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Studies on Multiple Drug Resistance (MDR) among *Pseudomonas aeruginosa* Isolates and their Virulence Factors

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MDR *Pseudomonas aeruginosa* strains have been isolated from clinical specimens with increasing frequency by various workers. The knowledge of prevalence of MDR isolates and screening of extended spectrum beta lactamase (ESBL) as mechanism of resistance and phenotypic expression of their virulence factors in the state of Himachal Pradesh is, therefore, essentially required. The aim of the study is to assess the prevalence of multidrug resistant isolates, extensive drug resistant (XDR), pan drug resistant isolates, ESBL producing isolates and to compare their virulence factors with those of sensitive strains. A total of 180 isolates recovered from patients at Indira Gandhi Medical College, Shimla were confirmed as *P. aeruginosa* isolates. These isolates were screened for their susceptibility to different antibiotics that are commonly used to treat infections due to this organism by *in vitro* antibiotic cultural sensitivity assay (Bauer, 1966). ESBL as a mechanism of resistance was confirmed by Double disc diffusion synergy test (DDST) and E test. Lipase, hemolysin, protease, gelatinase and biofilm formation were some of the virulence traits whose phenotypic expression *in vitro* was studied in respect of both MDR and MDS isolates. 27.22% (49/180) isolates of *P. aeruginosa* were recorded as MDR whereas 55% (99/180) as XDR and 1.11% (2/180) as PDR. Only 28.57% (16/56) ESBL producers were MDR also while rest 71.43% (40/56) ESBL producers were sensitive to multiple drugs. *P. aeruginosa* both resistant as well sensitive strains produced lipase, hemolysin, protease, gelatinase and biofilms. The proportions of strains expressing different virulence factors were comparable. The hospital based epidemiological data might have implications in better infection control as well as therapeutic strategies by virtue of the knowledge of antibiotic resistance patterns in this geographic region as well as prevalence of ESBL producers.

Key words: Extensive drug resistant, Extended spectrum beta lactamase (ESBL), Multi drug resistant, Pan drug resistant, *Pseudomonas aeruginosa*, Virulence factors.

Pseudomonas is a genus of gamma proteobacteria, belonging to the larger family of *Pseudomonads*. *P. aeruginosa* is an opportunistic pathogen with innate resistance to many antibiotics and disinfectants. This organism is saprophytic and widely spread in nature, particularly in moist environments. *P. aeruginosa* is an aerobic gram negative rod, usually 1.5-5ìm in length and 0.5 to 1.0ìm in width and is motile due to the presence of single polar flagellum. It is an opportunistic pathogen often implicated in nosocomial infections and causes many severe and often fatal infections particularly in immunocompromised patients with severe burns or those with HIV infection[1].

Several virulence factors contribute to the pathogenesis of the infections due to this organism such as the formation of pyocyanin, hemolysin, gelatinase and biofilm. These factors result in increase tissue damage and protect *P. aeruginosa* against the recognition by the immune system and the action of antibiotics[2]. The pathogenesis of *P.*

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aeruginosa infections is multifactorial because it has wide array of virulence determinants such as pili, lipopolysaccharide (LPS), flagella, elastase, alkaline protease, siderophores, siderophore uptake systems and extracellular protein toxins[3].

P. aeruginosa can also harbor several mechanisms of resistance which generate multi drug resistant isolates (MDR). The resistance to three or more classes of anti-*Pseudomonas* agents is used as a criterion for regarding an isolate as MDR. Extensively drug resistant (XDR) isolates describing a resistance profile which compromised most standard antimicrobial regimens or pan-drug resistant (PDR) isolates are resistant to all approved antimicrobial agents[4].

The selection of MDR has been taking place since the 1940s. The evolution and spread of resistance are relatively recent and have occurred mainly during past five decades. The first case of MDR *P. aeruginosa* strain was isolated in the hematologic unit in 1992[5]. Multidrug–resistant (MDR) *P. aeruginosa* strains have been isolated all over the world from clinical specimens with increasing frequency [6]. Increasing frequencies of MDR strains of *P. aeruginosa* in nosocomial infections all over the world require determination of correlation between the resistance to antibiotics and virulence of a pathogen involved. In general, this would depend on the interactions between the multiple factors associated with bacteria and their environments. The ultimate effect of the association between bacterial virulence and antimicrobial resistance depends mainly on four factors; (i) bacterial species involved, some microorganisms acquire antibiotic resistance mechanisms readily and evolve rapidly in response to antibiotic pressure (e.g. *P. aeruginosa*), (ii) specific virulence and resistance mechanisms, (iii) the environment or ecological niche, (iv) the immune system of the host.

