulase, lecithinase, pyocyanin and lipase when compare to other species like *E.coli*, *Proteus*, *Bacillus*, *Klebsiella*, *Staphylococcus* sp. These are all very low in prevalence of enzymes associated with bacterial pathogens.

***Keywords*:** Hospital waste, Improper disposal, Antibiotic resistant, Virulence characteristics, *Pseudomonas* sp

INTRODUCTION

Hospital wastes are one of the most dangerous causes of pollution. Hospital wastes are generated the diagnosis and treatment or immunization of human beings or animals. It is a universal set having subsets like infectious and hazardous wastes. Wrongly managed hospitals wastes can result in severe health hazards. Hospital wastes refer to all biological or non-biological wastes from hospitals, which are discarded directly to soil. This makes land pollution and environmental pollution in Hospital premises. Some of the pathogenic organisms are dangerous, because they may be resistant to treatment and possess high pathogenicity [1-3].

Hospital wastes are so infectious/hazardous that every means of improper disposal pose a threat to the environments. It is known for the treatment of sick persons but are unaware of the adverse effects of the garbage and filth generated by them on human body and environment. Mainly infection causing hospital dumping soil pathogens are Multi Drug Resistant pathogens. These drug resistant strains initially appeared in hospitals. There are many adverse and harmful effects to the environment and human being which are caused by the hospital waste generated during the patient care [4,5]. These resistance mechanisms have led to the appearance of MDR bacteria. These MDR bacteria, via multiple mutations, coupled with the acquisition of antibiotic resistance genes by HGT, have earned the name of 'superbugs' and have been responsible for many human infections, characterized by increased hospital admissions and increased mortality e.g. MDR *Pseudomonas* sp other 'superbugs' include, but are not limited to, some strains of *Escherichia coli*, *Bacillus* sp, *Klebsiella* sp, *Staphylococcus* sp and *Proteus* sp [6].

The main risk for public health is that resistance genes are transformed from environmental bacteria to human pathogens. As a result hospital waste dumping effluent could increases the number of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection of

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resistant bacteria reported by my study multidrug resistance profile of bacteria isolated from biomedical waste dumping site soil [7,8]. The aim and objective of the study is isolation, identification, enumeration and antibiotic profiling of microbes from soil contaminated with hospital waste dumping.

MATERIALS AND METHODS

Collection of Soil Sample: The soil samples were collected from two hospitals in Salem city. They are Gokulam private hospital and SKS hospital. The samples were collected from waste dumping area. Totally10soil samples were collected from different location around hospital environment. The soil samples were collected from depth of 5-6 cm from the hospitals waste dumpsites and transferred to sterile polythene bags labelled properly. All the soil samples were mixed together for further process.

Isolation of Bacterial Strains: One gram of mixed soil samples were poured into 100 ml of sterile distilled water in 250 ml conical flask and set as dilution factor 10^{-2}. 1 ml was taken from 10^{-2} dilution factor and transferred into tube containing 9 ml of blank and set as dilution factor 10^{-3}. Further 1 ml of aliquots was diluted up to 10^{-9}. Prepared the nutrient agar medium and sterilize the medium using 121°C for 20 minutes at 15 lbs. Taken 0.1 ml of aliquots from dilution 10^{-5}, 10^{-6} and 10^{-7} serially were by spread plate technique on nutrient agar plates. The nutrient agar plates incubated at 37°C for 24 h. After incubation, the typically different colonies were observed.

Enumeration of Bacteria: The isolated bacterial colonies were counted by using following Colony Forming Unit (CFU) formula and average counts for duplicate cultures were recorded as total viable bacteria in the samples.
Formula: CFU = Number of colonies x Dilution factor / Volume of sample

Pure Culture Maintenance by Streak Plate: The isolated organisms were purified through repeated subculture method. Streak plate methods were used for this purpose. Nutrient agar was used as media. When a plate yielded only one type of colony, the organisms were considered to be pure. The purification of the isolates was also confirmed by microscopic observation.

Identification of Bacterial Isolates: Different morphological and biochemical characteristics accompanied with colony characteristics on different selective medium were observed for the identification of bacterial isolates.

