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Microbiology of Ventilator-Associated Pneumonia

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Abstract

Ventilator-associated pneumonia (VAP) is a pulmonary infection that appears after 2 days of endotracheal intubation and when invasive mechanical ventilation is used. VAP is considered the most common nosocomial infection in the intensive care unit (ICU) and presents high morbidity and mortality rates, principally when caused by multi-resistant bacteria. Several risk factors are associated with VAP, including the microbiota, advanced age, immunocompromising conditions, pulmonary illness, length of mechanical ventilation, the aspiration technique, tracheostomy, supine positioning, enteral feeding, previous antibiotic exposure, among other endogenous and exogenous factors. The main pathogens are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and Enterobacteriaceae members, which are considered potentially multidrug-resistant pathogens. Conventional microbiology methods continue to be used for laboratory diagnosis. However, it is necessary to validate rapid and accurate laboratory methods, such as molecular assays that detect multiple gene sequences of a wide range of bacterial species and resistance markers. Therefore, the objective of this chapter is to review and update several aspects related to VAP, including risk factors, etiology, laboratory diagnosis, bacterial virulence and VAP severity, and antibiotic susceptibility.

Keywords: ventilator-associated pneumonia, respiratory infections, nosocomial infections, microbiology

1. Introduction

Pneumonia is a serious public health problem associated with high morbidity and mortality rates that leads to a significant increase in healthcare costs. It results from an infectious process of the lower airways through aspiration or inhalation of pathogenic microorganisms. It can be acquired in the community or in the hospital environment, after 48 h of admission [1].

Hospital-acquired infections usually have a high mortality rate (approx. 20%) when compared to the community acquisition (10%), this rate increases even more when it is associated with mechanical ventilation [2].

According to the guidelines of the American Thoracic Society, hospital pneumonia is divided into ventilator-associated pneumonia (VAP), which develops after 48–72 h of endotracheal intubation and the one that occurs in nonhospitalized patients, but that have constant contact with health services [3]. VAP is the infection of the pulmonary parenchyma with onset after 48–72 h of endotracheal intubation. Early-onset VAP occurs during the first 4 days of mechanical ventilation, whereas late-onset VAP occurs on 5 or more days of mechanical ventilation [4–7]. VAP corresponds to 70–80% of cases of hospital-acquired pneumonia in intensive care units [1].

VAP is characterized by the presence of new or progressive pulmonary infiltrates, systemic alterations such as fever and leukocyte alterations, altered sputum, and diagnosis of an infectious agent [8]. Mortality due to VAP is high, principally because of the association with multidrug-resistance (MDR) bacteria [9]. In pediatrics and neonatology, the frequency of VAP is 3–19%, with a mortality rate ranging from 10 to 20% of patients [10].

Many microorganisms can be involved in VAP. In this chapter, data on microbiology of VAP are reviewed, including risk factors, etiology, virulence features of main pathogens contributing to VAP severity, antimicrobial susceptibility, and laboratory diagnosis.
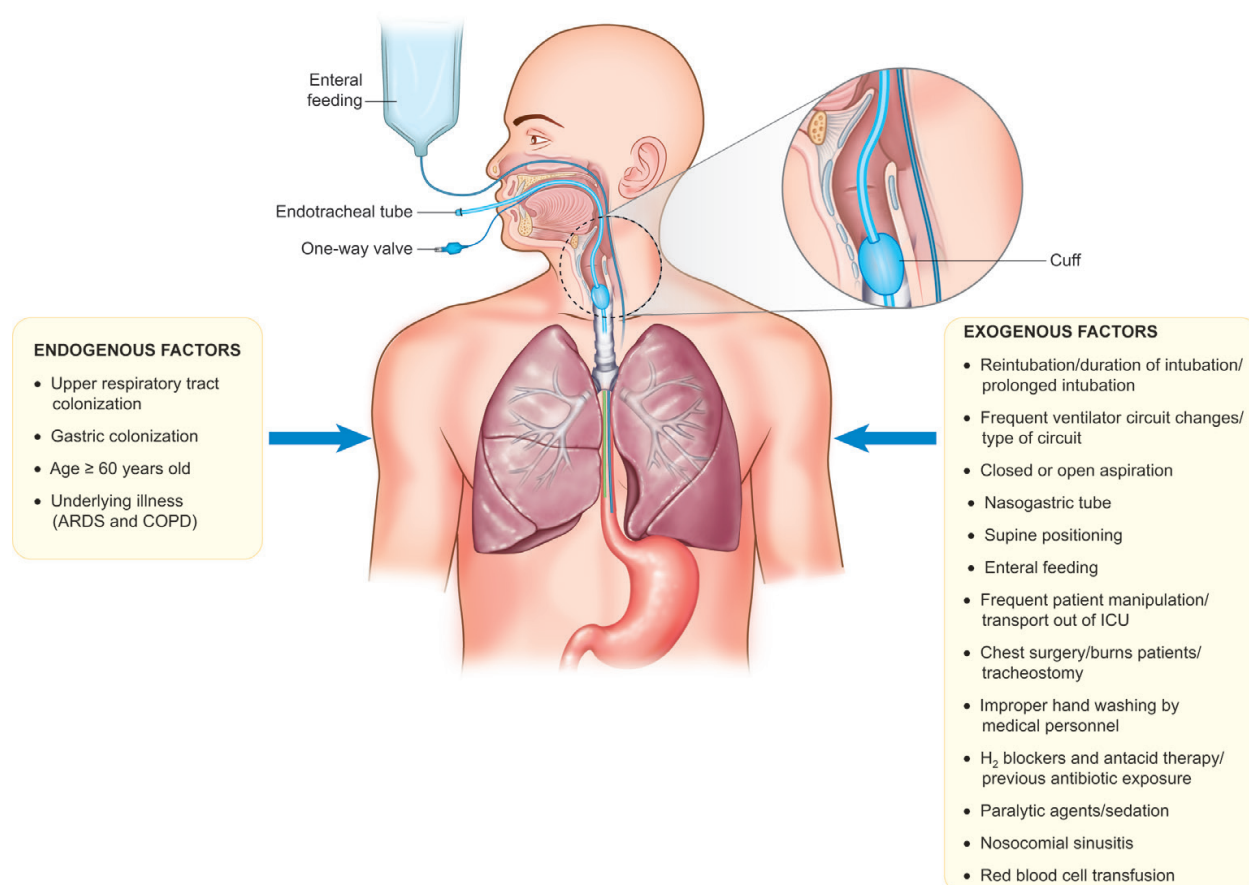
2. Risk factors for VAP