The present study aims at investigation of the antibiotic resistance among *P. aeruginosa* strains from hospital settings in Himachal Pradesh obtained during a period of one year and the presence of potential virulence factors present in or expressed by them and also to determine the correlation between the two with regard to sensitive and resistant strains.

MATERIALS AND METHODS

Collection and processing of clinical isolates

P. aeruginosa isolates (200 in number) recovered from human clinical cases and analyzed at Shoolini University in the Microbiology laboratory during period of one year. These isolates were subcultured in nutrient broth for enrichment and revived on slants of Pseudomonas isolation agar (Himedia, Mumbai). The confirmation was done on the basis of morphology, microscopic examination of Gram's stained preparations and standard biochemical tests. The purified colonies were preserved in 80% glycerol at -80^{0}C until used. *P. aeruginosa* strain ATCC 27853 was used as quality control strain.

In vitro cultural antibiotic sensitivity assay

The assay was performed following the standard CLSI guidelines 2013; using common anti-pseudomonad antibiotic discs following the disc diffusion method of Kirby Bauer[7]. The antibiotics used were: aminoglycoside: amikacin and gentamicin; cephalosporins: cefepime, ceftazidime and cefoperazone; quinolones: ciprofloxacin and levofloxacin; ureidopenicillins: piperacillin/ tazobactum and piperacillin; carbapenems: imipenem and meropenem; monobactam: aztreonam.The diameters of zones of inhibition were measured after 24h incubation at 37°C and the results interpreted according to criterion as described in CLSI guidelines[8]

Detection of ESBL

For preliminary screening of ESBL producing *P. aeruginosa*, the susceptibility of the isolates was determined against third generation cephalosporins following the method of Kirby Bauer[7]. ESBL positive isolates were confirmed by Double disc diffusion synergy test (DDST) and E test[9].

E.coli ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as quality control strains in the E test.

Determination of virulence factors

Gelatinase production

The protease test medium was prepared by adding 2% bacteriological gelatin (Loba Chemie, Mumbai) to nutrient agar medium (Himedia, Mumbai). *P. aeruginosa* strains were streaked on the medium for 24 h to 72 h at 37 °C. Zone of

clearance around the line of growth indicated gelatinase production.

Protease production

The protease test medium was prepared by adding 2% skimmed milk to nutrient agar (Himedia, Mumbai). Inoculum of the test organisms in a 30µl vol. was loaded in each well and incubated for 24 h to 72 h at 37 °C. Clear zone around bacterial colonies indicated protease production[10].

Hemolysin production

Blood agar was prepared aseptically by adding 5% of sterile blood to nutrient agar autoclaved at 121 °C for 15min which had been cooled to 45 °C and the contents mixed thoroughly. Blood agar plates were prepared and streaked with loopful bacterial cultures, incubated at 37 °C for 24 h. Clear zones around the line of bacterial growth due to destruction of RBCs indicated hemolysin production. The type of hemolysis was also recorded.

Lipase production

Egg yolk agar medium was prepared by mixing a sterile egg yolk 1:1 in physiological saline solution. 10% egg yolk was mixed with nutrient agar enriched with 1% NaCl. The contents were poured into petriplates and inoculum (30 µl) of the test organisms was placed in the wells and incubated at 37 °C for 1-4 days. Clear zone around the colonies indicated production of lipases by the organism.

Biofilm formation

The formation of biofilms by the *P. aeruginosa* strains was assessed by the tube test as well as tissue culture plate assays.

Tube test

This is a qualitative method of biofilm detection. Precisely, a loopful of each test organism was inoculated in 10 ml of trypticase soy broth containing 1% glucose + 2% sucrose in a test tube and incubated at 37 ºC for 24 h. The contents were then decanted off and the biofilm washed in phosphate buffer saline (pH 7.3), dried, and stained with crystal violet (0.1%). Excess of the stain was washed in deionized water. Tubes were kept in inverted position and dried completely11. The scoring in the stained tubes for biofilm formation was done by comparison with the quality control strain. Visible film lining the wall and the bottom of the tube was considered positive. The weak biofilm formation was scored as +, moderate ++ and strong as +++. The experiment was performed in triplicate and repeated thrice.

