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# Molecular characterization of *Acinetobacter baumannii* isolated from MDR-TB patients of Northern Punjab, Pakistan

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## Abstract

Respiratory tract infections (RTIs) are the most common and severe infectious diseases in developing countries. *Acinetobacter baumannii* is the bacterium known as causative organism for respiratory tract infections in human populations. The Report suggests that co-infection of *Acinetobacter baumannii* with *Mycobacterium tuberculosis* together increases health complications in multidrug resistant tuberculosis (MDR-TB) positive patients and creates fatal damage to the ailing population. In our study, 106 sputum samples of MDR-TB positive patients from Northern Punjab were studied. The isolation of *A. baumannii* from sputum of MDR-TB patients was done on selective media and initially screened by Oxidase and Catalase based identification followed by microscopic examination. Afterward, only ten suspected isolates of *A. baumannii* were again selected for further characterization for MDR by using Disc diffusion method. Antibiograms against number of antibiotics were accurately determined. Of these 10 isolates, 8 sample were found resistant to levofloxacin and subjected to molecular characterization using *bla-OXA-51* primers. Only 3 out of 106 (2.83 %) isolates were confirmed as MDR strains of *A. baumannii*. These results show the coexistence of MDR *A. bauminnii* with MDR-TB patients of Northern Punjab, Pakistan. In Northern Punjab regions a higher percentage (3 cases) of MDR- TB were detected, which were co-infected with *Acinetobacter baumannii* among hospitalized patients. These findings may show unhygienic hospital environment or practices which leads to the co-infection.

## Introduction

According to physicians, the most common and severe infections are respiratory tract infections (RTIs) [1]. It has been revealed that the risk of RTIs declines with increasing age. Generally, the incidence of RTIs increases at a certain age in the subject group, i.e. smoking. There are two forms of RTIs, lower respiratory tract infection (LRTI) and upper respiratory tract infection (URTI) [2]. Lower respiratory tract infection may be caused by different microbes, including *Escherichia coli, Acinetobacter baumannii, Klebsiella pneumonia, Haemophillus influenza, Streptococcus pneumonia, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Staphylococcus aureus* [3]. *Acinetobacter baumannii* is one of the most important human pathogens [4]. In nature *A. baumannii* can easily be isolated from different sources; mostly in water and soil [5]. It can also be found in healthcare environments and sputum of infected patients. It can disseminate in of several ways, but the most common form of its dispersion is the hospital staff [6] [7]. The bacterium can be isolated from sputum samples using MacConkey Agar, Tryptic Soya Agar (TSA), and Sheep Blood Agar. It is a non-fermenting, non-spore forming and gram-negative *coccobacilli*. *OXA-51* gene identification has been used for the molecular confirmation of *A. baumannii* [8].

Over the last 15 years, its clinical significance has increased due to its ability to rapidly acquire resistance against antibiotics [9]. In humans, most of the intensive care units (ICUs), it is abundantly present in the normal flora of the skin and mucous membrane and causes different types of infections including urinary tract infection, upper respiratory tract infections, ventilator-associated pneumonia, meningitis and septicemia [10] , [11].

*A. baumannii* resists environmental as well as chemical stress including UV rays, disinfectants, detergents and dehydration [12]. This resistance has made *A. baumannii* a dominant nosocomial pathogen [13]. The risk of its colonization increases during ICU stays [14].

The health care workers should have high-quality training in all hospital departments to prevent further dispersion of *A. baumannii* infections to others [15]. Pneumonia caused by MDR strains of *A. baumannii* has become a severe health issue. Risk factor for two types of patients recognized to pneumonia infection *Acinetobacter* hospital-associated pneumonia (AHAP) and *Acinetobacter* ventilator-associated pneumonia (AVAP), by univariate analysis before ceftazidime treatment, it is to be reported that potential risk factors for AVAP include imipenem resistance, long duration of hospital stay and fluoroquinolones exposure [16], [17].

*Acinetobacter baumannii* may be isolated from the sputum of 1-10% known cases of MDR TB patients [18]. *Mycobacterium tuberculosis* is the second leading infectious agent after HIV that causes AIDS. TB cases began to rise worldwide, and in 1993 WHO declared the first time that TB was a global emergency. In 2015 according to a survey, about 1.8 million people died, and 1.4 million fell ill with TB [19]. In ICU, there is 60% mortality due to pulmonary tuberculosis and 25 % mortality due to Acinetobacter hospital-associated pneumonia (AHAP) [20] .

The objective of this research was to confirm clinically diagnosed MDR TB cases with GeneXpert and to screen these cases for co-infection with *A. baumannii* by traditional methods and the presence of the *OXA-51* gene.