The use of mechanical ventilation is a significant risk factor for hospital-acquired pneumonia associated with aspiration, lowering of consciousness level, excessive management and patient transport, and chronic lung disease. The risk of VAP increases by 3% in the first 5 days of ventilation, 2% in 5–10 days, and 1% in 10 days of ventilation [11].

Although other routes may lead to VAP, such as hematogenous spread, inhalation of contaminated air, and also by extension of an infection of the pleural space, the main entry of pathogens into the lower respiratory tract occurs by aspiration of secretions containing microorganisms (from oropharynx or reflux of the stomach). Pathogens that cause VAP may be part of the upper airway microbiota or are acquired exogenously after hospital admission [8].

Figure 1 shows the different risk factors that are associated with VAP. Among risk factors inherent to the host (endogenous), it was observed that patients with advanced age, immunosuppressed individuals or pulmonary diseases have an increased risk for the development of VAP [4, 12, 13]. In a multicenter cohort study that analyzed the frequency of VAP among middle-aged, elderly, and very elderly patients, it was concluded that the highest frequency of VAP was in elderly patients (16.6%), associated to increased mortality among the elderly and very elderly (51%) when compared to middle-aged patients (35%) [4].

Long-term mechanical ventilation in patients with acute respiratory distress syndrome (ARDS) increases the risk of VAP [5]. Evaluation of the association between ARDS and VAP found that 55% of patients with ARDS developed nosocomial infection compared to 28%

Enteral feeding

Endotracheal tube

One-way valve

Cuff

ENDOGENOUS FACTORS

- Upper respiratory tract colonization
- Gastric colonization
- Age ≥ 60 years old
- Underlying illness (ARDS and COPD)

EXOGENOUS FACTORS

- Reintubation/duration of intubation/ prolonged intubation
- Frequent ventilator circuit changes/ type of circuit
- Closed or open aspiration
- Nasogastric tube
- Supine positioning
- Enteral feeding
- Frequent patient manipulation/ transport out of ICU
- Chest surgery/burns patients/ tracheostomy
- Improper hand washing by medical personnel
- H_2 blockers and antacid therapy/ previous antibiotic exposure
- Paralytic agents/sedation
- Nosocomial sinusitis
- Red blood cell transfusion

Figure 1. Main endogenous and exogenous risk factors for VAP.

without the syndrome [14]. Cigarette smoking, inhibition of mucociliary function, and reduction of cough reflex due to obstruction of airflow make chronic obstructive pulmonary disease (COPD) patients more susceptible to ventilation-associated infections [5].

The exogenous risk factors are due to the interventions undergone by the patient in intensive care units (ICUs) (**Figure 1**). Mechanical ventilation equipment is a primary source of infection, in which respiratory circuit condensations can be sources of microorganisms [8]. The endotracheal tube, as well as other invasive devices, promotes bacterial colonization of the trachea. Bacteria may have access to the lower respiratory tract through a partial blockage around the cuff or through the lumen of the endotracheal tube.