Tissue culture plate assay

The biofilm adherent on all sides of the wells was uniformly stained with crystal violet. Optical density (OD) of the stained adherent bacteria was determined with a microplate reader at a wavelength of 590nm[12]. The results were recorded after subtracting the reading of the blank (TSB plus glucose and sucrose, without bacterial cells). A three-grade scale was used to evaluate the biofilm producing ability of the test strains as follows; no or weak: OD<0.120; moderate: 0.120<OD<0.240; high: OD>0.240 as stated by Christensen *et al.*[11].

Statistical Analysis

Chi square $(\chi)^2$ test was applied to determine the significance of difference between the virulence factors of MDR and MDS isolates using SPSS (Statistical Package for the Social Sciences software, version 20. A p value of < 0.05 was considered significant

RESULTS

Confirmation of *P. aeruginosa* isolates and their susceptibility to different antibiotic groups

A total of 180 isolates were confirmed as *P. aeruginosa* isolates. The isolates screened for their susceptibilities to different antibiotic groups that are commonly used to treat infections due to this organism by *in vitro* antibiotic cultural sensitivity assay. The results are presented in (Fig. 1 and 2, Table 1). *P. aeruginosa* strains (39.44%) were resistant to aztreonam & 31.11% to imipenem whereas low proportion of resistant strains was observed in case of piperacillin/tazobactum (10%) and levofloxacin (7.78%). Out of 180 isolates examined 27.22% were found resistant to three or more antibiotic groups and such isolates were regarded as multidrug resistant (MDR). On further adding the strains having intermediate resistance to antibiotics, this figure increased to 75 (41.67%). Further, 55% isolates were found to be XDR while only 1.11% isolates were found to be PDR. The pattern of occurrence of MDR, XDR and PDR in *P. aeruginosa* isolates has been shown through Fig. 3.

Phenotypic detection of ESBL producers

A total of 171/180 (95%) isolates of *P.*

aeruginosa were resistant to one or more third generation cephalosporins used in the screening test. Following DDST, ESBL as a mechanism of resistance was confirmed in 56/171 (32.75%) isolates. Majority isolates, 67.25% were non ESBL producers. In E test, only 30 DDST positive isolates were tested. Only 8 / 30 (26.67%) isolates were ESBL positive by this test. Out of 56 ESBL positive isolates, only 16 (28.57%) isolates were MDR while rest 40 out of 56 (71.43%) ESBL producers were sensitive to multiple drugs.

Table 1. Antibiotic Resistance pattern of *P. aeruginosa* isolates to different antibiotics

Antibiotics	Sensitive isolates	Resistant
Piperacillin /tazobactum	162 (90%)	5+13*=18 (10%)
Aztreonam	109 (60.56%)	58+13*=71 (39.44%)
Piperacillin	135 (75%)	16+29* = 45 (25%)
Cefepime	143 (79.44%)	25+12* = 37 (20.56%)
Gentamicin	126 (70%)	54+0* = 54 (30%)
Cefoperazone	123 (68.33%)	28+29* = 57 (31.67%)
Ciprofloxacin	109 (60.56%)	37+34* = 71 (39.44%)
Amikacin	149 (82.78%)	24+7* = 31 (17.22%)
Levofloxacin	166 (92.22%)	10+4* = 14 (7.78%)
Imipenem	124 (68.89%)	40+16* = 56 (31.11%)
Ceftazidime	121 (67.22%)	50+9* = 59 (32.78%)
Meropenem	155 (86.11%)	13+12* = 25 (13.89%)

* Intermediate resistance

Table 2. Comparative analysis of virulence factors in MDR and MDS isolates of *P. aeruginosa*

Virulence factors	MDR [n=30]			MDS [n=30]		
	W[+]	M[++]	S[+++]	W[+)]	M[++)]	S[+++]
Haemolysin	9	8	1	3	17	2
Protease	10	11	0	8	18	1
Gelatinase	12	13	2	4	20	1
Lipase	12	5	3	7	8	2
Biofilm						
a) Tube test	15	9	1	4	11	2
b) TCP method	25	5	0	28	1	0

W: weak; M: moderate; S: strong

Table 3. Values used in χ^2 calculation

Virulence factors	MDR (n=30)	MDS (n=30)
Haemolysin	18	22
Protease	21	27
Gelatinase	27	25
Lipase	20	17
Biofilm		
a) Tube test	25	17
b) TCP method	30	29

