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A Study of Ventilator Associated Pneumonia Caused by Multidrug Resistant Pathogens With Special Reference to ESBL, AMPC And MBL.

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ABSTRACT

Ventilator Associated Pneumonia is the most frequent intensive care unit acquired infection with increasing incidence of multidrug resistant organisms. To know the bacterial pathogens causing Ventilator Associated Pneumonia and detect the presence of ESBL, AmpC, MBL and MRSA among the isolates. This study included adult patients on mechanical ventilation for more than 48hrs. Ventilator Associated Pneumonia was diagnosed using Clinical Pulmonary Infection Score ≥ 6. Quatitatitive cultures of Endotracheal Aspirate was performed and colony count > 10^5cfu/ml was considered. Antimicrobial susceptibility testing was done. Resistant organisms were further tested for ESBL, AmpC, and MBL & MRSA by conventional methods and E-test was done. Descriptive statistics like percentage, mean, standard deviation were applied. 478 adult patients received mechanical ventilation for > 48hrs. The incidence of Ventilator Associated Pneumonia was 59%. Enterobacteriaceae (32.21%), *Acinetobacter baumannii* (22.12%), *Pseudomonas aeruginosa* (19.3%) and *Staphylococcus aureus* (11.29%) were common pathogens isolated. Among the isolates 6.01 % were MBL, 45.83% ESBL, 1.01% AmpC and 9.01% MRSA respectively. Production of β-Lactamases among isolates is a major problem for treatment. Faster diagnosis and proper hygiene in ICU settings can reduce the burden of MDR and management of Ventilator Associated Pneumonia.

Keywords: Ventilator Associated Pneumonia(VAP), Multidrug-resistant (MDR), Extended Spectrum β lactamase (ESBL), Amp C β lactamase (AmpC), Metallo-β-lactamase (MBL), Methicillin Resistant *Staphylococcus aureus* (MRSA).

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INTRODUCTION

Ventilator Associated Pneumonia (VAP) is defined as pneumonia occurring more than 48hrs after endotracheal intubation and initiation of mechanical ventilation (MV) [1]. VAP is the most frequent intensive care unit (ICU) acquired infection occurring in 9 - 24% of patients intubated longer than 48hrs [2].

Early onset VAP occurs within the first 5 days of intubation and infection after 5days are called late onset VAP. Early onset VAP are caused by antibiotic sensitive pathogens, less severe and associated with better prognosis. Late onset VAP are caused by multidrug resistant (MDR) pathogens and are responsible for increased morbidity and mortality [3].

A number of studies in India have investigated the causative organisms of VAP. *Pseudomonas sps*, *Acinetobacter sps*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were identified as common VAP pathogens with varying prevalence [4]. Upto 40% of these are polymicrobial flora. *Pseudomonas sps* , *Acinetobacter spp*, Enterobacteriaceae are MDR due to production of extended spectrum β Lastamase (ESBL), AmpC β lactamase(AmpC), metallo-β-lactamase(MBL) and Methicillin Resistant *Staphylococcus aureus* (MRSA) [5]. The etiological agents of VAP needs to be studied with special reference to these MDR's.

The objective of the study was to know the bacterial pathogens causing VAP and to detect the presence of ESBL, AmpC, MBL, and MRSA among these pathogens.

MATERIALS AND METHODS

The study was conducted during the period 2006 - 2009 in different Health care centers of Tumkur. Adult patients admitted to the ICU and on MV for more than 48hrs were included in the study group. Informed consent was obtained next of kin. A criterion for diagnosis of VAP was made as per modified Clinical Pulmonary Infection Score ≥6 (CIPS) [6]. Patients with fever, leukocytosis, purulent sputum, hypoxia and CPIS ≥ 6 after 48hrs on ventilator support were included. The diagnosis was confirmed by performing a quantitative culture of endotracheal aspirate (EA) and observing $>10^5$cfu/ml [7, 8].

The organisms isolated were identified by standard microbiological techniques [9]. Antibiotic susceptibility was performed to routinely used antibiotics by Kirby Bauer disk diffusion method on Mueller Hinton Agar (MHA) as per CLSI guidelines [10]. Aminoglycosides, 2nd & 3rd generation cephalosporins, carbapenems, and fluoroquinolones were included. Organisms resistant to cephalosporins, carbapenems were further tested for ESBL, AmpC, & MBL respectively. Cefoxitin resistant *Staphylococcus aureus* were considered as MRSA.

