Original Article

Antimicrobial Susceptibility Pattern and In-vitro Biofilm Forming Abilities of Potential Pathogens Isolated from Inner Lumen of Endotracheal Tubes of Patients Admitted in Critical Care Units

PK Khatri[1], Vikrant Negi[2], Namit Mathur[3], Naveen Kishoria[4]

[1]Senior Professor, [2]PhD Scholar, Department of Microbiology; [3]Assistant Professor, [4]Senior Professor, Department of Medicine, Dr SN Medical College and Associated Group of Hospitals, Jodhpur, Rajasthan, India

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ABSTRACT

Introduction: The potential pathogens of ventilator associated pneumonia (VAP) have the ability to form biofilm within the endotracheal (ET) tube inserted in a patient. These pathogens can lead to onset of VAP once they are flushed into the lungs during mechanical ventilation.

Methodology: A prospective study was conducted where ET tubes were collected from the patients in ICU after more than 48 hours of intubation. ET tube was cut into sections and inner lumen was scrapped. The material was inoculated on 5% sheep blood agar and MacConkey agar. The isolated pathogens were tested for identification and antibiotic susceptibility testing. Bacterial isolates were also tested for in-vitro biofilm formation in microtiter plate by 1% crystal violet assay.

Results: Among the 100 ET tubes, 93 microorganisms were isolated which include 79 (84.9%) gram negative bacilli (GNB), 10 (10.8%) gram positive cocci (GPC), and 4 (4.3%) yeasts. Most common isolates were *Acinetobacter species*, *Klebsiella species*, *Pseudomonas species*, and *E coli*. Out of 10 GPC isolates, six were identified as Methicillin resistant strains including 4 (66.7%) coagulase negative *Staphylococcus species* and 2 (33.3%) *S aureus*. Out of 79 GNBs, 16 (20.3%) were extended spectrum beta lactomase (ESBL) producers, 14 (17.7%) AmpC, and 16 (20.3%) were carbapenemase producers. Carbapenemase production was further identified as metallo beta lactamase (MBL) producers (n=10) and serine carbapenemase producers (n=6). Out of 89 bacterial isolates, 82 (92.1%) were tested positive for in-vitro biofilm production while 07 (7.9%) were identified as non-adherent.

Conclusion: Most of the ET tubes inserted for more than 48 hours inside a patient have a high risk of getting infested with potential biofilm producing pathogen. There is high rate of multi drug resistant (MDR) strains among these potential pathogens. The biofilm formed inside the ET tube possess the risk of onset of VAP.

Keywords: Carbapenemase, Crystal violet assay, Multi-drug resistance, Nosocomial infections, Ventilator associated pneumonia.

INTRODUCTION

Ventilator associated pneumonia (VAP), the commonest nosocomial infection in critical care units, is a lung infection (pneumonia) that develops in a patient who is on mechanical ventilation for more than 48 hours.[1] About 8-28% of critical care patients who receive mechanical ventilation develops VAP and the rate of occurrence varies with duration of endotracheal (ET) tube intubation.[2,3]

The ET tube provides the surface for potential pathogens to attach and form biofilm. The biofilm formation phenomenon has been proven to occur on the inner surface of ET tube in intubated patients and the subsequent dislodgement of bacteria present in the biofilm to the lungs is thought to be a significant factor in the pathogenesis of VAP.[4,5] During the mechanical ventilation, suctioning can result in the dislodgement of the bacterial aggregates from biofilms into the lower airways.[4] Furthermore, 70% of VAP patients had identical pathogens present within the ET tube biofilm as encountered in the lung[6], indicating that the biofilm is a major and persistent source of pathogenic bacteria.

Antimicrobial tolerance is another issue associated with biofilm. Biofilm-specific characteristics impede antimicrobial agent diffusion and activity, which can also add to resistance.[7] An antimicrobial agent needs to overcome certain obstacles such as an increased number of resistant mutants, higher cell density, exchanges at molecular level, substance delivery, efflux pump, and persistent cells so that it can work more efficiently against bacteria residing within the biofilm.