In vitro phenotypic expression of virulence factors of MDR and MDS isolates

Thirty out of 49 MDR isolates and 30 out of 131 sensitive isolates studied for six different virulence factors are presented in Table II, Fig IV. 27 MDR isolates (90%) showed the gelatinase activity while 25 MDS isolates (83.33%) exhibited gelatinase activity. 18 MDR isolates (60%) and 22 MDS isolates (73.33%) produced hemolysin as evidenced by hemolysis on blood agar. 21 MDR isolates (70%) and 27 MDS isolates (90%) were found to express protease activity while 20 MDR isolates (66.67%) and 17 MDS isolates (56.67%) possessed lipase activity. Biofilm as formation was observed in all the 30 MDR isolates examined and 29 MDS isolates (96.67%) by tissue culture plate (TCP) method while 25 (83.33%) MDR isolates and 17 (56.67%) MDS isolates showed biofilm (50.9%).[25] In another study conducted in Turkey, Tamurkaynak *et al* (2006), found colistin to be the most effective drug against MDR *P. aeruginosa in vitro*[25]. Dash *et al*; 2014 observed 77.7% strains resistant to ceftazidime, followed by cefepime 64.8%, piperacillin 45%, ciprofloxacin 38.9%, levofloxacin 36.1%, gentamicin 37.3% and amikacin 30%[18]. Similar resistance patterns of *P. aeruginosa* have been found by other workers in India[27,28]. However, this comparative evaluation between antimicrobials was impaired because there

production by tube test.

Statistically all MDR and MDS isolates had no significant difference towards all virulence factors studied by us (Table 3), significant difference was, however found for protease and biofilm formation as analysed by tube test (Table 4).

DISCUSSION

There is a tremendous increase in the prevalence of multi drug resistant *P. aeruginosa* strains in all parts of the world. Using the criterion of multidrug resistance (resistance to 3 or more groups of antimicrobials) to regard *P. aeruginosa* isolates as MDR, we recorded 27.22% clinical isolates as MDR in hospital setting at Shimla (H.P). These isolates were recovered during one year period (December, 2012 to December, 2013). Such criterion to regard an isolate as MDR has also been described by other workers[13,14]. However, some workers have used different criteria for regarding an isolate as multi drug resistant. Some authors define MDR isolate as the one which is resistant to two or more drugs or drug classes of therapeutic relevance[15,16] whereas others regarded those strains as MDR which were resistant to four or more classes of antimicrobial agents[17].

Our study revealed, 49/180 (27.22 %) *P. aeruginosa* isolates as resistant to multiple drugs, 55% (99/180) as XDR (extensively drug resistant) and 2/180 (1.11%) as PDR (pan drug resistant) isolates. Dash *et al.* (2014) from Odisha reported a high percentage (84.7%) of MDR *P. aeruginosa* strains and moderate percentage of (35.7%) XDR *P. aeruginosa* strains[18]. These workers did not observe any PDR isolate. Chauhan and Sharma (2013) recorded 69.50 % *P. aeruginosa* isolates as multidrug-resistant[19]. Mohanasoundaram *et al.* (2011) in Tamil Nadu, India reported the occurrence of 71% while Gill *et al.* (2011) in Rawalpindi, Pakistan recorded 22.7% isolates as MDR, while 11% and 4.3% were XDR and PDR, respectively[14,20]. Farhatullah *et al.* (2009) reported a frequency of 29% MDR, in the burn patients, in Peshawar[21]. A frequency of 14% MDR was found in Houston (US) by Tam *et al* in 2010 which is lower than our study[4]. In another study, Aloush *et al* (2006) have reported an incidence of 14 MDR strains of *P. aeruginosa* per 10,000 hospital admissions in Israel[22]. A study conducted in Japan, in 2008, revealed an incidence of 1.1% of MDR *P. aeruginosa* while, defining MDR *P. aeruginosa* isolates as those resistant to carbapenems, amikacin and fluoroquinolones[23].

The antimicrobial susceptibility profiles observed in this study revealed that 92.22% isolates of *P. aeruginosa* were sensitive to levofloxacin and 90% to piperacillin/tazobactum followed by 86.11% to meropenem, 82.78% to amikacin, 79.44% to cefepime, 75% to piperacillin, 70% to gentamicin, 68.89% to imipenem and 68.33% to cefoperazone. Lesser proportions of the isolates were susceptible to ceftazidime (67.22%), ciprofloxacin (60.56%) and aztreonam (60.56%).