Antibacterial Sensitivity Test By Disc Diffusion Method (Kirby Bauer Method): Antibacterial Susceptibility of the isolated strains of *Staphylococcus* sp, *E. coli*, *Bacillus* sp, *Klebsiella* sp and *Pseudomonas* sp to various antibiotic discs were carried out according to standardized disc diffusion method. Overnight broth cultures of all selected bacterial isolates were swabbed separately on the surface of Muller Hinton Agar (MHA) plates. Then commercially available antibiotic discs were placed on the MHA plates with the help of sterile forceps.

The following antibiotic discs were used: Gentamycin, Cefoxitin, oxicilin, Ciprofloxacin, Penicillin, Ampicillin, Chloramphenicol, Tetracycline, Erythromycin, Vancomycin, Methicillin, Amoxicillin, Co-triamoxazole, Nalicidic acid. These plates were incubated at 37°C for 24 hours. After incubation, the degree of Resistant was determined by measuring the zone of inhibition of growth around the discs [6,9,10].

Virulence Test

Protease assay: The protease enzymes producing organisms are highly virulence pathogen. Proteases which are disrupt the proteins in human body. All the isolate bacterial cultures were screened for their ability of protease enzymes production on skim milk agar plate. A loopful of overnight bacterial culture was streaked separately on skim milk agar plates and incubated 37°C 24 hrs. After incubation period, plates were observed.

Lecithinase assay: Lecithinase was measured by utilising an egg yolk agar base. A loopful of isolated bacterial colonies were inoculated on the surface of egg yolk agar and allowed to incubate for 72 hours at 37°C. After incubation time, plates were observed.

Haemolysis assay: The haemolysin is one of the virulence factors which destroy Red Blood Cells (RBC) in human. Five percent defibrinated sheep blood agar in a trypticase soy agar base was the substrate. A loopful of bacterial colonies was inoculated on to the surface of the agar with incubation at 72 hours at 37°C. After incubation observed plate.

Coagulase assay: Rabbit citrated plasma containing EDTA (Ethylene diamine tetra acetate) was dispensed in 1ml aliquots; a loopful of bacterial colony was inoculated for 48 hrs at 37°C. Then the plates were observed after incubation.
Lipase Assay: Trypticase soy agar plates supplemented with 1% tween 80 (poilyoxyethylenesorbitanmonooleate) served as a substrate. Colonies were inoculated on the surface

of the agar plate and allowed to incubate for 72 h at 37ºC. The appearance of a turbid halo around the inoculum spot was taken as evidence of a positive test.

Pyocyanin assay: Production of pyocyanin from *Pseudomonas aeruginosa* was determined utilising Pseudomonas agar. A loopful of bacterial colonies inoculated on the surface of Pseudomonas agar plate and allowed to incubate for 24 hours at 37°C. After incubation, plates were observed [11,12].

RESULTS AND DISCUSSION

Isolation and Enumeration of Bacterial Colonies: Bacteria isolated from mixed hospital waste dumping soil collected from different hospitals in and around Salem, Gokulam private hospital Salem and SKS hospital. Totally around 177 colonies (Table.1) were isolated on nutrient agar plate.

Identification of Bacterial Isolates: 15 predominant individual colonies were selected and identified on the basis of Morphological characteristics, Gram staining and biochemical characteristics according to the key of Bergey's manual of Determinative Bacteriology. All the 15 selected bacterial colonies were examined under microscope after the Gram staining, Spore staining and Motility test. The results were observed and recorded (Table.2). Among the 15 isolates approximately 67% of Gram Negative and 35% of Gram of Gram Positive bacterial strains were observed.

All the selected 15 bacterial strains were characterized by the following biochemical analysis: Indole test, Methyl red test, Voges-proskauer test, Citrate test, Catalase test, Oxidase test, Triple sugar iron test, Urease test, Carbohydrate fermentation test. Biochemical analysis results were observed and recorded (Table.3).

The moisture content of the hospital wastes dump sites ranged from 3.8 to 7.5%. Many of the bacterial isolates found in the waste soil samples reported here are also known to cause hospital acquired or nosocomial infections. In the present study, the morphological analysis revealed that of the virulent pathogens were isolated. *Escherichia coli* were the leading etiologic agent and second most predominant ones were *Klebsiella* sp, *Pseudomonas sp*, and *Proteus* sp. The least etiologic agent is *Bacillus* sp, *Staphylococcus* sp.