## Materials and Methods

### Bacterial Isolation

This research was performed to evaluate the rate of occurrence and co-infection of *Acinetobacter baumannii* in the sputum of MDR-TB Patients of Northern Punjab in 2018. A total of 106 sputum samples were collected from MDR-TB patients of both genders (male and female) of different age groups, in 15 ml falcon tubes from three major cities of Punjab Lahore, Faisalabad and Sargodha. These samples were labeled correctly and stored at 4°C before processing. MacConkey agar and Tryptic soy agar were used for the cultivation of *Acinetobacter baumannii*. Both media were prepared according to manufacturer's instructions with the addition of 2.2 ml gentamycin (0.4g in 50 ml NS) per 1 liter of medium to make the medium selective for gentamycin resistance before use in each case. Pale yellow to greyish, smooth, and little mucoid colonies on these media, indicative of *Acinetobacter baumannii* were selected. Oxidase production and citrate utilization tests were used for biochemical identification. Christian Gram's Staining was used to stain only those positively cultured plates that were gentamycin resistant, oxidase negative and catalase positive [9].

### Antimicrobial sensitivity test

Antimicrobial susceptibility tests (AST) were performed to demonstrate that *Acinetobacter baumannii* isolated from the sputum of MDR-TB patients were also MDR *Acinetobacter baumannii*. AST was performed by Kirby's disc diffusion method using discs of antimicrobials ampicillin, colistin, erythromycin, levofloxacin, clarithromycin, augmentin and gentamycin.

### Molecular Characterization

The Gene sequence of the *OXA-51* gene was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). The sequence was employed to design the primer set for the *OXA-51* gene using Primer3 software (http://simgene.com/primer3). The sequence of primers is given in **Table 1**.

**Table 1:** List of primers For PCR for detection of *bla*-OXA-51 gene

| Sr. # | Primer name | Primer sequence (5´ to 3´) | Size (bp) |
|---|---|---|---|
| 1 | OXA-51 F | TAATGCTTTGATCGGCCTTG | 353 |
| 2 | OXA-51 R | TGGATTGCACTTCATCTTGG | |

**Note:** F=Forward, R=Reverse, OXA=Oxacillinase, bp=base pair``

### Polymerase Chain Reaction

DNA was extracted by using the boiling method [8]. A 25 µL reaction mixture consisted of 7.5 µL of deionized water, 2 µL of extracted DNA, 1.5 µL of forward and 1.5 µL of reverse primer then added 12.5 µL of Green Taq PCR Master Mix (QIAGEN). Thermal cycler conditions were: initial denaturation at 95°C centigrade for 3 minutes followed by 35 cycles with denaturation at 94°C centigrade for 45 seconds, annealing at 54°C centigrade for 45 seconds, extension at 72°C centigrade for 1 minute, and a final extension at 72°C centigrade for 5 minutes. The PCR products were electrophoresed at 110 V for 30 minutes using 1.2% agarose gel.

## Results

### Isolation and identification

Within 24 to 48 hours after inoculation on TSA or MacConkey Agar, 35 out of 106 sputum samples gave positive growth on Petri plate which were proceeded for *Acinetobacter baumannii* identification using biochemical and microscopic examination followed by DNA based confirmation using qPCR (**Fig. 1**). A total of 15 out of total suspected 35 samples which were grown on Petri plates were tested gram-negative by using Christian's Gram staining, oxidase-negative and catalase-positive non motile coccobacilli (**Table 2**).

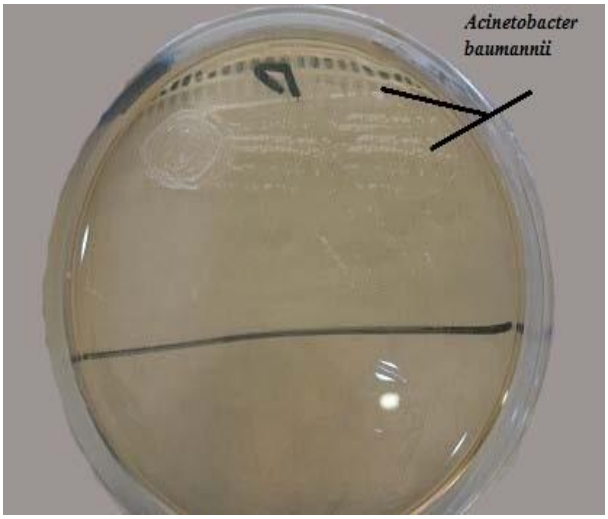


**Fig. 1:** *A. baumannii* cultured Petri plate showing mucoid growth, positively isolated from MDR-TB Patients Samples.

### Antimicrobial susceptibility Test (AST)

AST analysis by the disc diffusion method using AMP (15µg), Col (25µg), Ery (15µg), Cla (15µg), Lev (5µg), Aug (30µg), Gen (10µg) was done. Only 8 out of 10 positively selected samples were selected on the base of resistance to levofloxacin (**Table 3**) and (**Fig. 2**) because MDR *Acinetobacter baumannii* isolates are Levofloxacin resistant.