Gul *et al* (2007) reported that more than 90% of isolates were sensitive to ciprofloxacin[24]. Amutha *et al* reported the highest resistance of *P. aeruginosa* strains to ampicillin (85%) followed by amikacin (62.2%), gentamicin (48%), imipenem (5%), meropenem (17%) and ciprofloxacin

Table 4. Virulence factors of MDR and MDS isolates of *P. aeruginosa* and associated χ^2 values with 1 degree of freedom. Yates correction applied

Virulence factors	MDR (n=30)	MDS (n=30)	χ^2 .	Significant at alpha 05	.01
Haemolysin	18	22	1.2	Yes	Yes
Protease	21	27	4.75	No	Yes
Gelatinase	27	25	0.576	Yes	Yes
Lipase	20	17	0.632	Yes	Yes
Biofilm					
a) Tube test	25	17	5.078	No	Yes
b)TCP method	30	29	1.016	Yes	Yes

was variation in the choice of antibiotic tested in different studies. A recent study conducted by Tam *et al* (2010) on MDR *P. aeruginosa* demonstrated the highest susceptibility to colistin (97%)[4]. However, 100% resistance to carbapenems and quinolones, 91% resistance against penicillins / cephalosporins and 21% against aminoglycosides was observed by these workers.

The prevalence of extended spectrum beta lactamases as a mechanism of resistance is on increase all around the world. In the present study, we observed 28.57% ESBL positive MDR *P. aeruginosa* isolates and rest 71.43% ESBL positive MDS isolates. ESBL producing strains are usually found in those areas of hospitals such as ICUs where use of antibiotics is frequent and the patient's condition is critical. In India, prevalence rate of ESBLs ranging from 28% to 84% has been reported from various parts of the country. Higher rate to the tune of 64% has been reported by Mathur *et al* (2002) from South India[12]. Bakshi *et al*., 2013, reported high prevalence of ESBL (50%) among *P. aeruginosa* at Patiala (Punjab)[29] and 20.27% from Haryana (Aggarwal *et al.*, 2008)[30].

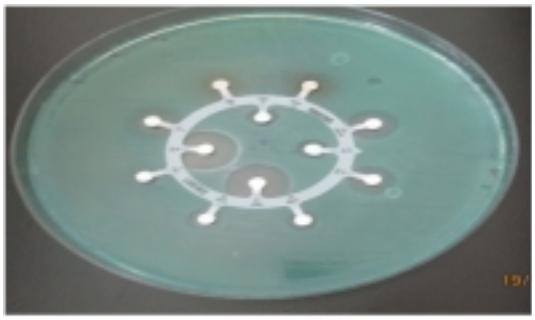

Fig.1. Isolate showing susceptibility to different antibiotics

The pathogenesis of *P. aeruginosa* is due to several virulence factors which are produced under certain environmental conditions. This organism produces several extracellular products that after colonization can cause extensive tissue damage, bloodstream invasion and dissemination. Proteases are assumed to play a major role during acute *P. aeruginosa* infection. The in vitro phenotypic expression performed in this study revealed production of all the six virulence factors by majority of isolates. It is well established that the bacteria growing in biofilms are more resistant to antimicrobial agents than their planktonic counterparts. The biofilm is composed of alginate and confers a mucoid consistency to *P. aeruginosa* isolates, acting as a protecting niche for the bacterium against the recognition of the immune system and the action of antibiotics. All these factors increase the possibility of chronic infections. We observed nearly all MDR isolates as biofilm producers and very few nonproducers. Deptula and Gospodarek (2010) observed even smaller frequencies, 9.3% multidrug susceptible