The present research was intended to isolate the potential pathogens present inside the inner lumen of the ET tube of critically ill patients and demonstrate their antibiotic resistance pattern and their ability to form in vitro biofilm.

METHODS

The current experimental prospective study was conducted at a tertiary care government teaching hospital, Jodhpur. An approval for conducting the study was taken from the ethical committee of the institute (No. F.1/Acad/MC/JU/18/5170). The patients admitted in the ICU of the institute, above the age of 16 years, and inserted with an endotracheal tube for more than 48 hours were included in the study. A written consent was taken from the relatives of the patients included in the study before collecting the samples.

Any patients who have acquired pneumonia prior to hospitalization were not included in the study. Clinical criteria for the suspicion of VAP include a new and persistent (>48 hours) or progressive radiographic infiltrate plus any two of the following: temperature of >38°C or 11,000 cells/ml or <4000 cells/ml, purulent secretions, gas exchange degradation and microbiological evidence in endotracheal aspirate. The clinical pulmonary infection score (CPIS) of 6 or more was clinically diagnosed as VAP.

The intubated ET tubes were collected only upon removal as guided by the treating physician. The ET tubes were collected, placed in sterile zip-lock bags, and transported to the Microbiology department without any delay. The ET tubes were cut into parts from three distinct portions, each sizing 1 cm in length. The inner lumen of each part was scraped off with the help of sterile scrapper and added to 1 ml sterile saline, followed by 10-20 seconds of vortex mixing. The vortexed sample was inoculated on 5% sheep blood agar and MacConkey agar. The streaked plates were incubated for 24 hours at 37°C. The colonies isolated on the culture plates were tested for Gram's staining and motility test. They were further identified using a battery of biochemical tests as per standard laboratory protocol. All the potential pathogens identified were also investigated for antimicrobial susceptibility test as per CLSI guidelines 2017.[8] Colistin susceptibility was tested in minimum inhibitory concentration (MIC) by using microbroth dilution method (MBD) as per CLSI guidelines 2017.[8] A MIC value ≤2 was considered susceptible while a MIC value ≥4 was considered as resistant. The gram negative bacteria were also tested for extended spectrum beta lactamase (ESBL), Amp C, and carbapenamase production. All the potential pathogenic bacterial isolates were tested for in vitro biofilm production using 1% crystal violet assay. The data were entered in MS Excel sheet and analyzed using SPSS version 21. A p value of <0.05 was considered statistically significant.

RESULTS

A total of 100 ET tubes were collected from 100 individual patients. Among these, 18 (18%) had a CPIS score of 6 or more (i.e. suggestive for VAP) while 82 (82%) had CPIS< 6 (i.e. not suggestive for VAP). The ET tubes were collected from the patients intubated for a period of 48 hours or more, upon extubation as indicated by the attending doctor. Most of the patients admitted in the ICUs of study settings were intubated for shorter durations as per the medical need.

Out of 100 ET tubes processed in the study, no growth was observed in 23 (23%) ET tubes, single type of potential pathogen was grown in 63 (63%) ET tubes while growth of more than one microorganism was found in 14 (14%) ET tubes.