Based on the microscopic examination and biochemical analysis according to the key of Bergey's manual of Determinative Bacteriology all selected 15 bacterial isolates were identified: S1, S8 and S13 as *Pseudomonas* sp, S4 and S12 as *E .coli*, S2, S5 and S10 as *Klebsiella* sp, S9 and S15 as *Bacillus* sp, S3, S6 and S11 as *Staphylococcus* sp, S7 and S14 as *Proteus* sp. All these only 6 identified bacterial strains (Fig.1) were used for further process.

The following isolated six bacterial strains were streaked on the different agar plates (Fig. 2). In cetrimide agar plates *Pseudomonas* sp only produce bluish green pigmentation others did not produce the pigmentation. In EMB agar plates *E. coli* only showed the dark greenish metallic sheen colonies and *Klebsiella* produced pink colour colonies, MSA plates were showed the *Staphylococcus* only produced golden yellow colour colony and the MacConkey agar plates were showed the *E. coli*, *Proteus* sp and *Klebsiella* sp positive results to conformed the lactose fermentation other isolates were considered as a non-lactose fermentation. In this study, the pathogenic bacteria isolated from the biomedical wastes were *E. coli* (18.6%), *Enterobacter* sp (14.5%), *Shigella* sp (8.9%), *Proteus* sp (9.7%), *Pseudomonas* sp (12%), *Serratia* sp (1.6%), *Staphylococcus aureus* (16.1%), *Klebsiella* sp (6.5%),*Citrobacter* sp (2.4%), *Bacillus* sp (4.0%) and *Salmonella* sp (5.7%). Some of the samples however, did not show any growth on the various media used. From the result, more gram negative organisms (especially members of the enterobacteriaceae) were isolated than gram positive organisms. *E. coli* (amongst the gram negative) and *Staphylococcus aureus* (amongst the gram-positive) were isolated in higher concentrations from the samples collected for this study [13-17].

Antibiotic Susceptibility Test of Soil Sample Associated Pathogens: Out of the thirteen antibiotics used in antimicrobial activity (Kirby-Bauer method); it was observed that *Pseudomonas*, *Klebsiella*, *Bacillus* showed against resistance to maximum antibiotics; whereas *Proteus* showed sensitivity to almost all the antibiotics used (Fig. 3 and Table.4). Characterization of all isolates gave surprising result showing emergence of *Neisseria* sp, *Alkaligenes* sp, as MDR pathogens. The cause of increasing resistant among the bacteria might be due to development of MDR due to antibiotic efflux pump against that drug due to its prolonged exposure at contaminated hospital dumping sites, due to mixing of both MDR and non MDR strains of pathogens at hospital waste disposal site resulting in genetic recombination of plasmids between two bacteria thriving at same place one of which might be MDR or induction of multi drug

resistance by proteins secreted by MDR bacteria. Protein source supplement like beef extract, yeast extract are the best source required for bacterial growth, even they can support growth without any other additional nutrient supplement required. Bacteria usually produce its secondary metabolite in stress conditions in order to survive. Carbohydrate, amino acids, multivitamin capsules etc. and metal ions acts as growth elicitors in case of many bacterial strains. The place of emergence of new and pathogenic strains of MDR bacteria can be hospital itself if not taken care of hospital dumped wastages [6,18-20].

Virulence Test: Virulence characteristics such as Haemolysis, Protease, Coagulase, Lecithinase, Pyocyanin, Lipase test were performed for all the 6 identified bacterial strains. The results were observed and recorded (Fig.4 and Table.5). *Pseudomonas, Bacillus and Staphylococcus* showed positive result for β-haemolysis compared to other microorganisms selected. The Blood agar plates were observed with zones around the colonies. Zone formation was observed in Skim milk agar plates with cultures namely *Klebsiella, Pseudomonas, Bacillus and Proteus* signifying the presence of protease enzyme. *Pseudomonas and Staphylococcus* showed positive result in Coagulate blood plasma. Only *Pseudomonas* gave positive result for Lecithinase enzyme test indicated by a white colour precipitation around the colony. This signified the presence of Lecithinase enzyme. *Pseudomonas* produced pyocyanin pigment giving a greenish colour to the culture in Pseudomonas Agar plates. *Pseudomonas and Bacillus* produced zone in Trypticase soy agar plate suggesting the presence of Lipase enzyme.

