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ORIGINAL ARTICLE

Clinical and Microbiological Characteristics of *Metallo β-lactamase* producing *Pseudomonas aueroginosa* in a sample of Egyptian Patients with Bronchiectasis

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ABSTRACT

Background*: Metallo-β-lactamases producing Pseudomonas aueroginosa represents a challenging concern especially in chronic colonization of bronchiectasis cases owing to progressive tissue damage, inflammation, frequent exacerbation and lack of response to antimicrobial agents.* ***Objectives:*** *This cross-sectional study was conducted to high-lighten the antimicrobial and molecular pattern of Pseudomonas aueroginosa isolates from bronchiectasis cases and assess their clinical outcome.* ***Methodology:*** *ninety-eight sputum and endotracheal aspirates were collected from bronchiectasis inpatients of Chest Department, Zagazig University Hospitals. Conventional identification and API20 NE were performed. Combined disc test and E-test were done, confirmed by PCR, to detect metallo-β-lactamases.* ***Results****: A total of 43 (43.9%) Pseudomonas were isolated, 16 (37.2%) were metallo β-lactamases and multidrug resistant. E. test had the best sensitivity and specificity. Both IMP-1 and VIM-1 genes were detected. The majority of isolates were IPM-1 positive.* ***Conclusion****: P. aeruginosa, is blamable for infection in more than 40% of bronchiectactic patients being a high-risk group. Combined disk test is a reliable, cheap and simple screening tool especially in low-resources laboratories. This study raised the concern of co-existing mechanism for both quinolone and carbapenem resistance for more investigation. Current results linked lab detection of MβL P. aeruginosa with inferior spirometry and lengthy hospital stay.*

INTRODUCTION

Bronchiectasis is a persistent and progressive condition characterized by dilated and thick-walled bronchi. The lower airways in patients with bronchiectasis are often colonized with pathogenic microorganisms in particular with *P. aeruginosa*, associated with pulmonary infections and secretion of inflammatory mediators that can lead to progressive tissue damage and airway obstruction leading to frequent exacerbations of bacterial infections and decreased pulmonary function [1,2].

Bronchiectasis is a vicious cycle of bacterial colonization, airway inflammation and airway structural damage. Inflammation in bronchiectasis is predominantly neutrophil driven under chemotactic action of interleukin-8, tumour necrosis factor-α and interleukin-1β however, persistent bacterial infection ensues evading phagocytosis adapting to chronic infection [3,4].

The Prevalence of bronchiectasis in England and Wales among all age groups has been increased in females from 21.2/100,000 person-years in 2004 to 35.2/100,000 person-years in 2013, and in males from 18.2/100,000 person-years in 2004 to 26.9/100,000 person-years in 2013 [5].

Pseudomonas aeruginosa producing metallo-beta-lactamase (MβL) has emerged as an alarming sign due to its multi-drug resistance pattern, compromising the use of carbapenem as the last β-lactams reserve for control of bacterial infections [6].

Metallo-β-lactamases (MβLs) are the most prevalent acquired carbapenemases enzymes. Particularly, the IMP- and VIM-type enzymes exhibit a worldwide distribution.[7] Moreover, SPM and GIM, have been reported in *P. aeruginosa* isolated from Brazil and Germany. [8]

Metallo-β-lactamases E test MBL strip, based on a combination of a β-lactam substrate and a β-lactam/metallo-β-lactamase inhibitor, was specifically designed to detect as many clinically relevant metallo-β-lactamases as possible[9].

Molecular detection of metallo-β–lactamase genes carried out by PCR including imipenemase (IMP) gene and Verona imipenemase (VIM) gene being the widely distributed world-wide. However, many other genes have been identified in *P. aeruginosa*in certain localities [10]. Objectives of this study were to underscore the antimicrobial and molecular pattern of *P. aeruginosa* isolates from bronchiectasis cases and to assess their clinical outcome.

METHODOLOGY

Objectives:

This research was a two-year cross-sectional study conducted on inpatients in Chest Department and Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University.

An informed written consent was obtained from all subjects included as stated by approval of the Institutional Review Board of Zagazig University Hospitals.

Current study included 98 inpatients complaining of exacerbation of bronchiectasis which was defined (according to British Thoracic Society 2010) as either a change in one or more of the common symptoms of bronchiectasis (increasing sputum volume or purulence, worsening dyspnea, increased cough, declining lung function, increased fatigue/malaise) or appearance of new symptoms (fever, pleurisy, requirement for antibiotic treatment) [11]. The demographic data is shown in table (1)

Table 1: Demographic data of the studied patients.