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Detection of ESBL by Disk approximation method [11]

The isolates that exhibited intermediate/resistance to 3rd generation cephalosporins were screened to detect ESBL production. A modified double disk synergy test (disk approximation test) first described by Jarlier[12] was carried out. Amoxycillin+clavulanic acid (20µg+10 µg) disk was placed in the centre and the ceftazidime (30µg) and cefotaxime (30µg) disks were placed on either side at a distance of 15mm center to center from the amoxicillin + clavulanic acid disk. Plates were incubated at 35°C for 18-20hrs and the pattern of zone of inhibition was noted. Isolates that exhibited a distance shape/size with potentiation towards amoxicillin+clavulanic acid disk were considered potential ESBL producers. *Escherichia coli* ATCC 25922 & *Klebsiella pneumoniae* ATCC 700603 were used as negative & positive controls respectively.

Detection of AmpC β lactamase by AmpC Disk test [13]

A lawn culture of *Escherichia coli* ATCC 25922 was prepared on MHA plate. Sterile discs (6mm) were moistened with sterile saline (20µl) and inoculated with several colonies of the test organism. The inoculated disk was then placed beside a cefoxitin disk (almost touching) on the inoculated plate. The plates were incubated overnight at 35°C. A positive test appeared as a flattening or indentation of the cefoxitin inhibition zone near the vicinity of the test disk. A negative test had an undistorted zone.

Detection of MBL by Modified Hodge Test [14]

Modified Hodge test (MHT) was carried out for MBL detection. The imipenem resistant strains were subjected to modified Hodge test for detection of carbapenemases. An overnight culture suspension of *Escherichia coli* ATCC 25922 McFarland standard was inoculated on the surface of MHA. After drying 10µg imipenem disk was placed at the center of the plate and the test strain was streaked from the edge of the disk to the periphery of the plate in four directions. The presence of a clover shaped zone of inhibition was considered as MBL positive. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* 25922 were used as controls.

Detection of MRSA [15]

Staphylococcus aureus resistant to cefoxitin by disc diffusion method was considered as MRSA [6].

Oxacillin Screen Agar

MHA plates containing 4% NaCl and 6µg/ml oxacillin were prepared. Plates were inoculated with 10 µl of 0.5 McFarland suspension in one quadrant and incubated at 35°C for 24hrs. Plates were observed for growth. Any growth after 24hrs was considered oxacillin resistant.

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Cefoxitin disc diffusion test

Isolates were subjected to cefoxitin disc diffusion test using a 30 µg disc. A 0.5 McFarland standard suspension of the isolate was made and lawn culture done on MHA plate. Plates were incubated at 37°C for 18hrs and zone diameters were measured. An inhibition zone diameter of ≤ 21mm was reported as oxacillin resistant and ≥ 22mm was considered as oxacillin sensitive.

Methicillin Sensitive *Staphylococcus aureus* (MSSA) ATCC 25923 & methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300 were used as negative & positive controls.

E-Test MIC test [16, 17]

ESBL are plasmid encoded cephalosporinases that are inhibited in vitro by clavulanic acid, which generally belong to TEM and SHV family of β lactamase. E-test employs strips coated with the relevant antibiotics, which form a gradient after placing them on agar plates. E-Test ESBL, AmpC, MBL, MRSA strips (AB Biodisk, Sweden) are double-ended strips with antibiotic and antibiotic inhibitor gradients. One end of the strip contains the cephalosporin and the other end the cephalosporin/clavulanic acid combination. Lawn culture of test organism 0.5 McFarland turbidity was made on MHA and the E-strips were placed. After overnight incubation the MIC values of the test isolate with the potential ESBL/AmpC/MBL are read from the strips. These strips yield the minimum inhibitory concentration (MIC) which determines the presence of ESBL (Table. 1). A decrease in MIC by 3 doubling dilutions in the presence of clavulanate indicates ESBL production or MIC value for antibiotic divided by the MIC value to antibiotic+clavulanic acid combination or phantom zone or distortion of the cefotaxime, ceftazidime, imipenem (CT, TZ or IM) inhibition eclipse confirms ESBL production (Ab-Biodisk, Sweden).