Among 6 identified bacterial strains, *Pseudomonas* sp was most virulent compared to all other bacterial strains. Most of the isolates possessed a positive virulence characteristic for each water source. Also included are the results of the acid liability test. Except for the enzyme proteinase, there was a very low prevalence of enzymes associated with bacterial pathogenesis from any of the water sources. In all cases, except for proteinase (produced by 20 per cent of isolates), less than 6 per cent of bacterial isolates produced a single extracellular enzyme associated with virulence. It is generally considered necessary to contain more than one extracellular enzyme in order for a microbe to have sufficient armaments to be virulent [11,21-23]. In this present study, *Pseudomonas* sp only possessed a positive virulence characteristic such as haemolysis, protease, coagulase, Lecithinase, pyocyanin and lipase when compare to other species like *E.coli, Pseudomonas, Proteus, Bacillus, Klebsiella, Staphylococcus* sp. These are all very low in prevalence of enzymes associated with bacterial pathogens.

In this present study hospital, using infection hazard wastes are not properly disposed in hospital environment soil. Hospital waste is a potential health hazards to the health care workers and public acquired infection, transfusion transmitted diseases. Increasing land and water pollution lead to increasing possibility of contracting many diseases. Study has compelled the authorities to think seriously about hospital wastes and the disease transmitted through their improper disposal.

CONCLUSION

Finally concluded that, in the present study most of the predominant organisms present in the hospital waste dumping site area. In the study area were virulent possess up antibiotic sensitivity potential source and reservoir of multiple drug resistance organisms. The presence of high number of pathogenic organisms resident in the hospital waste dumping site area is a treat to such environment and has a serious public health implication. It is imperative that all hospital wastes should be incinerated and treated properly before discharge into the environment and there should be an efficient waste management practices such as landfill where wastes could be channelled in both public and private hospitals.

Table.1: Enumeration of Bacterial Isolated Colonies

S.No	Dilution Factor	No. of Colonies (CFU/ ml)
1.	10^{-5}	74
2.	10^{-6}	65
3.	10^{-7}	38

Table-2: Microscopic Examination of Bacterial Isolates

Bacterial isolates	Microscopic examination		
	Gram staining	**Spore staining**	**Motility test**
S1	Gram Negative, Rod	Non-spore forming	Motile
S2	Gram Negative, Rod	Non-spore forming	Motile
S3	Gram Positive, Cocci	Non-spore forming	Non-motile
S4	Gram Negative, Rod	Non-spore forming	Motile
S5	Gram Negative, Rod	Non-spore forming	Non-motile
S6	Gram Positive, Cocci	Non-spore forming	Non-motile
S7	Gram Negative, Rod	Non-spore forming	Motile
S8	Gram Negative, Rod	Non-spore forming	Motile
S9	Gram Positive, Cocci	Spore forming	Motile
S10	Gram Negative, Rod	Non-spore forming	Motile
S11	Gram Positive, Cocci	Non-spore forming	Non-motile
S12	Gram Negative, Rod	Non-spore forming	Motile
S13	Gram Negative, Rod	Non-spore forming	Motile
S14	Gram Negative, Rod	Non-spore forming	Motile
S15	Gram Positive, Cocci	Spore forming	Motile

Table.3: Biochemical analysis of isolated Bacterial Strains

Strains No	Indole	MR	VP	Citrate	TSI	Oxidase	Catalase	Urease	Carbohydrate fermentation test
S1	_	_	_	+	G+,H_2S	_	+	_	_
S2	_	_	+	+	G+,H_2S-	_	+	_	+
S3	_	_	_	_	G+,H2S-	+	+	+	+
S4	+	+	_	_	G+,H2S-	_	+	_	+
S5	_	_	+	+	G+,H_2S-	_	+	_	+
S6	_	_	_	_	G+,H2S-	+	+	+	+
S7	+	+	_	+	G+,H2S-	_	+	+	+
S8	_	_	_	+	G+,H_2S	_	+	_	_
S9	_	+	+	+	G+,H2S	_	+	_	_
S10	_	_	+	+	G+,H_2S-	_	+	_	+
S11	_	_	_	_	G+,H2S-	+	+	+	+
S12	+	+	_	_	G+,H2S-	_	+	_	+
S13	_	_	_	+	G+,H_2S	_	+	_	_
S14	+	+	_	+	G+,H2S-	_	+	+	+
S15	_	+	+	+	G+,H2S	_	+	_	+