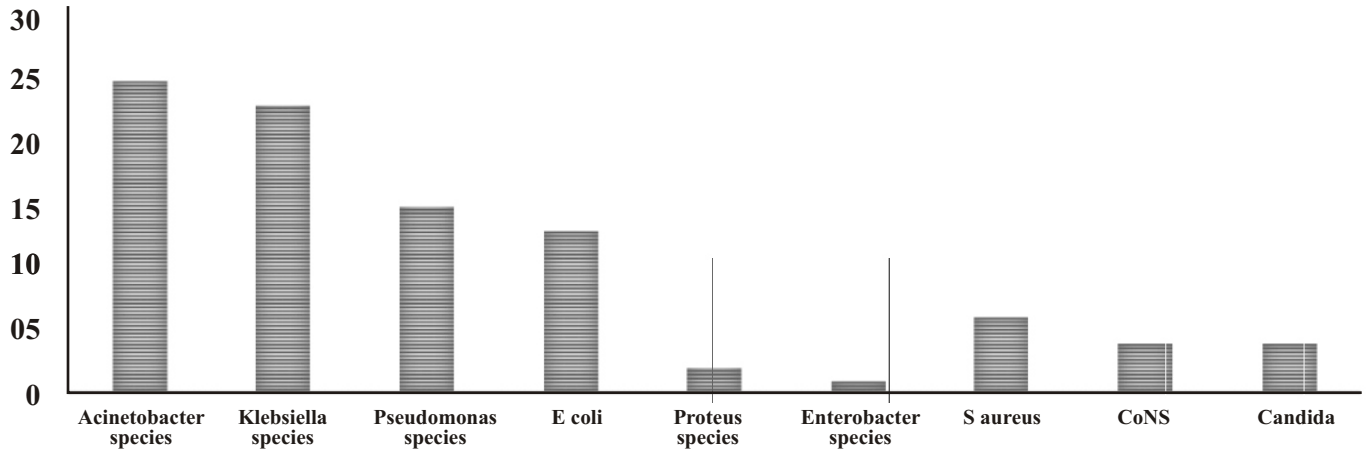
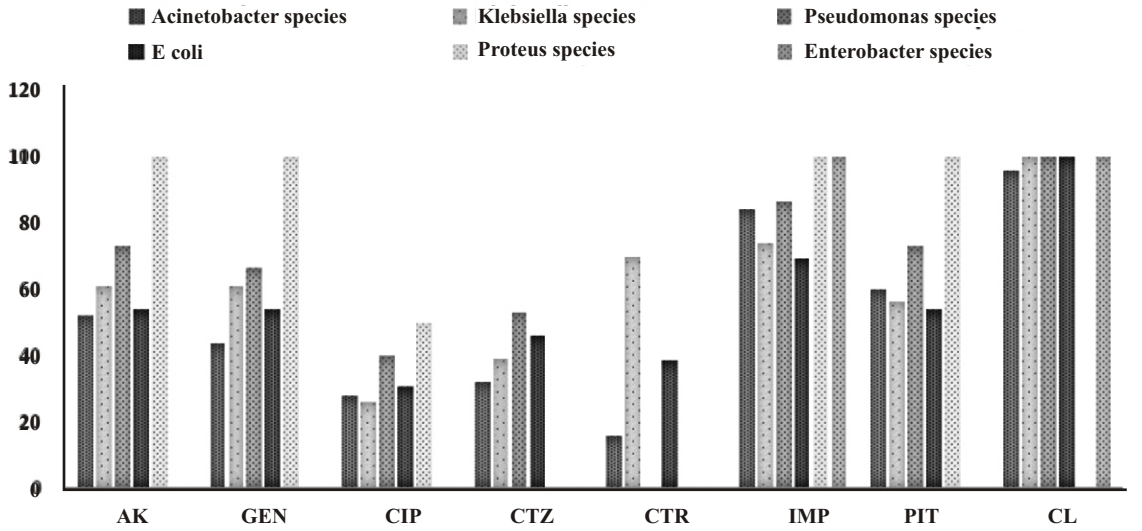
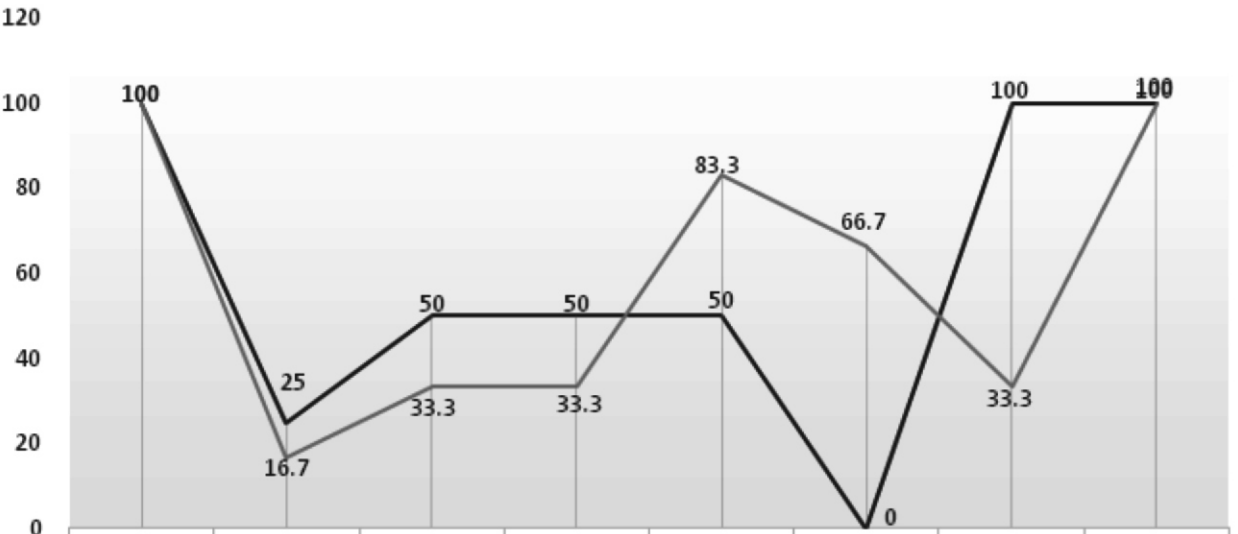