Table/Fig. 1 : MIC E-strips used for ESBL, AmpC, MBL & MRSA

Sl.no	Enzyme detected	Antibiotic combination	abbreviation	MIC range
1.	ESBL	Cefotaxime/cefotaxime +clavulanic acid	CT/CTL	0.25-6µg/ml & 0.016-1µg/ml
2.	ESBL	Ceftazidime/ceftazidime+clavulanic acid	TZ/TZL	0.50-32µg/ml & 0.064-4µg/ml
3.	Amp C	Cefotetan+cefotetan+clavulanic acid	CN/CNI	0.50-32µg/ml &0.50-32µg/ml
4.	MBL	Imipenem+imipenem+clavulanic acid	IP/IPI	4-256µg/ml & 1-64µg/ml
5.	MRSA	Oxacillin	OXA	0.016-256µg/ml

Statistical Analysis

Statistical analysis was done by using MS excel sheet. Descriptive statistics like percentage, mean, standard deviation were applied.

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RESULTS

478 adult patients develop VAP out of 806 patients on Ventilator support, the remaining were weaned out of ventilator support within 48hrs. The incidence of VAP was 59%. Quantitative cultures of EA from patients with CIPS score ≥ 6 yielded >10^5cfu/ml. *Acinetobacter baumannii* 106/478 (22.17%) was the commonest pathogen isolated followed by *Pseudomonas aeruginosa* 91/478 (19.03%), Enterobacteriaceae 154/478 (32.21%) and *Staphylococcus aureus* 54/428 (11.29%). Polymicrobial flora was seen in 194/478 (40.58%) of cases.

The percentage of resistance to Ampicillin was 100%, among *Acinetobacter baumannii*, 87.8% in *Pseudomonas aeruginosa* & *Klebsiella pneumoniae* 91.2% among *Escherichia coli*. Resistance to Amikacin was seen in *Acinetobacter baumannii* (48.1%), *Pseudomonas aeruginosa* (43%), *Klebsiella pneumonia* (26.4%) and *Escherichia coli* (26.3%) respectively. Increased resistance to 2nd & 3rd generation cephalosporins (ceftazidime & cefotaxime) were seen among *Acinetobacter baumannii* (78%), *Pseudomonas aeruginosa* (47.25%), *Klebsiella pneumoniae* (40.2%) & *Escherichia coli* (68%). Resistance to carbapenems (imipenem & meropenem) was 8% & 3% in *Acinetobacter baumannii* & *Pseudomonas aeruginosa* respectively. High resistance to fluoroquinolones was also observed among the isolates. 30% of non-fermenters & 17% among Enterobacteriaceae were resistant to fluoroquinolones. Isolates resistant to 3rd generation cephalosporins were tested for ESBL & Amp C, those resistant to carbapenems were screened for MBL production & oxacillin resistant *Staphylococcus aureus* were screened for MRSA (Table/Fig. 2). We did not isolate any carbapenemase producing *Klebsiella pneumoniae*. Among non-lactose fermenters 6.09% (12/197) were MBL producers, 45.83% (66/144) were ESBL producers, 1.01% (2/197) were AmpC-β- lactamase producers and 9.25% (5/54) were MRSA. Isolates that were positive for ESBL, AmpC, MBL and MRSA were subjected to confirmation by E-test respectively. In our study we got similar results by standard detection methods for ESBL, AmpC, MBL, MRSA and MIC E -strip test. When the value of antibiotic / antibiotic inhibitor combination was ≥ 8 or the presence of phantom zone, it was considered as a β-lactamase producer (AB-biodisk instructor manual). Phantom zone was exhibited by 14.70% (10/68) of isolates and were confirmed as ESBL. Isolates that were MBL positive had MIC values between 16µg/ml for IP and 2µg/ml IPI for *Acinetobacter baumannii* isolates & *Pseudomonas aeruginosa* isolates. ESBL MIC between 0.25 µg/ml /0.016 µg/ml (CT/CTL), >32 µg/ml /, 0.38 µg/ml (TZ/TZL) for *Eschreichiacoli*,0.26 µg/ml /0.16 µg/ml (CT/CTL) *Pseudomonas aeruginosa,* >32 µg/ml /0.75 µg/ml (CN/CNL) for Amp C among *Pseudomonas aeruginosa* & 6 µg/ml/0.56 µg/ml for *Acinetobacter baumannii,*16 µg/ml /2 µg/ml for MBL among *Acinetobacter baumannii* & *Pseudomonas aeruginosa*. The MIC for MRSA was 8 µg /ml. The MIC E- strip results were as follows (Table/Fig3).