Prolonged intubation may promote the formation of a layer of microorganisms adhered to the inner surface of the endotracheal tube. This formation, known as biofilm, represents an important virulence mechanism and contributes to pathogen persistence as well as therapeutic failures, since microorganisms in the biofilm state are more resistant to host defenses and also metabolically less active, therefore, are more resistant to antibiotics [8, 15]. To inhibit the biofilm formation on the surface of polymeric medical devices, many studies have focused on the development of new biomaterials with modifications that alter the biophysical interactions of the cell surface or impede biofilm growth. A wide range of new coatings with antimicrobials, cationic antimicrobial peptides, or metal nanoparticles (e.g., copper, gold, iron,

magnesium, silver, titanium, or zinc) have been applied to medical devices such as endotracheal tubes [16, 17]. Other approach consists in the use of polymers that exhibit antimicrobial activity by themselves, with positively charged active groups (biguanide, cyclic *N*-halamine, quaternary ammonium, pyridinium or phosphonium salts, and polyionenes) or other polymers, such as synthetic poly(phenylene ethynylenes), polynorbornenes, and polymethacrylates that display similar antimicrobial activities of human peptides [17]. Both types of devices display advantages and disadvantages, but in the near future one expects to have nontoxic and biocompatible products available, which display broad-spectrum antibiofilm activities for the prevention of biofilm formation on endotracheal tubes [18].

The aspiration technique of endotracheal secretions also plays an important role as a risk factor for the establishment of VAP. The open method where a sterile aspiration probe is introduced has disadvantages such as loss of oxygenation, since the patient is temporarily disconnected from the ventilator and the system is opened with exposure of the patient, and the maximum duration of use of each circuit is not known [5].

Tracheostomy is an indicated procedure after 2 weeks of translaryngeal intubation of critically ill patients. Apparently, early tracheostomy may be associated with a lower incidence of pneumonia when compared to the late procedure or nonprocedure [19]. Frequent reintubations are also associated with VAP because of the risk of aspiration of gastric contents through the use of the nasogastric tube, subglottic dysfunction, and lowering of the level of consciousness [5].

The VAP prevention guidelines recommend the placement of the patient in the bed between 30 and 45° semi-reclined [20]. The supine position to which the patient is subjected may lead to lesions such as atelectasis in the dorsal lung region, barotrauma in the ventral lung region [5]. Experiments performed on rats proved the advantage of lateral decubitus in improving gas exchange, reducing gastroesophageal reflux, and avoiding pulmonary infection by gastric aspiration due to gravity [21]. Recently, the semi-decubitus position (30–60°) was shown to reduce the risk of VAP compared to supine positioning (0–10°) [20].

Nasal feeding by nasogastric tube increases gastric secretions and pH, leading to colonization by Gram-negative bacilli. Aspiration of this gastric content increases the risk of VAP. The use of sedative medications used in therapeutic procedures can cause prolonged relaxation of the muscles, increasing the risk of aspiration [5].

In addition, in the neonatal intensive care unit (NICU), some risk factors are associated with characteristics peculiar to this age group, including: length of stay in the NICU, enteral and parenteral feeding, blood transfusion, low birth weight, prematurity, and bronchopulmonary dysplasia [10, 13].

3. Etiology of VAP

VAP is usually caused by bacteria, whereas fungi and viruses are rarely involved [3, 6]. Generally, early-onset VAP is caused by pathogens more susceptible to antibiotics, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, and methicillin-susceptible *Staphylococcus*

aureus. On the other hand, late-onset VAP is usually caused by antibiotic-resistant bacteria, such as *Pseudomonas aeruginosa*, *Acinetobacter* spp., methicillin-resistant *S. aureus* (MRSA), and extended-spectrum β-lactamase producing Enterobacteriaceae, such as *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter* spp., among others [5–7]. However, some studies have reported that both susceptible and antibiotic-resistant microorganisms can have similar frequencies in early and late-onset VAP [22, 23].

In many cases, VAP can be caused by more than one pathogen (polymicrobial infection). This fact can be ignored sometimes when isolates are reported only as a percentage of the total number of isolates. In a recent study, performed in medical and surgical ICUs of a hospital in Spain, of 147 VAP patients, 32 (21%) had more than one pathogen associated. Interestingly, the clinical outcomes were not influenced by the polymicrobial etiology, when appropriate antibiotic therapy was administered [24].

The etiology of VAP varies in different countries and even between ICUs of the same city, distinct patients groups (like the ARDS patients, immunocompromised, and so on), or settings of the same hospital [25]. However, among Gram-negative bacteria, a high frequency is generally reported for *P. aeruginosa*, *Acinetobacter* spp., and Enterobacteriaceae members. Among Gram-positive isolates, *S. aureus* and *Streptococcus* spp. are considered as important pathogens [3, 5, 14, 25–27]. **Table 1** shows a list of the most frequently and also some uncommon microorganisms detected in VAP patients.