Parameter	*Patients no = 98*
Age (yrs) (M±SD)	25 -76 (51.56 ±15 years)
Sex:	
Male (No,%)	41(42%)
Female (No,%)	57(58%)
Etiology:	
Post infectious (No.,%)	25(26%)
COPD bronchiectasis (No.,%)	39(40%)
Miscellaneous known causes (No.,%)	16(16%)
Idiopathic (No.,%)	18(18%)
No. exacerbation/year (%)	
<3	51(52 %)
≥3	47(48 %)

Exclusion criteria were: acute on top of chronic respiratory failure, hemoptysis, heart failure, systemic corticosteroids and/ or antibiotics intake in the previous 4 weeks, active tuberculosis, interstitial lung diseases (clinical and radiological diagnosis).

All patients were subjected to thorough medical history with assessment of dyspnea by modified Medical Research Council (mMRC) dyspnea scale, clinical examination (general and radiography (plain chest-X ray posteroanterior view and high resolution chest computerized tomography with contrast), spirometry [Forced expiratory volume in 1st second (FEV1), Forced vital capacity(FVC), Forced expiratory volume in 1st second / Forced vital capacity (FEV1/FVC) percentage of predicted] after withdrawal of Short-acting bronchodilators for at least 6 hours arterial blood gases analysis, bronchoalveolar lavage with fibro optic bronchoscopy.

Specimens Collection:

Sputum and bronchoalveolar lavage (BAL) specimens (mostly after chest physiotherapy) were collected from all patients. Specimens were subjected to direct Gram stained films, followed by Zeihl Neelsen stain to exclude MTB. Culture on nutrient agar and blood agar was performed, *Pseudomonas spp* were recovered from culture plates.

Bacterial strains: *Pseudomonas* isolates were identified by conventional methods (oxidase test) confirmed by API 20 NE (bioMerieuxMarcy l'Etoile, France) [12].

Antibiotic susceptibility testing using Mueller Hinton (MH) agar plates was performed. The following antimicrobials were selected for *Pseudomonas* isolates fulfilling the range of treatment in clinical practice. Aztreonam (30), Ceftazidime (30), Cefotaxime (30), Ceftriaxone (30) Cefepime (30), Ertapenem (10) and Imipenem (10). Doxacycline (30), Ticarcillin/clavulanic acid (75/10), piperacillin/tazobactam (100/10), amikacin (30), gentamicin (120), ciprofloxacin (5) and gatifloxacin (30) (Oxoid) [13].

Metallo-β-lactamases *MBL Phenotypic detection:*

Imipenem EDTA (IPM–EDTA) combined disk test *was selected for detection of MBL production (according to Franklin et al., 2006).* Two IPM disks (10µg), the first one contains 10 µl of 0.1 M (292 µg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO), were placed 25 mm apart (center to center) in a single agar plate. An increase in zone diameter of >4 mm around the IPM-EDTA disk compared to that of the IPM disk alone was reflected as being positive for an MBL [14].

E-test MBL: (AB Biodisk), Colonies were picked from 18-h plates and suspended in saline solution to a turbidity of a 0.5 McFarland standard, as stated in the manufacturer's instructions.

PCR analysis: was performed for confirmation of MBLs detected by phenotypic tests using IPM-1 and VIM-A metallo-β-lactamases

DNA was extracted from overnight cultures using the QIAnhamp DNA Mini Kit (QIAGEN, USA), according to the manufacturer's instructions. DNA was quantified following the instructions and recommendations stated in[15].

The following primers were used:

IMP-F 5′-GGAATAGAGTGGCTTAAYTCTC-3′

IMP-R 5-GGTTTAAYAAAACAACCACC-3′

detected at 232 bp.

VIM-F 5′-GATGGTGTTTGGTCGCATA-3′ VIM-R 5'-CGAATGCGCAGCACCAG-3', detected at 390 bp. Thermal cycling conditions were as follows 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min lasting for 30 cycles. Amplicons were analyzed on agarose gels (1.5%) with appropriate DNA markers.

RESULTS

Out of 98 specimens collected, a total of 43 non-duplicate *Pseudomonas* was isolated representing 43.9%.

MDR isolates acquired non-susceptibility to at least one agent in three or more antimicrobial categories as follows [16]: anti-pseudomonal cephalosporins, carbapenem, aminoglycosides, tetracyclines and/or fluoroquinolones

Eight strains were metallo β-lactamase, ciprofloxacin resistant and aminoglycosides resistant. Six strains were metallo β-lactamase, ciprofloxacin resistant and β-lactam-β-lactamase combination-resistant. Two strains were metallo β-lactamase, ciprofloxacin resistant and doxacycline resistant.

Regarding Metallo β-lactamase production (table 2) by combined disk test: 18 strains were positive out of 43 (41.8%). By E test: 16 isolates were positive out of 43 (37.2%). PCR revealed: 16 (37.2%) were positive for metallo β-lactamase genes, 8 isolates had IMP genes alone, 5 had VIM gene and 3 strains harbored both genes.