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Table/Fig. 2 : ESBL, AmpC, MBL & MRSA isolated among the isolates.

Sl No.	Bacteria	ESBL	AmpC	MBL	MRSA	**TOTAL**
1	Non-Fermenters					
	Acinetobacter baumannii	0	1	9	NS	**10**
	Pseudomonas aeruginosa	2	1	3	NS	**06**
2	Enterobacteriaceae					
	Klebsiella pneumonia	28	0	NS	NS	**28**
	Escherichia coli	38	0	NS	NS	**38**
3	Gram Positive Cocci					
	Staphylococcus aureus	NS	NS	NS	055	**05**
	TOTAL	**68**	**02**	**12**	**05**	**87**

NS- Not Screened

Table 3/Fig 3: MIC values for MDR isolates

Enzyme- organism	**CT/ MIC**		**TZ/ MIC**		**CN/MIC**		**IM /MIC**		**OXA /MIC**
	CT	**CT+CTL**	**TZ**	**TZ+ TZL**	**CN**	**CN+CNI**	**IM**	**IMI**	-
ESBL- *Escherichia coli*†	0.32* ± 0.01	0.021 ± 0.09	>75 ± 0.1	0.26 ± 0.09	-	-	-	-	-
ESBL-*Klebsiella sps*‡	6* ± 3	0.053 ± 0.01	10.9 ± 2	0.28 ± 01	-	-	-	-	-
ESBL-*Pseudomonas sps*	0.25	0.016	16	0.38	-	-	-	-	-
AmpC -*Pseudomnoas sps***	-	-	-	-	>32	0.75	-	-	-
AmpC- *Acinetobacter sps*	--	-	-	-	6	>0.50			
MBL – *Acinetobacter* sps††	-	-	-	-	-	-	12 ± 4	1.5 ± 0.5	
MBL-- *Pseudomonas* sps							16	2	
MRSA ll	-	-	-	-	-	-	-	-	8

Mean ± SDev, * Phantom zone exhibited by few isolates, † Extended Spectrum β Lactamase, ‡ Amp C β Lactamase, ††Metallo- β Lactamase

ll Methicillin Resistant β Lactamase

DISCUSSION

VAP is the most dreaded infection among critically ill patients receiving MV. The incidence of VAP in our study was 59% such high incidence of VAP was seen in some studies across India [5]. Dey Arindam and Indira Bairy recorded the incidence of VAP in mechanically ventilated patients as 45.5% & a still higher occurrence was observed by Mukhopadhyay et al [18]. The high incidence of VAP may be due to patients on prior antibiotic therapy, previous surgeries, improper use of antibiotics and improper barrier nursing. No restriction of patient attendant entry into the ICU also increases the occurrence of VAP due to MDR pathogens.

We found that non-Lactose fermenters were the predominant isolates (49.21%) followed by Enterobacteriaceae (32.21%). *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were commonly isolated (78.3%). *Acinetobacter baumannii* has replaced *Pseudomonas aeruginosa* [19, 20]. *Acinetobacter baumannii* has emerged as one of the most troublesome pathogens in health care settings locally and globally. Its remarkable ability to develop or acquire multiple antibiotic resistance and propensity to survive for long periods

under wide range of environmental conditions, make it a frequent cause of hospital outbreaks and an endemic health care associated pathogen. Its common targets are, most vulnerable hospitalized and critically ill patients with breaches in skin integrity who require airway protection, causing pneumonia, urinary tract infection, wound infection and bacteremia. In a study by Vishal et al [19] *Acinetobacter* VAP patients were found to be associated with various underlying clinical conditions like head trauma, cerebral hemorrhage and chronic obstructive pulmonary disease [20].