Microorganisms	Frequency (%)
(1) Gram-positive bacteria	
Staphylococcus aureus	20–32
Streptococcus spp.	2–8
Coagulase-negative staphylococci	1–2
(2) Gram-negative bacteria	
Pseudomonas aeruginosa	20–28
Acinetobacter spp.	4–13
Klebsiella pneumoniae	8–12
Escherichia coli	4–10
Haemophilus influenzae	4–8
Enterobacter spp.	6–7
Serratia spp.	2–4
Neisseria spp.	2–3
Stenotrophomonas maltophilia	2–3
(3) Other bacteria	<1 each
Anaerobes	
Corynebacterium spp.	

Microorganisms	Frequency (%)
Enterococcus spp.	
Moraxella spp.	
(4) Viruses	<1 each
Influenza virus	
Herpes simplex virus	
Cytomegalovirus	
(5) Fungi	<1 each
Aspergillus spp.	
Candida spp.	
Pneumocystis carinii	

Table 1. Frequency of etiologic agents of VAP.

4. Virulence of major pathogens and VAP severity

Clinical outcomes of VAP depend on a variety of factors, which are inherent to the patient, the hospital assistance, and also the microorganism, including host immune system status, underlying diseases associated, appropriate antibiotic therapy, accurate and rapid clinical and laboratory diagnosis, antimicrobial susceptibility, and virulence of the pathogen. Antimicrobial susceptibility is discussed in Section 5. Here, we present significance of major virulence factors associated with VAP severity of four selected pathogens: *Acinetobacter baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*.

4.1. *Acinetobacter baumannii*

There are more than 20 *Acinetobacter* species, with *A. baumannii* being the most commonly isolated in clinical settings, in which it represents important emerging nosocomial pathogen. *A. baumannii* is a Gram-negative bacterium, strictly aerobic, nonfermentative coccobacillus, nonmotile, nonpigmented, and catalase-positive. It is ubiquitous in nature and has been recovered from soil, water, and animals and found as part of the normal skin, throat, and rectal flora of human. Although a frequent colonizer, *A. baumannii* can be the cause of severe and sometimes lethal infections, frequently of nosocomial origin, principally VAP. A survey in U.S. hospitals showed that the majority of the isolates (57.6%) were from the respiratory tract, and *Acinetobacter* species ranked fifth as the causative organism of VAP (6.6%) [28–30].

In recent years, it has been designated a "red alert" human pathogen and has caused considerable concern in the medical community. This pathogen can adhere to surfaces, and it specifically targets moist tissues such as mucous membranes or skin that has been exposed due to accident or injury, and can cause a wide variety of infections. Most of the cases involve the respiratory tract, but bacteremia, meningitis, and wound infection may also occur. A recent systematic review and meta-analysis showed that some invasive procedures frequently used

in the ICU increase the risk of *A. baumannii* bacteremia: mechanical ventilation, central venous or urinary catheterization, and nasogastric tube use [31, 32].

The virulence of *A. baumannii* can be attributed to several factors: capacity to form biofilms; its ability to adhere, to colonize, and invade human epithelial cells; its antibiotic resistance mechanisms; and its ability to acquire foreign genetic material to promote its own survival under antibiotic and host selection pressures. Approximately 30% of *Acinetobacter* strains also produce an exopolysaccharide, which is a major virulence factor protecting bacteria from host defenses [28, 31].

4.2. *Klebsiella pneumoniae*

K. pneumoniae is generally considered an opportunistic pathogen that affects mainly immuno-compromised individuals. It can be found normally in the intestine, oral cavity, and skin, as well as in hospital settings and medical devices [33]. *K. pneumoniae* is able to form biofilms in catheters and endotracheal tubes, which represent major sources of infection in patients with invasive devices [34].

Infections by *K. pneumoniae* that involves biofilm formation tend to be persistent or chronic, since the biofilm protects the pathogen of the host immune response and also of the antibiotic action [35]. An additional risk factor for chronic infections caused by nosocomial strains includes resistance to multiple antibiotics, making difficult for the choice of suitable antibiotics for the treatment [36].

K. pneumoniae has about 78 capsular serotypes (or K antigens) [37]. Some of them present an increase in the production of the capsule and present very viscous colonies, which are called hypermucoviscous. Such isolates have also been considered to be hypervirulent because capsule is the most important virulence factor of *K. pneumoniae* [38].

A practical way to check if an isolate is hypermucoviscous is by using the string test. If there is the formation of a viscous chain greater than 5 mm after touching bacterial growth on agar and try to stretch it with a platinum loop. The degree of mucoviscosity correlates with the establishment of invasive infections. Hypervirulent *K. pneumoniae* is highly invasive and can affect previously healthy persons, causing fatal infections, including severe pneumonia among them [38]. The bacterium with the phenotype of hypermucoviscosity is capable of spreading from one organ to other organs (metastatic spread) [39].