Table 2: Different lab methods for detection of *MβL-P. aueroginosa*

Test \ *No.*	*MBL*
CDT	18
E-test	16
PCR genes	
IMP-1 alone	8 (50%)
VIM-1 alone	5 (31.25%)
Both IMP-1&VIM-1	3 (18.75%)

Besides, metallo β-lactamase infected patients have more complications in the form of hemoptysis, pneumonia, chronic respiratory failure, corpulmonale), prolonged length of hospital stay > 7 days and deteriorated breathlessness scored by mMRC dyspnea scale with statistical significant difference($p<0.05$). The results are shown in table (3)

Table 3: Clinical outcome of studied patients.

	MβL (16)	*Non-MβL (27)*	*P value*
Dyspnea scale (mMRC)	2.9 ±0.6	2.1 ±0.7	<0.05*
Associated complications	14	12	<0.01**
Length of hospital stay	1	20	<0.001**
< 7 days	15	7	
> 7 days			
Prior antibiotic use	16	20	<0.05*
Ciprofloxacin intake	16	9	<0.001**

Regarding spirometry; *MβL* producing *Pseudomonas aueroginosa* group showed a significant worsening of *FEVI, FVC* and *FEV1/FVC* than non-*MβL* producers with statistical difference *($p<0.05$).* (Table 4)

Table 4: Assessment of pulmonary function tests

Spirometry	*MβL (No= 16)*	*Non- MβL (No= 27)*	*P- value*
FEV1 (% pred.)	62.9±18.5	71.7±15.3	<0.05*
FVC (% pred.)	79.3±22.5	83.9±18.5	>0.05
FEV1/FVC (%)	74.7±11.8	78.2±12.4	>0.05

DISCUSSION

Bronchiectasis is a disorder of persistent lung inflammation and recurrent infection, ended by irreversible bronchial dilatation. An interplay between immunogenetic susceptibility, immune dysregulation, bacterial infection, and lung damage occurs. The damaged epithelium impairs mucus removal, eases bacterial infection with increased cough, sputum production, and airflow obstruction. These patients are vulnerable to bacterial and fungal pathogens and exposed to heavy antibiotic treatment. The capability of *P. aeruginosa* survival in the chronically infected lung is often associated with an increased resistance to antimicrobial agents [17,18].

Two decades ago, Wilson addressed that *P. aeruginosa* bronchiectactic patients have poorer lung function and worse quality of life [19].

This study was designed to underscore the antimicrobial and molecular pattern of *P. aeruginosa* isolates from bronchiectasis cases and assess their clinical outcome. Nevertheless, during interpretation of the results, a lack of detailed research work discussing some microbiology lab procedures in the literature, that enforced the authors to compare current microbiologic results with *Pseudomonas* isolates from other patients' groups.

Current study revealed that *P. aeruginosa* represented 43.9% of all isolates recovered from bronchiectasis cases that seems to be the highest percent recorded compared with other studies on bronchiectasis patients (28%) [20].

The most striking point denoted in antibiotic susceptibility results that all IPM-resistant isolates were multidrug resistant which were at the same time resistant to ciprofloxacin. This observation was demonstrated by other researchers in their separate research studies that all their carbapenem resistant *P. auroginosa* and other non-fermentative Gram-negative bacilli isolates were likewise resistant to fluoroquinolones [21-23].

But then again, this observation still in need of additional investigations to underpin if there is a shared molecular mechanism of gene transfer. A high statistical

significant association was observed between *MβLs* and ciprofloxacin intake P <0.001 (table 3)

In the meantime, overexpression of efflux pumps that expel carbapenems, possibly will proceed to carbapenem resistance linked with multidrug resistance (MDR), since quinolones, penicillins, cephalosporins and aminoglycosides are common efflux pump substrates [24].

In current study, PCR was set as a gold standard for detection of *metallo β-lactamases.* Eighteen of our isolates (41.8%) were *MβLs* by combined disc method. With the best Sensitivity 100% and a specificity of 93.1 in comparison with E-test *MβL* and PCR. This is in line with results of an earlier study, comparing combined-disk test with a sensitivity of 100% and a specificity of 98% [14]. Whereas the double disc synergy test DDST had a sensitivity of only 79% and a specificity of 98%. Current results potentiate that of another study to recommend combined-disk CDT being satisfactory for screening even though its low specificity because of an easiness and simplicity [25]. However other researchers argued EDTA-based MβL detection methods for being non-specific with high false-positive rate [26].