Table.4: Antibiotic Susceptibility Test for the six isolates

Antibiotics	Zone of Inhibition					
	Pseudomonas	***Klebsiella***	***Bacillus***	***E.coli***	***Staph.***	***Proteus***
Amoxicillin (30 mcg)	_ (R)	_ (R)	_ (R)	_ (R)	_ (R)	22mm (S)
Cotrimazazole (25 mcg)	_ (R)	_ (R)	15mm (S)	30mm (S)	15mm (S)	32mm (S)
Ciprofloxacin (10 mcg)	16mm (R)	16mm (R)	10mm (R)	26mm (S)	10mm (R)	24mm (S)
Gentamycin (10 mcg)	21mm (R)	16mm (R)	10mm (S)	18mm (S)	11mm (R)	24mm (R)
Nalidixicacid (30 mcg)	11mm (R)	10mm (R)	_ (R)	10mm (I)	_ (R)	14mm (S)
Ampicillin (10 mcg)	_ (R)	23mm (S)	_ (R)	_ (R)	_ (R)	22mm (S)
Oxicilin(1mcg)	_ (R)	17mm (S)	_ (R)	_ (R)	_ (R)	19mm (S)
Erythromycin (15 mcg)	_ (R)	8mm (R)	14mm (R)	15mm R)	14mm (I)	28mm (S)
Methicillin (5mcg)	_ (R)	_ (R)	_ (R)	_ (R)	_ (R)	14mm (S)
Tetracycline (30 mcg)	10mm (R)	_ (R)	14mm (R)	15mm R)	24mm (S)	17mm (I)
Vancomycin(30mcg)	_ (R)	_ (R)	4mm (R)	_ (R)	18mm (S)	19mm (S)
Chloramphenical (10 mcg)	14mm (R)	15mm (S)	4mm (R)	12 (R)	_ (R)	_ (R)
Cefatazidime (30mcg)	_ (R)	_ (R)	_ (R)	_ (R)	_ (R)	_ (R)

[R] - Resistant[S] - Sensitive [I] - Intermediate

Table.5: Comparison of virulence characteristics of bacterial isolates from the hospital wastes dumping site soil.

S.NO	Characteristics	Result					
1	Haemolysis	_	_	+	_	+	+
2	Protease	_	+	+	+	+	_
3	Coagulase	_	_	+	_	_	+
4	Lecithinase	_	_	+	_	_	_
5	Pyocyanin	_	_	+	_	_	_
6	Lipase	_	+	+	+	+	+