The drug resistance of isolates in early onset VAP and late onset VAP did not show any difference. Even the American Thoracic Society Guidelines supports the same reasoning suggesting that patients with early onset VAP who have received prior antibiotics or who have had hospitalization are at a greater risk for infection with MDR pathogens and should be treated similar to patients with late onset VAP [21].

MDR pathogens were resistant to macrolides, aminoglycosides, fluoroquinolones, 2nd & 3rd generation cephalosporins & carbapenems with no choice for treatment of these pathogens. Piperacillin–tazobactum & colistin were the only drugs for treatment of these isolates [2]. *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are noted for their intrinsic resistance to antibiotics and their ability to acquire genes encoding resistance determinants. Foremost among the mechanism of resistance in both of these pathogens is the production of β-lactamase and aminoglycoside-modifying enzyme, diminished expression of outer membrane proteins, mutations in topoisomerases, and up-regulation of efflux pumps. The accumulation of these multiple mechanism of resistance leads to the development of MDR or pan-resistant strains [22].

MBL and Amp C were frequently isolated among non-Lactose fermenters, ESBL and Amp C in Enterobacteriaceae, MRSA among *Staphylococcus aureus*. Numerous β lactamases have been described in *Acinetobacter baumannii.* The chromosomally encoded cephalosporins (Amp C type) is common to all strains *Acinetobacter baumannii* [23]. The occurrence of OXA enzymes confers β lactamase resistance. Two major metallo-β-lactamase reported are IMP and VIM type. TEM, SHV & CTX-M type ESBLs are common among *Pseudomonas aeruginosa* and Enterobacteriaceae that confer drug resistance in strains [24]. Joseph et al in their study observed that AmpC-β-lactamases were most commonly produced in non-fermenters, while MBL among *Pseudomonas aeruginosa* [2]. The findings in our study were reversed by MBL being more common in *Acinetobacter baumannii*. Production of ESBL, AmpC, MBL, and MRSA were responsible for MDR among isolates. VAP caused by *Staphylococcus aureus* are not frequently reported by studies in India [2, 5]. This emphasizes the need for judicious selection of patients for antibiotic therapy. The prophylactic use of antibiotics is therefore not recommended and exposure to antibiotics is a significant risk factor for colonization and infection with nosocomial MDR pathogens as observed by many authors [2, 24]. The rational use of appropriate antibiotics may reduce patient colonization and subsequent VAP with MDR pathogens. Unnecessary prolonged hospitalization of patients should be avoided as far as possible. Knowledge of the risk factors should suggest the possibility due to MDR pathogens in patients developing VAP after hospitalization for 5 days or more.

There was no significant difference in the ESBL, AmpC, MBL & MRSA detected by conventional method and E strip test in our study. This may be because E-test were done only on selected isolates confirmed by conventional methods and we used a zone ratio for antibiotic /antibiotic inhibitor greater or equal to 8 or presence of phantom zone as indicative for a β-lactamase producer. Florijn A et al [17] noted that by double disk diffusion method all the four disks scored equally for recording ESBL isolates. Therefore the most commonly used practice to confirm β-lactamase enzymes by carrying out clavulanate synergy tests may no longer be sufficient in populations with a high prevalence of β-lactamases among isolates. It becomes more and more apparent that regular species identification forms the base on which accurate β-lactamase detection can be built. Studies have also been conducted by direct application of the E-strips on EA samples for early diagnosis and treatment of VAP [25].

Conventional methods, E-test or Vitek ESBL detection system for detection of ESBL, AmpC, MBL and MRSA can help in appropriate antibiotic therapy and reduce development of MDR.

CONCLUSION

VAP is increasingly associated with MDR pathogens. MDR isolates were the major cause of concern. Productions of ESBL, AmpC, MBL and MRSA confer drug resistance among isolates. Knowledge of the antibiotic susceptibility pattern will guide the choice of appropriate antibiotic. Studies suggests use of macrolide (IV) + either β lactam (IV) or antipneumococcal/ antipseudomonal β lactam (IV) or antipneumococcal quinolone (IV) + either β lactam (IV) or antipneumococcal/antipseudomonal β lactam (IV) has been proposed for treatment of ICU patients. Barrier nursing, following proper hand washing technique can reduce the MDR pathogens among VAP in ICU patients.

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