To overcome the great debate about the accuracy of cheap phenotypic methods; CDT and DDST both are restricted by several elements as temperature, aeration, pH and thickness of media. Meanwhile, the synergy between imipenem and imipenem+EDTA disks are inclined by diffusion. EDTA must diffuse close to the imipenem disk and achieve a concentration with effective chelating activity to demonstrate a synergy. This may explain the difference in results of CDT and DDST [27].

Current results verified the value-added regarding the use of *MβL*-E-test with high sensitivity (100%) and specificity (100%). That can qualitatively and quantitively detect *MβL* production, predicting treatment outcome.

Diverse studies acknowledged the use of Etest *MβL* as being a highly sensitive and specific test for detecting *IMP*-1and *VIM-2* allele-positive isolates of *Pseudomonas spp.*[9,28].

It is worth mentioning that, 16 (37.2%) of our isolates were positive for *MβL* production by both E-test and molecular methods. Half of these isolates harbored IMP-1 genes, 5 (31.25%) were *VIM*-1gene positive and the remaining isolates had both genes.

A previous Egyptian study detected *MβL* in 54.8% of their isolates from healthcare infections in Ismalia, 2016 [29]. However, the first Egyptian study reported the appearance of first *MβL* in Egypt, in 2014 [30].

Several studies were done world-wide for detection of *MβLPsudomonas,*in Saudi Arabia diverse genes were found, reportingthe most prevalent extended-spectrum *β*-lactamase and penicillinase: *Vietnamese extended-spectrum β-lactamase (VEB-1)* gene in 16/34 and *oxacillinase OXA-10* gene in 14/34[31]. In Iran, 42.85% of isolates were positive for *MBLIMP-1* and *VIM*-1 gene in two educational hospitals[32]. In addition to a study in Uganda, 33 % of *P. aeruginosa were* carbapenem-resistant harbored the following genes, *IMP-1, VIM-1, SPM 1 and NDM 1 in with IMP-1* responsible for more than one third of *MBL. VIM-1, VIM-2, VIM-4, VIM-11*, and *VIM-28* genes from different clinical samples[33]. Other M*β*Ls *VIM-15 and VIM-16* genes were detected in Bulgaria and Germany validating the high spotting the ongoing evolution and dissemination of this group of β-lactamases[34]. Meanwhile, *IMP-18* and *VIM-2* encoded metallo-*β*-lactamases in *P. aeruginosa* were recorded in Costa Rica[35]. Furthermore, *P. aeruginosa* isolates carrying both *VIM*-2 and *VIM-11*genes have been described in Mexico [36].

In the present study, *MβL P. aueroginosa* infected patients had inferior spirometry (FEV1, FVC and FEV1/FVC), in addition to worsening in dyspnea (lower mMRC dyspnea scale) with more complications than non-*MβL-P. aueroginosa* with statistically significant difference (p<0.05). Earlier studies concluded that *P. aueroginosa* has a biofilm forming capacity in airways that marks it challenging to be eliminated by antibiotics or immune system [37].

In addition to *P.aueroginosa* causes airway destruction that indirectly reflected by lung function impairments. This was partially concordant with the findings of other studies [38-40] that confirmed both worse spirometry and diffusing capacity in patients chronically colonized with *P. aueroginosa* had. Moreover, a higher residual volume than others whom intermittently or non-infected with *P. aueroginosa*. As well, BTS 2010 proved that colonization with *P. aueroginosa* is associated with reduced FEV1 which is correlated with breathlessness as assessed by the MRC dyspnoea score and extent of disease on high resolution computed tomography (HRCT) [11].

In the present study, bronchiectasis patients infected with *MβL-P. aueroginosa* associated with more complications and prolonged hospital stay more than 7 days (p <0.05). A similar observation was verified that *P. aeruginosa* had a significantly higher number of exacerbations (p= 0.008), greater number of hospital admissions (p= 0.007), a prolonged hospital stays (p < 0.05)[41]. Beside that *P.aeruginosa* infection is associated with significantly longer length of hospital stay (was seen to be 5 or more days) in COPD patients [42].

CONCLUSION

Current study high-lightened the problem of *P. aeruginosa*, blamable for infection in more than 40% of bronchiectactic patients being a high-risk group. The authors compared different lab techniques for accurate diagnosis of *MβL P. aeruginosa* and recommended combined disk test as a reliable, cheap and simple screening tool especially in low-resources laboratories.

MβL-P. aueroginosa accounted for 37.2% of isolates, denoting a warning sign for wise selection of antimicrobials.This study raised the concern of co-existing mechanism for both quinolone and carbapenem resistance for more investigation. *IMP*-1 gene was detected in half of isolates, 5 (31.25%) were *VIM*-1gene positive and the remaining isolates had both genes Current results linked lab detection with inferior spirometry of *MβL P. aueroginosa* infected patients and lengthy hospital stay with statistically significant difference.

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