

Atypical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience

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Abstract: Background Ventilator-associated pneumonia (VAP) is the most common hospital acquired infection seen in ICU in patients on mechanical ventilation. A diversity of microbes can cause VAP, causative agent differ according to patient populations and types of ICUs. Atypical bacteria not cultured by routinely used methods, have been implicated as causes of VAP, still no sufficient studies to assess size of their role as causative agent in VAP. In this study we aim at estimation of the potential role of atypical bacteria as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* in ventilator-associated pneumonia in the intensive care units of Alexandria Main University Hospital. Materials and methods: 60 endotracheal aspirates were collected from VAP ICU patients. Samples were subjected to routine culture as well as PCR amplification using specific primers for detection of the following atypical bacteria: *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila*. Results: Out of the 60 endotracheal aspirate, routine culture revealed growth of: enterobacteriae in 14 (23.3%) aspirate, pseudomonas in 13(21.7%), candida in 14(23.3%), and MRSA in 10 (16.7%). In 19 (31.7%) endotracheal aspirates, no growth was encountered on routine culture. PCR reaction was positive for Atypical bacteria in 9 (15%) out of 60 samples, five were positive for mycoplasma, three for Legionella, and only one was positive for Chlamydia. Atypical bacteria positive results were encountered in 4 (21%) out of 19 aspirates with no growth culture results. Conclusion: Our results point that atypical bacteria are not an uncommon cause for VAP. This finding has to be taken into consideration while tailoring the empiric antimicrobial coverage of patients diagnosed with VAP.

[Nadia Abdel-Salam Mokhless, Malaka Farouk El-Mofty, Nesrine Fathy Hanafi, Akram Muhammad Fayed and Sara Lotfy Asser. **A Typical Bacteria in Ventilator Associated Pneumonia; an Egyptian University Hospital Experience.** Journal of American Science 2010;6(12):1074-1079]. (ISSN: 1545-1003).
<http://www.americanscience.org>.

1. Introduction:

Ventilator-associated pneumonia (VAP) is considered as the most frequent ICU-acquired infection among patients receiving mechanical ventilation (MV). This kind of respiratory tract infection prolong the duration of Mechanical ventilation and delay the release from ICU. Most antibacterial chemotherapy prescribed in an ICU are administered for respiratory tract infections.⁽¹⁾

VAP can be caused by a large variety of microorganisms. the causative agent may differ according to the population of patients in the ICU, the durations of hospitalization and stay in the ICU. "Atypical" pneumonia differ from typical one in not to be associated with shaking chills⁽²⁾ and caused by atypical bacteria which cannot be grown by routinely used microbiologic culture media and techniques as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila*⁽³⁻⁵⁾.

Mycoplasma pneumoniae was first atypical pathogens to be identified as a cause of respiratory tract infection in 1944.^(6,7) Characterized by absence of a rigid cell wall, poor staining by Gram method,

and high nutritional requirements for its culture as it needs a high concentration (10-20%) of serum or supplements. This makes diagnosis of mycoplasma by conventional microbiologic examination difficult.

⁽⁷⁾ *Chlamydia pneumoniae* laboratory diagnosis depended on non-specific and technically demanding techniques till 1990 but now it has been replaced by detection of chlamydial antigens or detection of DNA by PCR.⁽⁷⁾ *Legionella pneumophila* are nutritionally fastidious, intracellular bacilli, gram negative organisms.⁽⁸⁾ Infection with *legionella is* associated with exposure to artificial water systems, condensers and respiratory therapy equipments.⁽⁹⁾ Use of PCR as a rapid and specific diagnostic method for legionella infection overcame the long culture time needed for its growth (3-5 days) and the need of media supplemented with iron and cysteine as well as difficult colonial identification in mixed cultures.⁽⁷⁾

Accurate diagnosis of VAP remains a difficult target to achieve, that relies mainly on clinical, microbiological and radiological diagnosis.^(10,11) Main clinical criteria for VAP

diagnosis have been reported to be new lung infiltrate on chest X-ray with fever, leukocytosis or leukopenia, and purulent secretions.⁽¹²⁻¹⁴⁾ Inadequate antibiotic treatment have been always reported by researchers to be related to poor prognosis of VAP.^(15,16) Microbiological culture and sensitivity results remains a gold standard for planning treatment for the VAP patient before empirical antibiotic administration⁽¹⁷⁾. This have been emphasized by the Guidelines from the Infectious Disease Society of America (IDSA).⁽¹⁸⁾

It is well established that Beta-lactams are not effective against such organisms because *Chlamydia pneumoniae* and *Legionella* species are intracellular organisms and *Mycoplasma pneumoniae* lacks a cell wall. In those cases Erythromycin and tetracycline can be useful. Other antibiotics effective against atypical bacteria, includes Macrolides, Doxycycline and Fluoroquinolones.⁽⁶⁾ Our study aims at assessment of the potential role of atypical bacteria as *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila* in ventilator-associated pneumonia in the intensive care units of Alexandria Main University Hospital.