Figure 1: Potential pathogens isolated from ET tubes.



AK: Amikacin; GEN: Gentamycin; CIP: Ciprofloxacin; CTZ: Ceftazidime; CTR: Ceftriaxone; IMP: Imipenem; PIT: Piperacillin-Tazobactam; CL: Colistin.

Figure 2: Antibiotic susceptibility pattern (Antibiogram) of the Gram-negative bacilli isolated from ET tube specimens.



	VSA	P	ERY	CD	DOX	CX	CIP	LZ
CoNS	100	25	50	50	50	0	100	100
S. aureus	100	16.7	33.3	33.3	83.3	66.7	33.3	100

VSA: Vancomycin Screen Agar; P: Penicillin; ERY: Erythromycin; CD: Clindamycin; DOX: Doxycycline; CX: Cefoxitin; CIP: Ciprofloxacin; LZ: Linezolid

Figure 3: Antibiotic susceptibility pattern (Antibiogram) of gram-positive cocci isolated from ET tube specimens.

A total of 93 different microorganisms were isolated from ET tube specimens. Among these microorganisms, *Acinetobacter species* were isolated from the majority of ET tube samples followed by *Klebsiella species, Pseudomonas species*, and *E coli.* Other than bacterial isolates, four *Candida species* were also isolated from the specimens (Figure 1).

High rates of antibiotic resistance were observed in gram-negative bacilli against Ceftriaxone, Ciprofloxacin, Amikacin, and Gentamicin. Imipenem and Colistin had demonstrated high susceptibility rates (Figure 2).

Vancomycin susceptibility was tested by using Vancomycin Agar Screen (VAS). Vancomycin and Linezolid were recorded as most susceptible antibiotics as no

resistance was found against both the antibiotics. Methicillin resis-tance was found in two strains of *S aureus* and four strains of coagulase negative *Staphylococcus species*.

All the isolated bacterial strains were tested for in-vitro biofilm formation by crystal violet assay and categorized as strong, moderate, weak biofilm forming and non-adherent (non biofilm forming) strains (Figure 4).

