Chapter 1

General introduction

1. History of bronchoalveolar lavage as a diagnostic and research tool

Since the introduction of the rigid bronchoscope by Dr. Jackson in 1904, bronchoalveolar lavage (BAL) had become an increasingly important tool in pulmonary diseases. Primary, BAL was used as a treatment for patients who suffered from diseases associated with accumulation of purulent secretions such as alveolar proteinosis, cystic fibrosis and bacterial pneumonia[1]. Large volumes of saline (15-30 l) were instilled over 2 to 3 hours to clear the lungs of excess secrete. This “large volume” BAL was commonly known as: bronchioloalveolar debridement[2].
Between 1960 and 1970, the introduction of “small volume” (instillation of 300 ml sterile saline) BAL followed. Tested on healthy volunteers, it was found to be a save method, providing extensive information on cellular components of normal BAL fluid (BALF)[3,4]. After the introduction of the flexible scope in 1970, a increase in implementation and use of BAL was seen. The increase in interest in BAL as a research tool was reflected by the increase in publications (40-fold) on BAL in the period 1970-1990[5]. In recent years, research output dealing with BAL has reached a plateau at approximately 500 papers annually[5].

2. Description of bronchoalveolar lavage

By definition BAL is a method for the recovery of cellular and non-cellular components from the lower respiratory tract (e.g. alveoli)[6]. It is a safe technique, with few major complications[7]. In many cases (e.g. pulmonary proteinosis, alveolar hemorrhage, eosinophilic pneumonia) BAL can replace lung biopsy[8]. Possible uses of BAL in diagnostics are summarized in Table 1.1.

2.1 Technical aspects of bronchoalveolar lavage

Guidelines and recommendations on the technical aspect of BAL have been published by the European Respiratory Society (ERS) Task Force in several reports[9,10]. Premedication usually consists of a sedating compound (diazepam) with a compound that causes dilatation of the bronchi (atropine) and local anaesthesia by application of lidocaine.

Table 1.1 Pulmonary diseases where BALF can be used to reach a diagnosis[5,72,107].

Non-infectious
Sarcoidosis
Hypersensitivity pneumonitis
Idiopatic lungfibrosis
Connective tissue disorders
Langerhanscell histiocytosis
Malignancies
Alveolar hemorrhage
Alveolar proteïnosis
Eosinofilic pneumonia
Bronchitis obliterans with organizing pneumonia
Asbestosis
Silicosis

Infectious
(Ventilator-associated) pneumonia
Pneumocystis pneumonia
Mycobacterial infection
Aspergillus fumigatus infection
Viral pneumonia
Toxoplasma pneumonia
Legionella infection
Mycoplasma pneumoniae pneumonia
Chlamydia pneumoniae pneumonia
Cryptococcal infection
Histoplasma infection
Strongyloïdes infection

2.2 Site of lavage

The site of lavage depends on the localization of the abnormalities. In case of localized disease, for instance an infection with a radiographically apparent infiltrate or a malignancy, the involved segment should be sampled[11]. In patients with diffuse lung disease, the middle lobe or lingula is most commonly used site to be lavaged[10] since anatomically this is the most accessible site and the fluid obtained at one site is representative of the whole lung in diffuse lung diseases (inflammation is not limited to one site)[10]. Using the method described, approximately 1.5-3% of the lung (approximately 1,000,000 alveoli) are sampled[6].

2.3 Fluid used

Usually, the lavage is performed using sterile saline (0.9% NaCl). Preferable the saline is preheated to body-temperature (37ºC) to help prevent coughing and to, slightly, increase cellular yield[12]. However, this is not necessary and therefore many institutions use saline at room temperature[13,14].

The volume of sterile saline instilled differs between institutions, the volume varies between 100 and 300 ml in aliquots of 20 to 50 ml[15], the ERS task force recommended the use of 200-240 ml divided in four aliquots.

2.4 Fluid instillation and recovery

The fiberoptic bronchoscope is wedged into a subsegmental bronchus. The fluid is instilled through the bronchoscope and almost immediately recovered by applying suction (25-100 mmHg). Each aliquot is aspired into a separate syringe or trap[6]. Recovered aliquots are consecutively numbered.
Usually 60-70% of the instilled volume is recovered in healthy volunteers[16,17], in smokers, patients with underlying pulmonary disease and ventilated patients, the recovery rate is lower[18,19].