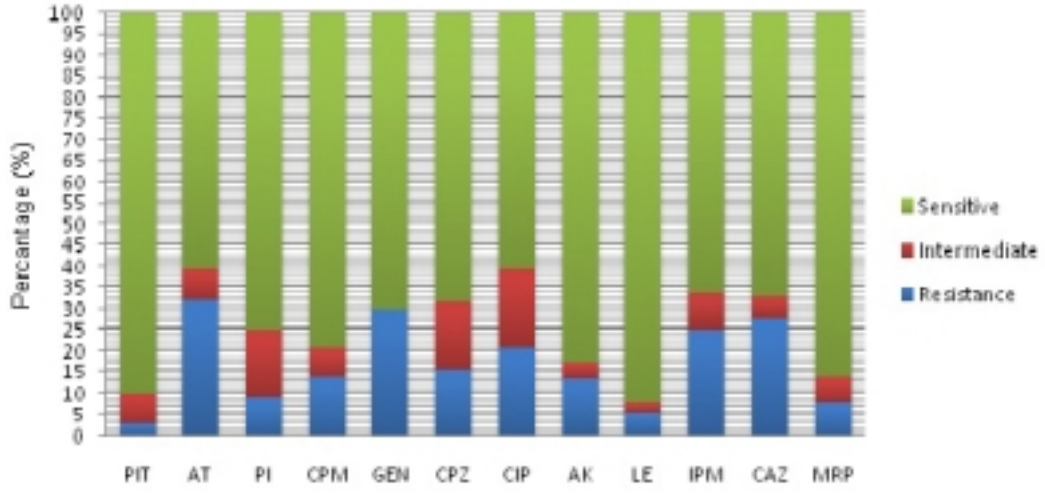

Fig. 2. Antimicrobial susceptibility profile of *P. aeruginosa* isolates; PIT: piperacillin/tazobactum, AT: azetronam, PI: piperacillin, CPM: cefepime, GEN: gentamicin, CIP: ciprofloxacin, LE: levofloxacin, AK: amikacin, IPM: imipenem, CAZ: ceftazidime, MRP: meropenem