Fig: 1 Percentage of Bacterial isolates

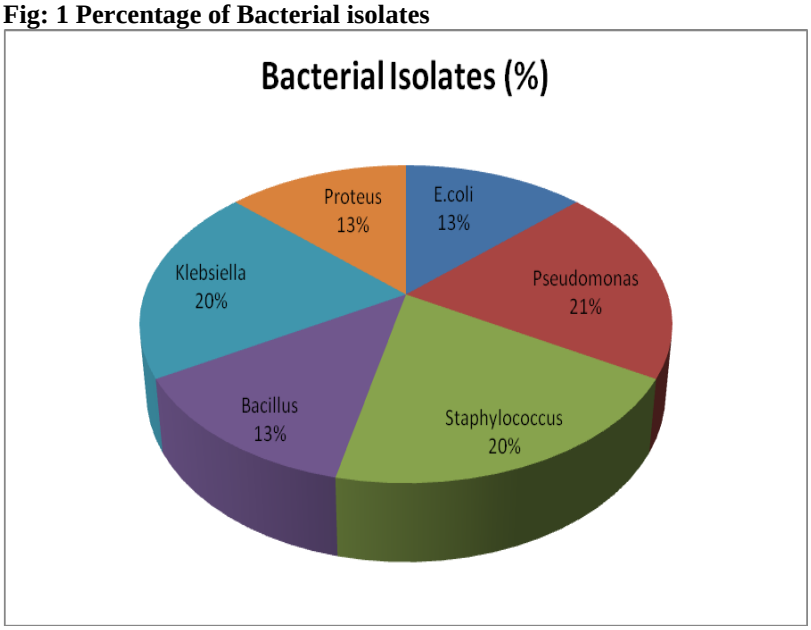
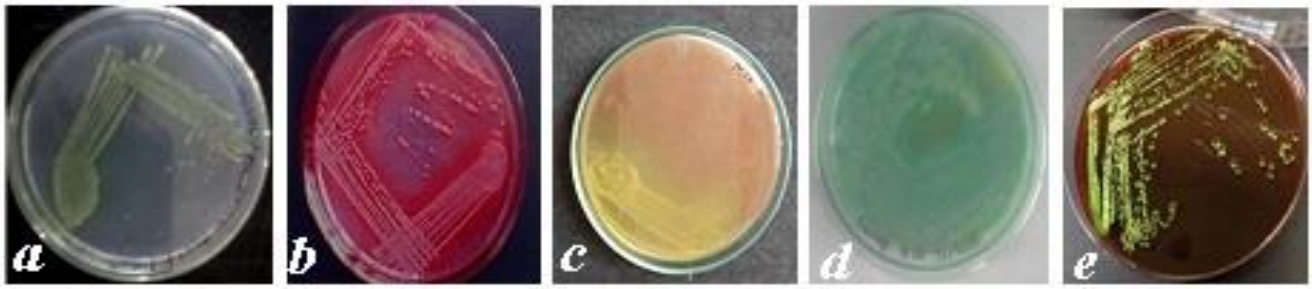
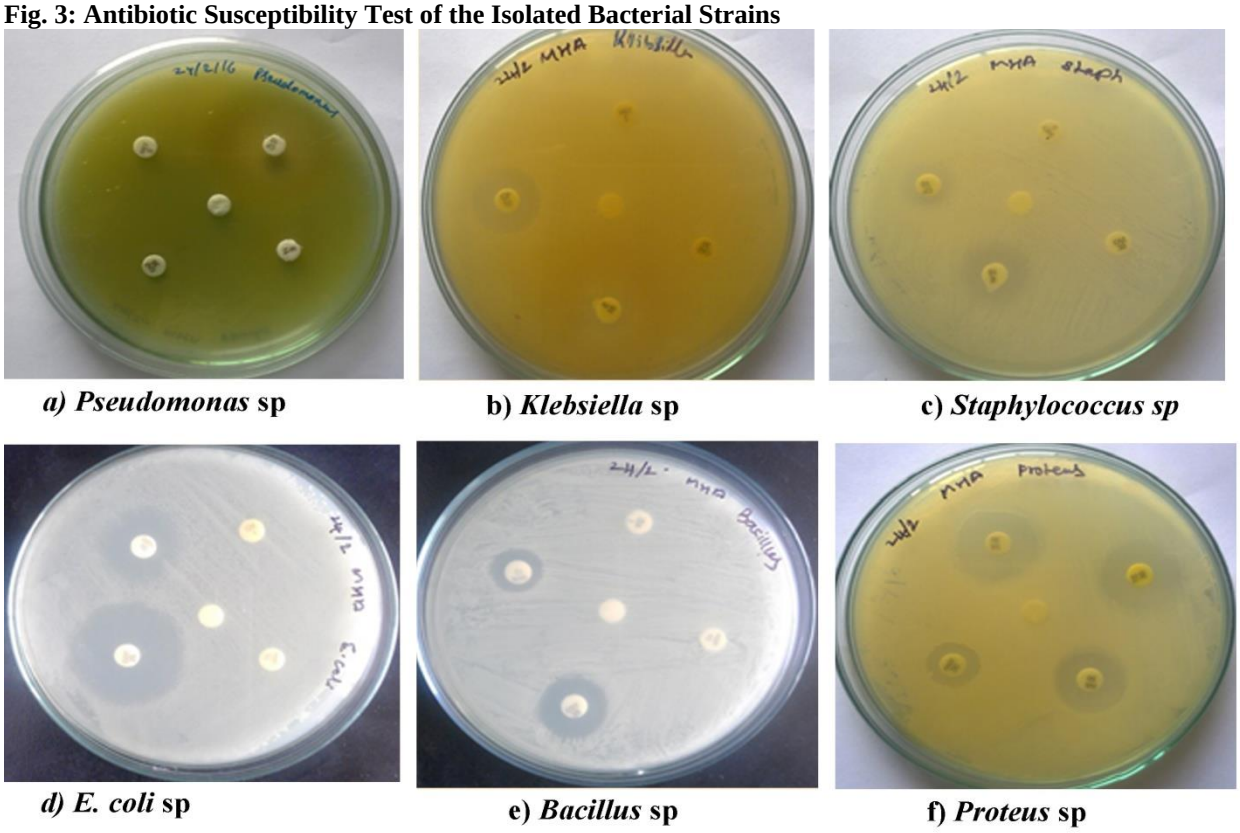