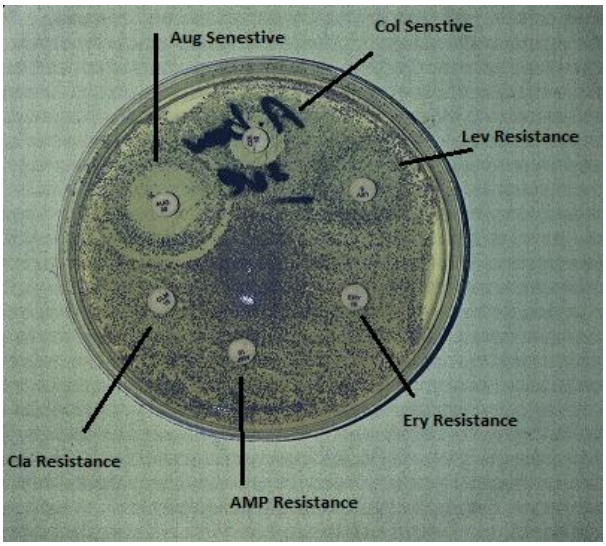


**Fig. 2:** AST showing antimicrobial resistance and sensitivity using disc diffusion method.

**Table 2:**15 out of 106 samples were screened as positive growth (Oxidase and Catalase specificity) and these were further processed using Gram's staining and only 10 were isolated as *A. baumannii* suspects that

| Sr # | Sample ID | Age | Gender | City | Oxidase | Catalase | Gram's Stain | Microscopy |
|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 35 | F | FSD | Negative | Positive | Positive | Cocci |
| 2 | 4 | 47 | M | FSD | Negative | Positive | Negative | Coccobacilli |
| 3 | 5 | 28 | F | FSD | Negative | Positive | Negative | Bacilli |
| 4 | 9 | 48 | F | FSD | Negative | Positive | Positive | Coccobacilli |
| 5 | 19 | 18 | F | LHR | Negative | Positive | Negative | Bacilli (Rod) |
| 6 | 35 | 20 | F | LHR | Negative | Positive | Negative | Coccobacilli |
| 7 | 36 | 20 | F | LHR | Negative | Positive | Positive | Coccobacilli |
| 8 | 61 | 63 | M | SRD | Negative | Positive | Negative | Coccobacilli |
| 9 | 76 | 60 | F | SRD | Negative | Positive | Negative | Coccobacilli |
| 10 | 90 | 20 | M | SRD | Negative | Positive | Negative | Coccobacilli |
| 11 | 93 | 24 | M | SRD | Negative | Positive | Negative | Coccobacilli |
| 12 | 98 | 50 | F | SRD | Negative | Positive | Negative | Bacilli (Rod) |
| 13 | 103 | 25 | F | SRD | Negative | Positive | Negative | Coccobacilli |
| 14 | 105 | 35 | M | SRD | Negative | Positive | Negative | Coccobacilli |
| 15 | 106 | 26 | F | LHR | Negative | Positive | Negative | Coccobacilli |

**Note:** Meet the criteria with Oxidase negative, Catalase positive and Gram's negative coccobacilli.
SRD=Sargodha, LHR= Lahore, FSD=Faisalabad, M=Male, F=Female

**Table 3:** AST analysis by disc diffusion method (using number of antibiotic discs) was done and only 8 out of 10 suspects were screened on the base of resistance to levofloxacin.

| Sr. # | Sample | Amp | Col | Ery | Cla | Lev | Aug | Gen |
|---|---|---|---|---|---|---|---|---|
| 1 | 4 | R | S (13mm) | R | R | R | S (25mm) | R |
| 2 | 61 | S (40mm) | R | R | R | R | S (45mm) | R |
| 3 | 76 | S (40mm) | S (40mm) | R | R | R | S (35mm) | R |
| 4 | 90 | R | R | R | R | R | S (28mm) | R |
| 5 | 93 | R | R | R | R | R | R | R |
| 6 | 103 | R | R | R | R | R | R | R |
| 7 | 105 | R | R | R | R | R | R | R |
| 8 | 106 | S (35mm) | S (20mm) | R | S (20mm) | R | S (45mm) | R |

**Note:** Amp=ampicillin, Col= Colistin, Ery= Erythromycin, Cla= Clarithromycin, Lev= levofloxacin, Aug= Augmentin, Gen= Gentamycin, R= Resistant, S= Sensitive

### PCR Amplification

The appearance of the beta lactamase *bla-OXA-51* gene with 353bp amplicon size was detected in three samples, 4, 90 and 106 (**Fig. 3**).