The capsule consists of polysaccharides and is generally constituted by repeating units of three to six sugars [38]. The main functions assigned to it include: (1) protection of *K. pneumoniae* against opsonization and phagocytosis [40]; (2) interference with dendritic cells (DCs) maturation and, consequently, in the production of pro-Th1 cytokines mediated by DCs [41]; (3) anti-inflammatory effect by the inhibition of IL-8 expression [42–44]; and (4) reduction of the amount of antimicrobial peptides reaching the bacterial surface, thus, promoting resistance to them [45].

In addition to capsule, iron acquisition is a virulence property that also contributes to the persistence of the microorganism in the patient body and, consequently, to VAP, since iron is essential for bacterial growth. Pathogenic members of the Enterobacteriaceae family usually display

a variety of iron uptake systems, of which at least 12 have been described in *K. pneumoniae*. Isolates associated with pulmonary infections also produce yersiniabactin and salmochelin, which are not sequestered by the host protein lipocalin 2 of the innate immune defense [46, 47]. Additionally, hypervirulent *K. pneumoniae* produces a higher amount and more active siderophore molecules than classical *K. pneumoniae*, which increases its pathogenic potential [48].

4.3. *Pseudomonas aeruginosa*

VAP caused by *P. aeruginosa* has been associated with higher case fatality rates than that by other bacteria. This pathogen is a noninvasivefermenting Gram-negative, aerobic, rod-shaped polar-flagella, with unipolar motility. *P. aeruginosa* is considered emerging as an important nosocomial pathogen worldwide and is responsible for an extensive spectrum of infections in humans associated with significant morbidity and mortality. It is an opportunistic pathogen that is normally found in plants, soils, and in a variety of aquatic environments. The adaptability and high antibiotic resistance allow it to survive in a wide range of other natural and artificial settings, including surfaces in medical facilities. In addition, *P. aeruginosa* is recognized for its ability to form biofilms and directly increase the VAP-induced lung injury. In the United States, *P. aeruginosa* is among the most common hospital pathogens and is the second most common pathogen isolated from patients with VAP and has been associated with prolonged hospitalization, increased cost, and mortality [49–52].

Cell surface virulence factors of *P. aeruginosa* play an important role in colonization of the lower respiratory tract. These factors include *flagellum*, *pili* or fimbriae, lipopolysaccharide (LPS), as well as type III secretion system (T3SS), which is a major determinant of virulence. The T3SS expression is frequently associated with acute invasive infections and has been linked to increased mortality in infected patients, and it is shared among many pathogenic Gram-negative bacteria as a means of injecting toxins directly into host cells [49, 53].

Additionally, several proteases are produced by *P. aeruginosa*. These proteases have established roles in distinct infectious process, such as hydrolysis of immunoglobulin, fibrin, fibrinogen, and also disruption of epithelial tight junctions. Main *P. aeruginosa* proteases include pyocyanin, which induces damage to the respiratory tract, such as epithelial necrosis and reduced ciliary movement; pyoverdin, its main secreted siderophore; protease IV, a serine protease responsible to degradation of complement proteins, fibrinogen, immunoglobulin G, and plasminogen; elastase and metalloproteinases that degrade elastin, collagen types III and IV, surfactant, immunoglobulins, complement factors, and cytokines; and exotoxin A, one of the most potent toxins with cytopathic activity, among others, such as *quorum-sensing*, a very sophisticated gene regulatory mechanism that allows bacteria to coordinate activity through the production of small diffusible molecules. These functions include the formation of biofilms, motility, secretion of virulent factors, and exopolysaccharide production [49, 54].

4.4. *Staphylococcus aureus*

S. aureus strains produce several virulence factors that contribute to the pathogenesis and severity of lower respiratory infections. Some of them can hinder host defenses, such as protein

A, coagulase, leukocidin, and γ-toxin [55]. Protein A is an important virulence factor in the pathogenesis of experimental staphylococcal pneumonia in mice [56]. Moreover, protein A mediates: (1) invasion across airway epithelial cells through activation of RhoA GTPase signaling and proteolytic activity; (2) binding to tumor necrosis factor receptor 1 (TNFR1) on lung epithelial cells, and (3) activation of a specific intracellular signaling causing the recruitment of neutrophils. These activities increase inflammation of the airway epithelium and, thus, contribute to tissue damage [57].

Cysteine proteases, in particular staphopain A (ScpA), cleave the pulmonary surfactant protein-A (SP-A), a major surfactant component with immune functions that is important during *S. aureus* infections [55]. Additionally, *S. aureus* releases enzymes with significant roles as virulence factors, including proteases, nucleases, lipases, hyaluronidase, and staphylokinase that facilitate the invasion of the infected tissue [58].