2. Materials and methods

Materials

The study was conducted on 60 patients on Mechanical ventilators for more than 48 hours and acquired VAP during their stay in the ICU of Critical Care Department in the Alexandria Main University Hospital in the period between April 2009 and April 2010. Cases included in this study have been informed and consented. Inclusion criteria were fever, leukocytosis, development of persistent radiographical pulmonary infiltrate during stay in the ICU and with no history of a previous pulmonary disease or pulmonary symptoms at the time of admission. Thirty ICU patients on ventilators for more than 48 hours without developing VAP, within the same period of time, were included as a control group.

Methods

Endotracheal aspirates as well as clinical data have been collected from patients and controls. Samples were subjected to routine culture as well as DNA extraction with subsequent PCR amplification for detection of specific DNA sequences of the following atypical Bacteria genus: *Mycoplasma pneumoniae*, *Chlamydia pneumoniae* and *Legionella pneumophila*.

The endotracheal tube was previously inserted guided by a laryngoscope. A sterile suction

catheter was introduced blindly into the endotracheal tube after disconnecting the ventilator. 2 ml of endotracheal aspirates were obtained by suction. The part of the catheters containing the aspirates were cut and placed in sterile test tubes and sent to the laboratory.

The endotracheal aspirates, within catheters in test tubes, were homogenized and liquefied by adding 2 ml sterile 1% N-acetyl L-cysteine (equal volume of the specimen). All tubes were centrifuged for 15 minutes at 4×10^3 rpm and then vortexed and left at room temperature for another 15 minutes.^(19,20) Each homogenized specimen was then divided into 2 equal portions in 2 sterile Eppendorfs. The first part was used for conventional microbiological studies while the other was kept at -20°C for PCR assay. Each aspirate was streaked on Blood agar, MacConkey's agar and Sabouraud's Dextrose agar plates. All plates were incubated aerobically at 37°C for 24 hours. Any growth was identified according to the conventional bacteriological and mycological techniques.^(19,20)

PCR assay:

Extraction of DNA was performed using QIAamp DNA blood mini kit (Qiagen). Separate PCR reactions were performed for amplification of each DNA sequence of each organism using Techne Progene thermal cycler. Reaction mixture consisted of 5 μl DNA extract, 25 picomoles of each of the forward and reverse oligonucleotide primers specific for *Mycoplasma pneumoniae*⁽²¹⁾, *Legionella pneumophila*⁽²¹⁾ and *Chlamydia pneumoniae*⁽²²⁾, 12.5 μl Taq PCR master mix (MBI Fermentas), and 4.5 μl nuclease free water. For detection of the amplified products:⁽²¹⁾ 10 μl of the amplification products were electrophoresed into 2% agarose in Tris-borate EDTA containing 0.5 $\mu\text{g/ml}$ ethidium bromide at 80 volts for 45 minutes. Revealed DNA bands were visualized on an ultraviolet transilluminator.

3. Results

The mean age of the VAP study group was 43.1 ± 24.68 (18-85) year, while that of the control group was 49.7 ± 20.5 (12-70). There was no statistically significant difference between them as regards age and gender: male to female ratio was 33:27(55:45%) in patient group while in the control group, the male to female ratio was 19:11 (63:36.6%). As regards causes of hospital admission for the 60 VAP patients and the control group It has been found that cardiac problems were the most commonly encountered among both patients (38%) and controls (28%), rest of causes for admission included accidents, poisoning, Renal and hepatic problems. In the endotracheal aspirates obtained from

the 60 VAP patients included in this study, 41 specimens were positive by conventional microbiological cultures and 19 were culture negative. In the control group, 11 were positive and 19 specimens were culture negative (See table 1).