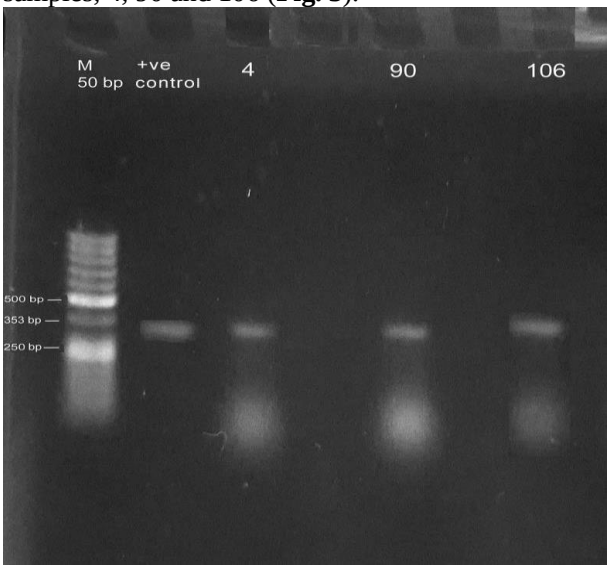


**Fig. 3:** Ethidium Bromide stained gel showing amplicons of *bla*-OXA 51 (353 bp).

## Discussion

This study focused on the isolation of *A. baumannii* from MDR-TB patients of Northern Punjab Pakistan. It is a novel study as no reference is found in the literature with similar work plan in Pakistan. In Germany, some researchers worked (in 2006) on hospitalized TB patients to find the spectrum of other respiratory bacterial pathogens. They isolated Acinetobacter species in 5.17% cases. In comparison, we found only a 2.83% occurrence of *Acinetobacter baumannii* in MDR-TB patients [20]. There are various reasons for this discrepancy. The referred report focused only on hospitalized cases of ICU, whereas in this study both hospitalized and community-associated MDR-TB patients were included.

In 2006 Erbes and his researchers isolated 5.1% included all *Acinetobacter* species, whereas our number of 2.83% solely represented *Acinetobacter baumannii.* All *Acinetobacter baumannii* isolates were MDR (multidrug-resistant), showing resistance

to levofloxacin, ampicillin, colistin, erythromycin, gentamycin, clarithromycin and amoxicillin by disc diffusion method [21]. These results are consistent with those reported by Shazly and his team in 2015, who reported all US isolates of *Acinetobacter baumannii* as MDR [22].

Finally, in this study, OXA-51 primer instead of primers for housekeeping genes were used for molecular confirmation of *A. baumannii* isolates. The *OXA-51* gene is present only in MDR strains of *A. baumannii*.

## Conclusion

In this study, the *Acinetobacter baumannii* was isolated from known cases of clinical MDR-TB patients of Northern Punjab. Amplification of the *OXA-51* gene by PCR was found in 2.83% isolates showing co-infection of *Acinetobacter baumannii* with *Mycobacterium tuberculosis* in MDR-TB hospitalized patients. All the *Acinetobacter baumannii* were MDR strains because the *OXA-51* gene is only present in MDR strains.

## Abbreviations

MDR, Multi Drug Resistant; TB, Tuberculosis; WHO, World health organization; RTI, respiratory tract infection; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection; ICU, intensive care unit; AHAP, *Acinetobacter* hospital-associated pneumonia; AVAP, *Acinetobacter* ventilator-associated pneumonia; TSA, Tryptic soya agar; NS, normal saline; PCR, polymerase chain reaction; bla, beta lactamase; OXA, Oxacillinase; AST, antimicrobial susceptibility resting; M; Male, F; Female, FSD; Faisalabad; LHR, Lahore; SRD, Sargodha; Amp, ampicillin; Col, Colistin; Ery, Erythromycin; Cla, Clarithromycin; Lev, levofloxacin; Aug, Augmentin; Gen, Gentamycin; R, Resistant; S, Sensitive.

## Conflict of interest

The authors declare no conflict of interest.

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