Interestingly, *S. aureus* display a great ability to subvert innate and adaptive immune responses to favor its replication [59]. In some situations, such as in immunocompromising conditions, there is a higher susceptibility to acquire *S. aureus* infection, mainly by hospitalized patients. In this context, *S. aureus* and especially the epidemic methicillin-resistant *S. aureus* strains cause severe necrotizing pneumonia by producing Panton-Valentine leukocidin (PVL) that has been reported to cause rapidly progressive necrosis of the lung tissue in young immunocompetent patients. The severity of disease, survival, and clinical outcome of VAP patients can also be associated with the presence of the Panton-Valentine leukocidin genes in MRSA [60]. The role of PVL in the pathogenesis of MRSA infection is not clear, but recently, it was demonstrated that the PVL have strong affinity for host extracellular matrix proteins being, therefore, implicated as a *S. aureus* adherence molecule. Moreover, PVL as a cytotoxin targets human polymorphonuclear neutrophils, and monocytes or macrophages, or both, leading to their apoptosis or necrosis as result of the Bax-independent apoptosis occurring by means of a novel pathway that presumably involved PVL-mediated pore formation in the mitochondria membranes.

5. Antimicrobial susceptibility and management of patients

Choosing an initial antibiotic for suspected VAP is a difficult task. A scheme of empiric antibiotic therapy must take into account that *S. aureus*, *P. aeruginosa*, *Acinetobacter* spp., and Enterobacteriaceae members together represent more than 80% of VAP cases worldwide and several strains are defined as MDR pathogens [26]. To provide suitable antibiotic exposure regarding the possibility of infection by MDR pathogens, the empiric therapy should contain multiple agents with broader spectrum of activity [25].

However, antibiotic choices should be based on local prevalence and the antimicrobial susceptibility profile of the usual pathogens, since data from guidelines or other hospitals can be ineffective [61]. For empiric MRSA coverage, vancomycin or linezolid are strongly recommended. On the other hand, if it is indicated as MSSA coverage, the following antibiotics should be used: piperacillin-tazobactam, cefepime, levofloxacin, imipenem, or meropenem. Suspected etiology for MRSA or MSSA should be based on the presence of risk factors [61].

It has been reported that more than 50% of MRSA are also resistant to macrolides, lincosamides, fluoroquinolones, and aminoglycosides. This high level of resistance not only impedes successful therapy but also allows the microorganism to persist in the hospital, expanding its reservoir. So, vancomycin is the first-line treatment to VAP patients caused by MRSA. Nevertheless, some studies have described *S. aureus* strains with decreased susceptibility to vancomycin (vancomycin intermediate-resistant *S. aureus*, VISA). The acquired-resistance of MRSA to vancomycin is related to acquire mutations that appear in MRSA during vancomycin therapy [62, 63]. More recently, studies describing MRSA strains with high-level vancomycin resistance (vancomycin-resistant *S. aureus*, VRSA) were described. The mechanism of resistance is associated to the presence of transposon Tn1546, acquired from vancomycin-resistant *Enterococcus faecalis*, which is known to alter cell wall structure and metabolism, but the resistance mechanisms in VISA and VRSA isolates are less well defined [62].

Antibiotic options for Gram-negative coverage are more varied and must contain two anti-pseudomonal antibiotics from different classes in the presence of risk factors for MDR pathogens for the initial treatment of suspected VAP. If the patient does not present risk factors for MDR pathogens, only one anti-pseudomonas drug should be prescribed [61].

The frequency of infections caused by *P. aeruginosa* has increased in combination with the morbidity and mortality among hospitalized patients, all of which are exacerbated by antimicrobial resistance. Studies have demonstrated that resistance to carbapenems, aminoglycosides, and fluoroquinolones has increased gradually over the past few years, as well as episodes caused by MDR strains. Many *P. aeruginosa* isolates display an intrinsic reduced susceptibility to several antibacterial agents, as well as a tendency to develop resistance during therapy, especially in carbapenem-resistant strains. The most common mechanism of imipenem resistance in *P. aeruginosa* is a combination of chromosomal AmpC production and porin alterations. It also produces extended-spectrum β-lactamases (ESBLs) and can harbor other antibiotic resistance enzymes such as *K. pneumoniae* carbapenemases (KPC) and imipenem metallo-β-lactamases. β-Lactamase production, especially ESBLs, remains the main factor to acquired β-lactam resistance [52, 64, 65].

K. pneumoniae may present two major types of antibiotic resistance: (1) expression of ESBLs, which make them resistant to cephalosporins and monobactams and (2) the expression of carbapenemases that make *K. pneumoniae* resistant to almost all available β-lactams, including carbapenems. The first reported of carbapenemase by *K. pneumoniae* was in the USA, in 1996, which was designated KPC. Currently four classes of carbapenemases (classes A–D) have already been described and KPCs are classified into class A. To date, 16 KPC class A variants have already been identified. In addition to KPCs, *K. pneumoniae* strains may carry other forms of carbapenemases, such as class B metallo-β-lactamases (such as New Delhi's metallo-β-lactamase NDM-1 enzymes) and OXA class. In addition to β-lactamases, mutations in outer membrane proteins (OMPs) may also make the bacterium more resistant to β-lactams, particularly if it was in combination with the expression of a carbapenemase [66].