Fig. 2: Isolated Bacterial Strains on Different Selective Media



a) Pseudomonas sp on Cetrimide agar plate, b) Klebsiellasp on MacConkey agar plate, c) Staphylococcus sp on Mannitol Salt agar plate, d) Bacillus sp on Bacillus cereus agar plate and e) E. coli on Eosin Methylene Blue agar plate.

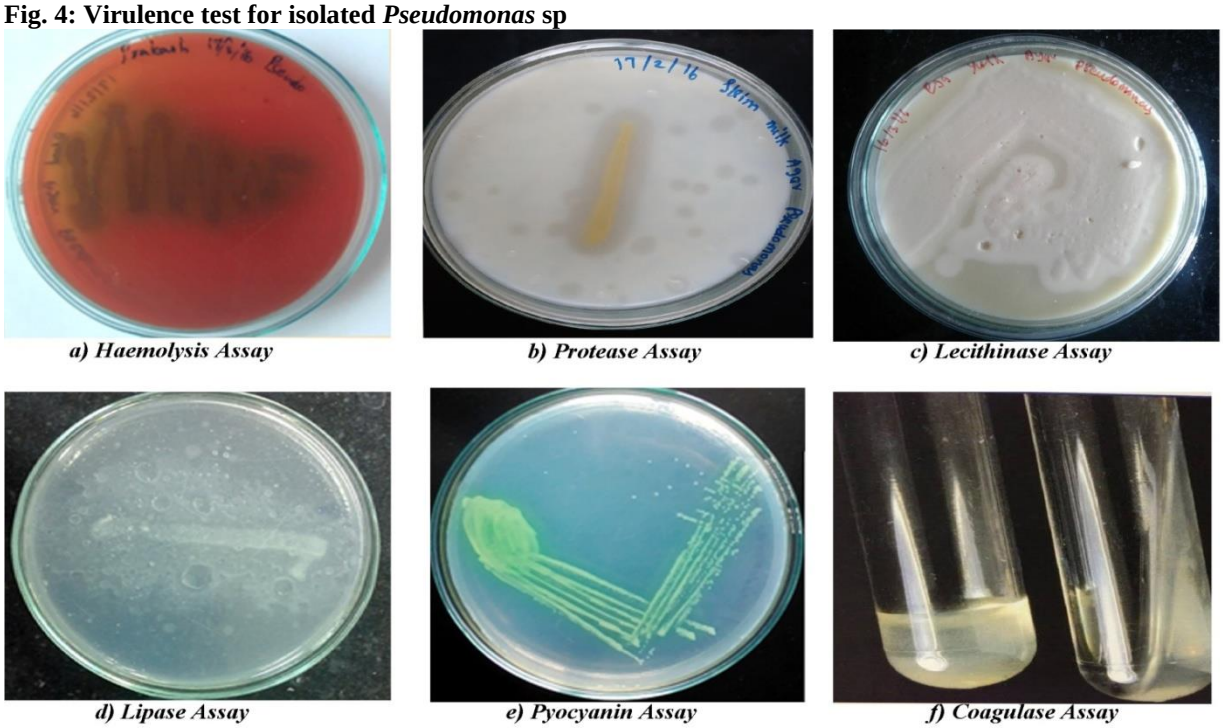
Fig. 3: Antibiotic Susceptibility Test of the Isolated Bacterial Strains



a) Pseudomonas sp b) *Klebsiella* sp c) *Staphylococcus sp*

d) E. coli sp e) *Bacillus* sp f) *Proteus* sp

Fig. 4: Virulence test for isolated *Pseudomonas* sp



a) Haemolysis Assay *b) Protease Assay* *c) Lecithinase Assay*

d) Lipase Assay *e) Pyocyanin Assay* *f) Coagulase Assay*

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