All the gram-negative bacteria were tested for enzyme production such as ESBL, Amp C, carbapenemase, metallo beta lactamase (MBL), and serine carbapenemase which are responsible for drug resistance (Table 1).

DISCUSSION

The indwelling medical devices have become an important tool in modern medicine which provides effective, low cost and seldom simple management solutions for critically ill patients to support normal physiological functions of the body. Their widespread use is compromised by aptness to become colonized by microorganisms. These microorganisms, mostly potential pathogens present in hospital environments, form biofilm within these medical devices

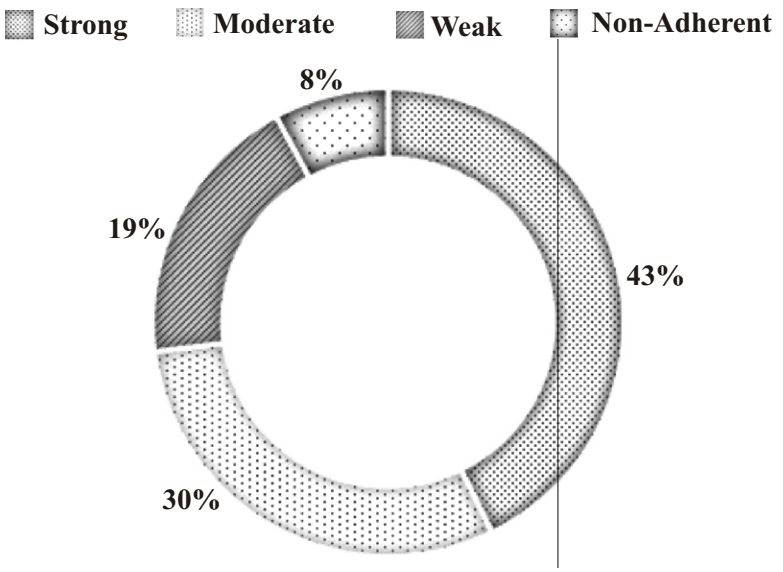


Figure 4: Distribution of in-vitro biofilm fo ming abilities of potential pathogen isolates.

Table 1: Distribution of various types of enzyme production responsible for antibiotic resistance by the gram-negative isolates

Variable		Non producers	Producers	Biofilm*			
				S	M	W	NA
ESBL	Count	63	16	9	3	3	1
	%	79.75	20.25	56.25	18.75	18.75	6.25
Amp C	Count	65	14	3	5	5	1
	%	82.28	17.72	21.43	35.71	35.71	7.14
Carbapenemase	Count	63	16	8	6	2	0
	%	79.75	20.25	50	37.5	12.5	0
MBL	Count	69	10	6	3	1	0
	%	87.34	12.66	60	30	10	0
Serine	Count	73	06	2	3	1	0
Carbapenemase	%	92.41	07.59	33.33	50	16.67	0

*Distribution of biofilm formation by MDR GNB (drug resistance enzyme producers) into four categories. (S: Strong; M: Moderate; W: Weak; NA: Non-adherent); ESBL: Extended spectrum beta lactamase; MBL: Metallo beta lactamase.

and thus lead to medical device associated infections.[9] Biofilm formation within ET tube may act as a reservoir for potential pathogens which may infect the lower respiratory tract of intubated patients.

In the present study, out of 100 ET tube samples, potential pathogens were grown in 77 (77%) samples. A similar yield rate from ET tube samples was reported previously by Crains et al[10] in their study from New Zealand. Mono-microbial growth (i.e. only single type of microorganism) was observed in 63/77 (81.8%) samples while poly-microbial growth (i.e. multiple organisms) were recorded in 14/77 (18.2%) samples in the current study. Similarly, a growth rate of 21% by mixed flora has also been reported previously by Ferrer et al[11] in their study. ET tube biofilms can be formed by various microorganisms from different sources such as oral flora, gastro-intestinal flora, or potential pathogens from hospital environment.[12] This can be the reason for different patterns of microorganisms isolated from ET tube samples.