A. baumannii is also considered an emerging cause of nosocomial outbreaks, especially by MDR strains in ICUs. The most significant mechanism of carbapenem resistance in *A. baumannii* is the production of carbapenemases, which can be either intrinsic or acquired. Carbapenems

have been considered the agents of choice for infections caused by susceptible pathogens, but the rapid increase in carbapenem resistance rates has complicated this issue. Other mechanisms include: changes in OMPs, penicillin-binding proteins, and efflux pumps; resistance to aminoglycosides, mediated by aminoglycoside phosphotransferases, acetyltransferases, and adenyltransferases; resistance to quinolones, polymyxins, tetracyclines, among others [28].

A recent cohort study of bacteremia associated with pneumonia found that inappropriate initial antibiotic treatment seems to be the most important independent determinant of mortality and is the only identified mortality predictor amenable to intervention [67]. These Gram-negative bacteria are responsible for increasing numbers of infections encountered in hospitals, particularly among immunocompromised patients, and community-acquired infections are also increasing in prevalence. Furthermore, the impact of *P. aeruginosa* and *A. baumannii* resistance on health systems is a major concern in hospitals worldwide.

6. Laboratory diagnosis

The diagnosis of VAP is usually based on clinical, radiographic, and microbiological criteria. Microbiological diagnosis is important in the management of VAP, since early diagnosis can influence clinical outcomes. The usual methods for microbiological diagnosis are based on quantitative or semiquantitative culture, but the results can take 48 h or more to be available. The Gram stain method has been used as screening of infection and to guide initial antibiotic therapy. However, utility of microscopy examination of respiratory secretions is still controversial.

Molecular methods can also be used to obtain results more quickly and initiate rational antibiotic therapy of patients with VAP. Many method formats are available for the detection of target genes for microbial identification and also for the detection of antimicrobial resistance genes.

6.1. Culture

Semiquantitative culture of endotracheal aspirates (ETA) is the recommended microbiological procedure to diagnose VAP, since it is more sensitive and can be done more rapidly. Other biological specimens have been used, including the ones obtained by invasive sampling, such as: bronchoalveolar lavage (BAL), blind bronchial sampling (mini-BAL), and protected specimen brush (PSB). Blood cultures should also be performed for all patients with suspected VAP. In all cases, samples should be obtained before the patients initiate antibiotic therapy [61].

The main problem with the semiquantitative culture of ETA is that its high sensitivity promotes the unnecessary prescription of antibiotics to some patients. In the case of quantitative cultures of lower respiratory tract secretions, the following threshold cut-offs are usually applied to diagnosis true infection: ETA 10^5–10^6, BAL 10^4, and PSB 10^3 CFU/mL. This strategy may lead to false-negative results and worse clinical outcomes in some patients [61].

6.2. Gram stain

The Gram stain of respiratory specimens can provide rapid information regarding morphological aspects of the bacterial pathogen and whether it is Gram-positive or Gram-negative. Additionally, microscopic examination may reveal whether the smear is suggestive of infection. It is generally accepted as active infection when the biological sample has more than 25 neutrophils and less than 10 epithelial cells per 10× low-power field.

A decision strategy based on the results of Gram stain was proposed to assist the clinician in the empirical prescription of antibiotics [68]:

- **If the Gram stain of the ETA is negative:** Antibiotic prescription can wait until the microbiological culture result is available, since it is very unlikely that the patient have VAP.

- **If the Gram stain of PSB is positive**: The antibiotic therapy can be initiated and based on the result of Gram stain, since it is very likely that the patient has VAP. Later, it can be adjusted according to the culture result.

- **If the Gram stain of PSB is negative and the Gram stain of the ETA is positive:** The antibiotic therapy may only be initiated depending on the severity of the patient's clinical condition or when the VAP is confirmed by the culture.

Nevertheless, the utility of Gram staining in the diagnosis of VAP and as a guide for the antibiotic empirical therapy of VAP is a very controversial subject. It is usually accepted that this old diagnostic tool has a high negative predictive value, i.e., VAP is unlikely with a negative Gram stain [69].

6.3. Molecular methods

Several molecular-based methods have been proposed for the detection of respiratory pathogens that offer a reliable diagnosis, with high sensitivity and specificity. Most of them are nucleic acid-based amplification methods that identify, simultaneously, multiple and specific target gene sequences (multiplex assays) of a wide range of bacterial species and resistance genes [70, 71].

Considering that the etiology of the VAP is very different from the community-acquired pneumonia, some main potential gene targets are *mecA* gene in *S. aureus*; bla_{VIM} and bla_{IMP} genes in *P. aeruginosa*; bla_{OXA} genes in *Acinetobacter* spp.; and bla_{KPC} gene in members of the Enterobacteriaceae family, in addition to the detection of *Stenotrophomonas maltophilia* [72].