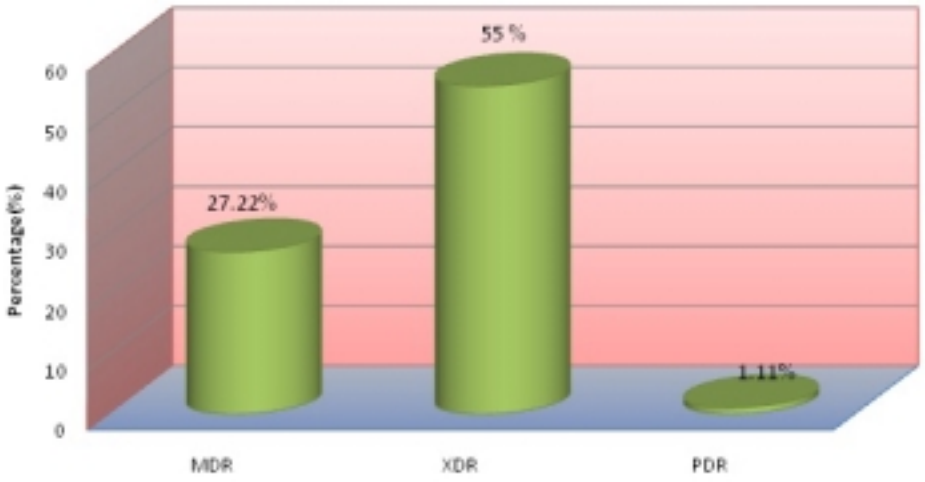

Fig. 3. Percentage of MDR, XDR and PDR *P. aeruginosa* isolates

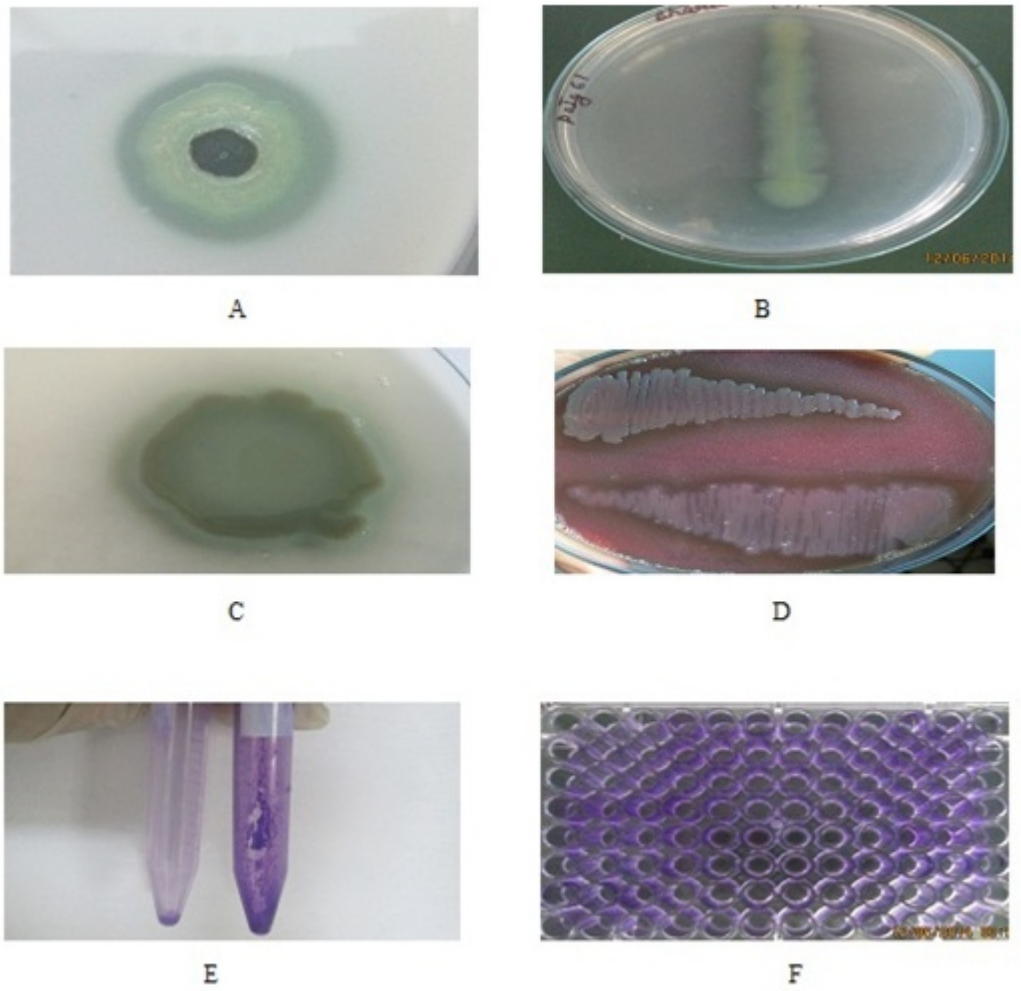

Fig. 4. Demostration of different virulence factors by in vitro tests: A- Protease test, B-Gelatinase test, C- Lipase test, D-Hemolysin test, E-Biofilm formation by tube test and by F-Tissue culture plate (TCP) method

and 8% multidrug resistant isolates as biofilm producers which is lesser as compared to our study[6].

We observed the production of hemolysin, which accounted for 60% of MDR isolates and 73.33% for MDS and production of gelatinase, which accounted for 90% of MDR isolates and 83.33% for MDS, corresponds to other virulence factors associated with tissue injury. Stehling *et al* (2008) compared the virulence traits of nonmucoid and mucoid isolates and did not observe any significant difference in the production of hemolysin and gelatinase. The average frequencies being 53.6% and 37.5%, respectively[31]. Jacome *et al* (2012) observed 93.4%, 72.1%, 34.4% strains having gelatinase and hemolysin, biofilm production activities respectively in *P. aeruginosa* isolates[32].

P. aeruginosa also produces some proteases (LasB elastase,LasA elastase and alkaline protease) which are able to destroy the protein elastin. This protein forms a bigger constituent of human lung tissue that is responsible for lung expansion and contraction. We observed 70% MDR isolates and 90% MDS isolates having a high proportion of protease activity. Similarly lipase as virulence factor was produced by 66.67% MDR and 56.67% MDS isolates respectively.

MDR and MDS (n=30) isolates have been statistically tested for comparative analysis of their virulence factors by using Chi $(\chi)^2$ test. All MDR and MDS isolates had no significant difference towards all virulence factors studied (Table 3), significant difference was, however found for protease and biofilm formation as analysed by tube test. Protease production was more in MDS isolates as compared to MDR isolates and biofilm formation (tube test) was more in MDR isolates as compared to MDS isolates (Table 4).

CONCLUSION

It may concluded from this study that *P. aeruginosa* can produce several virulence factors under *in vitro* conditions. These factors are often associated with emergence of multidrug resistant, extensive drug resistant and pan-resistant *isolates*, making the treatment of infections caused by this bacterium difficult. This study therefore emphasizes the need for proper and continuous surveillance of beta lactamase producing *P. aeruginosa* so that infection control measures in hospital settings can be implemented effectively.

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