A total of 93 microorganisms were isolated from ET tube samples including 4 (4.3%) yeasts. Among all the isolates, 89 (95.7%) gram negative bacteria out numbered 10 (10.8%) gram positive bacteria in terms of isolation from ET tube samples. High rates of antibiotic resistance against fluoroquinolones and cephalosporins were observed among GNB isolates. All the GNB isolates were observed susceptible to Colistin except one strain each of *Acineto-bacter species* and *Proteus species*, which is intrinsically resistant. Carbapenem was also found to be a very effective drug against the GNB isolates in the current study (Figure 2). These patterns of antibiotic susceptibility results are well supported by previous studies performed on lower respiratory tract pathogens.[13,14] Methicillin resistance was observed in two strains of *S aureus* and three strains of coagulase negative *Staphylococcus species*. Higher antibiotic resistance rates were recorded against Penicillin, followed by Erythromycin, Clindamycin, and Ciprofloxacin. Only one strain of *S aureus* demonstrated resistance towards Doxycycline. All of the GPC strains were susceptible to Linezolid as well as glycopeptides like Vancomycin and Teicoplanin.

There are many techniques to determine the in-vitro biofilm producing ability of the bacteria, 1% crystal violet assay (CVA) is one of the widely used among them. In the present study, all the bacterial isolates were tested for in vitro biofilm formation by using 1% CVA in microtiter plate. The intensity of the biofilm formation was determined by their optical density (O.D.) value using spectrophotometer and grouped as (i) strong, (ii) moderate, (iii) weak biofilm producers and (iv) non-adherent or non-biofilm producers. Majority of bacterial isolates in the present study were found as strong biofilm producers (43%) followed by moderate biofilm producers (30%). Only 8% of the isolates were not able to form biofilm in-vitro by 1% CVA method. A previous study by Gil-Perotin et al[15] suggested that 95% of ET tubes consist of bacterial biofilm if intubated for 24 hours or more.

Higher rates of ESBL and carbapenemase production (20.25%) were observed among GNB isolates. When tested further, MBL carbapenemase (12.66%) was recorded higher than the serine carbapenamase (7.59%) among the carbapenemase producers. Majority of these MDR organisms were also found to produce in-vitro biofilm. Only one ESBL producing and one AmpC producing bacteria were tested as non-adherent (i.e. non biofilm producing) strains while all other strains including carbapenemase producers formed biofilm. These results are well supported by findings of Baidya et al[16] who also reported the majority of VAP pathogens producing ESBL or/and MBL along with the in-vitro biofilm production in the microtiter plate. Since the isolated strains in the present study are exposed to hospital environments especially intensive care units and thus exposed to prior antibiotic usage which might have led to their MDR character. These were isolated from ET tube sections, i.e. inanimate surfaces, which also specify their biofilm forming ability. This combination of multidrug resistance and ability to form biofilm inside the ET tube can act as a serious problem in health deterioration as well as difficult management of patients admitted in ICU and under mechanical ventilation.

CONCLUSION

The biofilm formation by the potential pathogens within the ET tube inserted in the patients admitted in critical care units brings the risk of VAP onset. These potential pathogens are mostly multi drug resistant in nature. The MDR and biofilm forming ability of these potential pathogens helps them to escape antibiotic treatment which may further complicate the management of patients. It is important to understand the nature of these potential pathogens and develop strategies to prevent the biofilm formation within ET tubes.

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Corresponding Author

Vikrant Negi, Department of Microbiology, Dr SN Medical College and Associated Group of Hospitals, Jodhpur, Rajasthan, India.

email: negi.vikrant@gmail.com

ORCID ID: https://orcid.org/0000-0002-3634-8696