The conventional microbiological culture revealed that among cultures of the patients group, *Candida* spp was the commonest organism isolated accounting for 23.3%. Only 16% were of significant count ($\geq 10^5$ CFU/ml). This was followed by *Pseudomonas aeruginosa* (21.6%) and 15% were of significant count, then the polymicrobial growth 20% and 16% with significant count, *Staphylococcus aureus* 16% and 10% with significant count, *Acinetobacter* spp 8.3% and 6.6% with significant count, *Proteus* spp 6.6% and 5% with significant count, *Klebsiella* spp 6.6% and 5% with significant count, *E-coli* 5% and 1.6% with significant count, *Coagulase-negative staphylococci* 1.6% which were of significant count and *Diphtheroids* 1.6% which were also of significant count. It is also to be noted that 31.6% of the total endotracheal aspirates of the 60 VAP cases were negative by conventional microbiological culture.

Table 1: The results of conventional microbiological culture of the endotracheal aspirates from the 60 VAP patients and the control group

	Conventional microbiologic Culture results*	
	Positive	Negative
Cases n=60	41 (68.3%)	19 (31.7%)
Controls n=30	11 (36.6%)	19 (63.3%)
Total n=90	52 (57.7%)	38 (42.2%)

*P value = 0.004

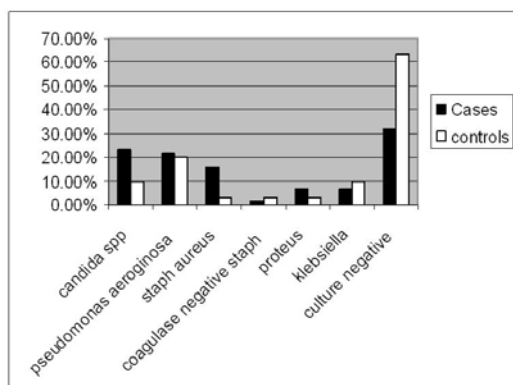


Figure 1: The conventional microbiological culture results for cases & controls group.

While in the control group, bacteria revealed from cultures were *Pseudomonas aeruginosa* accounted for 20%, *Klebsiella* spp 10%, *Candida* spp 10% also, then *Staphylococcus aureus* 3.3%, *Coagulase-negative staphylococci* 3.3% and *Proteus* spp 3.3%. In addition, 10% were culture negative and 13.3% were polymicrobial. The growth counts of the endotracheal aspirates of the control group were insignificant ($\leq 10^5$ CFU/ml). Atypical bacteria DNA detection by PCR was positive in 9 (15%) out of 60 samples, the majority of them (5) were mycoplasma, 3 were positive with Legionella, and only one sample was positive with Chlamydia. The Atypical bacteria positive results was encountered in 4 (21%) out of 19 aspirates with no growth culture results. See figure 1

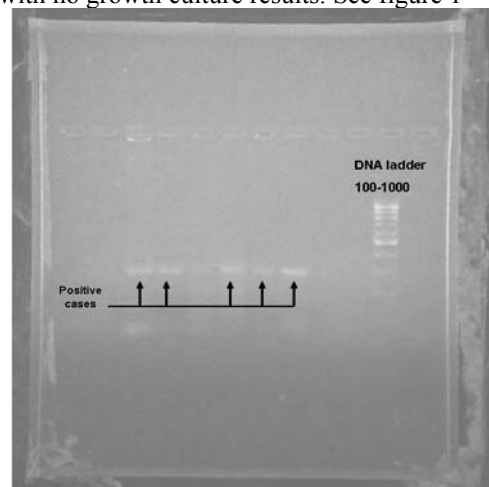


Figure 2: Gel electrophoresis showing 5 positive Mycoplasma pneumoniae cases by PCR.

Table 2: PCR and culture results of cases and control

	Culture**		PCR*	
	Culture positive	Culture negative	PCR positive	PCR negative
Cases	41 (68.3%)	19 (31.7%)	9 (15%)	51 (85%)
Controls	11 (36.6%)	19 (63.3%)	0 (0%)	30 (100%)

* PCR was done using primers for Mycoplasma pneumonia, Legionella pneumophila, Chlamydia pneumonia.

**All specimens were cultured on Blood agar, MacConkey's and SDA.

It was found that 5 specimens were positive for *Mycoplasma pneumoniae*, three were positive for *Legionella pneumophila* and only 1 was positive for

Chlamydia pneumoniae. Among the 5 positive specimens for *Mycoplasma pneumoniae*, 3 were positive by conventional microbiological cultures and grew other associating microorganisms, while 2 were culture negative. The 3 specimens that were positive for *Legionella pneumophila*, only 1 grew other microorganism by conventional cultures and the other

2 were culture negative. The only positive specimen for *Chlamydia pneumoniae* did not grow any microorganisms by conventional microbiological cultures. As for the control group included in this study, none of their DNA extracts were positive in the PCR assay for atypical bacteria.