Currently, a variety of platforms or systems are available to identify respiratory pathogens using distinct technologies. Some molecular diagnostic systems detect a small number of microorganisms, such as GeneXpertMRSA/SA that detects MRSA and MSSA. On the other hand, IRIDICA and MALDI-TOFI can detect a wide range of pathogens and resistance markers. **Table 2** shows the major commercial systems available to detect respiratory pathogens, including bacteria, viruses, and fungi.

Depending on the methods, the advantages of molecular methods include rapid results; detection of very low amounts of gene sequences; target sequences to identify the agent and/

Systems	No. of pathogens/markers	Technology
Abbot IRIDICA System	780 bacteria, 200 fungi, 13 viruses, and 4 resistance markers	PCR/ESI-MS
Accelerate PhenoTest™ BC kit	27 bacteria and 2 yeasts and AST	FISH
Amplidiag® CarbaR+VRE	5 carbapenemase and 2 vancomycin-resistance markers	Multiplex RT-PCR
CE-IVD HAI BioDetection kit	12 most common nosocomial pathogens and 15 resistance markers	NSG
Curetis Unyvero™	16 bacteria, 1 fungus, 18 resistance markers	PCR
FilmArray® Respiratory Panel	17 viruses and 3 bacteria	Multiplex RT-PCR
FTD Bacterial pneumoniae HAP	Detection and quantification of *K. pneumoniae* and *P. aeruginosa*	Multiplex RT-PCR
GeneXpertMRSA/SA	Only MRSA and MSSA	RT-PCR
MALDI-TOFI	Wide spectrum of bacteria and fungi	MS
NxTAG® Respiratory Pathogen Panel	18 viruses and 3 bacteria	Multiplex RT-PCR
R-Biopharm RIDA® GENE-kits	*mecA/mecC*, SCCmec cassette, and *S. aureus*	Multiplex RT-PCR
Verigene® Respiratory Pathogens Flex Test	Up to 13 viruses and 3 bacteria (customized)	Multiplex RT-PCR

AST: antimicrobial susceptibility testing; FISH: fluorescence *in-situ* hybridization; MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight; MRSA: methicillin resistant *S. aureus*; MSSA: methicillin sensitive *S. aureus*; MS: mass spectrometry; NGS: next-generation sequencing; PCR: polymerase chain reaction; PCR/ESI-MS: PCR-electrospray ionization mass spectrometry; RT-PCR: real time-PCR.

Table 2. Commercial molecular systems for detection of respiratory pathogens and resistance markers.

or the resistance gene markers; possibility to search for multiple agents and resistance markers; direct detection in clinical specimens; and higher sensitivity. On the other hand, among important drawbacks are: most of them are qualitative, risk of contamination, high costs, and lack of validation.

6.4. Exhaled breath metabolomics

Recent advances in diagnostic technologies have pointed to metabolomics as an emerging and faster method to aid in the diagnosis of various diseases, such as cancer, asthma, among others. The procedure can be performed with samples such as plasma and also with noninvasive samples, such as exhaled air and saliva. Results can return within a matter of hours, compared with days of conventional culture. In the case of exhaled air, the method consists in determining the profile of volatile organic compounds (VOCs) emitted by the patient through respiration [73]. These metabolic degradation products present in the expired air are derived from the patient and the pathogen. The VOC profile is detected through sensitive procedures such as nuclear magnetic resonance spectroscopy [74] and gas chromatography-mass spectrometry [75]. Studies in patients with VAP have allowed the determination of distinct VOC patterns in clinical cases associated to different pathogens, showing good correlation with the microbiological culture and offer great potential as biomarkers [76, 77].

Major drawbacks of this method include: (i) the sampling methodology, which should enable to sample from beyond the endotracheal tube and hence to exclude air from the upper respiratory tract; (ii) discovery of more pathogen-specific metabolites; and (iii) the need of trained personnel to operate the analytical methodology by gas chromatography-mass spectrometry apparatus.

7. Final considerations

One of the main problems of VAP is the lack of a gold standard for rapid and reliable diagnosis. Mortality associated with VAP remains high, mainly because of the increasing prevalence of MDR pathogens and their resistance profiles vary depending on the patient group and the hospital setting. However, significant progress has been obtained in the development of systems or platforms for molecular detection of respiratory pathogens, which are feasible to be applied to the routine diagnosis of VAP. Additionally, metabolic profiling of exhaled breath will aim to speed up the process after refinement of the sampling methodology and discovery of highly discriminatory biomarkers. With the validation and implementation of these methods for diagnosis, probably a more adequate control of VAP will be obtained. Especially, because the early and appropriate use of antibiotics may result in reduced mortality among patients under mechanical ventilation. Although, it is important to observe the guidelines for patient management, antibiotic therapy must be based on local prevalence and microbiology data.

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