Table 3: Results of PCR assay in relation to conventional microbiological culture results in VAP patients.

PCR assay	Conventional microbiological culture		Total
	Positive (n= 41)	Negative (n= 19)	
Mycoplasma pneumoniae	3(7%)	2 (10%)	5 (8.3%)
Legionella pneumophila	1 (2.4%)	2 (10%)	3 (5%)
Chlamydia pneumoniae	0	1 (5%)	1 (1.6%)
Total	4(9%)	5(26%)	9 (15%)

4. DISCUSSION

VAP complicates the course of 9 – 20% of mechanically ventilated patients and mortality varies greatly from 8 to 76%. Once pneumonia is suspected, bacteriologic confirmation should be obtained and empiric therapy must be instituted as soon as possible, as a delay in therapy or inappropriate therapy greatly increases mortality.⁽²³⁾ Awareness of the potential microbial causes of VAP and confirmation of the specific cause in an individual patient are essential to guide optimal antibiotic therapy.⁽²⁴⁾

Endotracheal aspirates, chosen in this study as the respiratory specimen, are used more frequently as a diagnostic method in intubated patients with suspicion of pulmonary infection, because of its simplicity and minimal training required, but the fact that the culture also contains other non-pathogenic organisms from the upper respiratory tract flora, results in a low positive predictive value of this test. However, this can be avoided by the use of the semiquantitative method of culture of the obtained specimen, with a designated threshold value above which diagnosis of VAP can be established.^(25,26) Cultures revealed that *Candida* was the commonest organism isolated accounting for 23.3%, while 16% only were of significant count ($\geq 10^5$ CFU/ml). This was followed by *Pseudomonas aeruginosa* 21.6% and 15% were of significant count, then the polymicrobial growth 20% and 16% with significant count, *Staphylococcus aureus* 16% and 10% with significant count,

The results of our study agree with the results of several previous studies.⁽²⁷⁻³¹⁾ where *Pseudomonas aeruginosa* was the predominant organism isolated by endotracheal aspiration and bronchoalveolar lavage, followed by *Staphylococcus aureus* and *Klebsiella pneumoniae*.⁽³²⁾ The significantly high rate of Gram negative bacilli in our study and many other studies probably indicates the

high incidence of prolonged hospital stay and the prolonged duration of mechanical ventilation that predisposes the patients to acquire infections from the multidrug-resistant pathogens. In contrast, other authors reported other bacterial strains as *Acinetobacter baumannii* and *Streptococcus*.⁽³³⁻³⁴⁾

The results of PCR assay for atypical bacteria of the DNA extract of the endotracheal aspirates of the 60 VAP patients revealed a total of 9 positive cases (15%) for the tested microorganisms, 5 cases were positive for *Mycoplasma pneumoniae* (8.3%), 3 cases were positive for *Legionella pneumophila* (5%) and only 1 case was positive for *Chlamydia pneumoniae* (1.6%).

Many were studies conducted for detection of atypical bacteria by PCR. Hassan et al reported detection of legionella and Chlamydia pneumoniae in VAP cases while no cases were positive for *Mycoplasma pneumoniae*.⁽²⁸⁾ Moreover, Bachinskaya et al reported that 9% of their patients were positive for *Mycoplasma pneumoniae* and 9% were also positive for *Chlamydia pneumoniae*.⁽³⁵⁾ In another study by Apfalter et al, where real time PCR was used as a fast diagnostic tool for non-conventionally cultured microorganisms, they reported that 3% of their cases were positive for *Mycoplasma pneumoniae* and 2% of cases were positive for *Chlamydia pneumoniae*. They concluded that *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* should be considered as causative agents in critically ill patients who develop early-onset nosocomial ventilator-associated pneumonia. Thus, empirical antimicrobial regimens should cover *Chlamydia*, and *Mycoplasma*.⁽³⁶⁾ Furthermore, El-Ebiary et al also diagnosed six cases of *Legionella pneumoniae* among patients with definite VAP. Using specific culture for *Legionella* and serology for *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Coxiella burnetii*, and *Chlamydia pneumoniae*, only

Legionella was diagnosed in 2 patients by serology and in 4 patients by culture.⁽³⁷⁾ Our results draw attention towards the possibility of these rarely diagnosed agents as being not infrequent causative agents for VAP. The prevalence of such atypical pathogens is to be taken into consideration while tailoring the empiric antimicrobial coverage of patients diagnosed with VAP.

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