3. Laboratory processing of BALF

BALF is processed immediately upon arrival at the laboratory. Since the first aliquot is usually poorly recovered and reflects a disproportionate amount of bronchial material[20], this fraction is used it for mycobacterial investigation only. The other three fractions are pooled and processed. Complicated and time-consuming procedures, such as filtration, lysation and resuspending of BALF can be avoided in order to sustain the cell morphology and to facilitate 24-hour service from the laboratory. Equal parts of fractions I, II and III are pooled and used for the further investigation, such as total cell count, cytospinpreparations, quantitative culture and determination of soluble factors. When indicated additional tests can be included such as: detection of fungi, *Legionella pneumophila*, viruses and detection of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* by means of polymerase chain reaction (PCR).
The total cell count is performed by using a Fuchs-Rosenthal hemocytometer, every nucleated cell is counted. A BALF sample retrieved from a healthy person contains 150.000 tot 300.000 cells per ml.

3.1 Quality control of BALF

To ensure that the obtained material represents the situation in the alveoli, a number of criteria have been established. A BALF is regarded non-representative if it fulfills one of the following criteria: i) volume <20 ml, ii) total cell count <60.000 cells/ml, iii) presence of >1% squamous epithelial cells, iv) presence of >5% bronchial epithelial cells, v) presence of extensive amounts of debris, vi) severely damaged cell morphology.

3.2 Cytocentrifugation

All preparations are made by cytocentrifugation using a Thermo-Shandon Cytospin 3 (Thermo Electron's Anatomical Pathology Group, Astmoor, England) following a standardised protocol[21,22] using pre-cleaned slides. The amount of BALF (in drops) used is dependent on the number of cells per ml in the pooled fraction (Table 1.2). The program used follows a centrifugationspeed of 650 rpm ($\approx 40 \times g$) with a low acceleration for 10 minutes. Using cytospinpreparations shows a few advantages over smear preparations. First of all, the cytospinpreparations are dry when they are taken out of the centrifuge, and can be fixed and stained immediately. Secondly, due to the monolayer, the preparation will stain equally and the contents of the cell and the nucleus are perfectly visible (Figure 1.1). The third advantage lies in the fact that a relatively small and easy-view preparation needs to be examined, making it easy to investigate the whole preparation. However, there is a small disadvantage, BALF differential cell counts on cytocentrifuged preparations can underestimate the proportion of lymphocytes[23].

Table 1.2 Cytocentrifugation parameters of the Cytospin 3 in the work-up of BALF.

Cells/ml	Number of drops	Acceleration	Speed	Time
< 50.000	7	low	650 rpm	20 min
50.000 – 100.000	5 – 4	low	650 rpm	10 min
10.000 – 200.000	4 – 3	low	650 rpm	10 min
200.000 – 300.000	3	low	650 rpm	10 min
300.000 – 400.000	3 – 2	low	650 rpm	10 min
400.000 – 500.000	2	low	650 rpm	10 min
> 500.000	Dilute with NaCl 0.9% to 3 drops final volume	low	650 rpm	10 min
BALF with excess amount of blood	use 1 drop less than mentioned above	low	650 rpm	10 min

(rpm = revolutions per minute)

3.3 Stains performed on BALF

Many stains can be used in the work-up of BALF. After extensive research the following stains were implemented in our hospital described below. A number of stains are routinely performed on each BALF sample. These include the Gram-stain (one preparation) and the May-Grünwald Giemsa (MGG) (three preparations) stain. Before Gram-stain is performed there is a two minute fixation step with absolute methanol. Unfixated preparations are used for the MGG stain. After staining, each preparation is sealed by using a drop of Shandon-mount (Thermo Electron's Anatomical Pathology Group) and a covering slip. This ensures that the quality of the preparation does not

deteriorate by the oil used for the oil-immersion lens, and that no damage occurs during use so that they can be stored indefinitely. Besides the standard stains, a number of stains can be added to the work-up upon clinical indication. For example: Grocott (methenamine-silver)(fungi/*Pneumocystis jiroveci*), Auramine-Rhodamine, Ziehl-Neelsen (acid-fast bacteria), Legionella immuunfluorescence (*Legionella spp*), Acridine-orange (micro-organism in general) and the iron stain.

Figure 1.1 Gram stained preparations of a BALF sample, showing that by using cytospin preparations (left) the nucleus and the cell contents are better visualized compared to smear preparations (right). Magnification 400×.

3.4 Cytological composition of BALF

In a healthy individuals the cell population of BALF consists of 80 to 90% alveolar macrophages, 5 to 10% lymphocytes and the remaining 1 to 2% are polymorphonuclear granulocytes, eosinophils and mast cells.

3.4.1 Alveolar macrophages

Alveolar macrophages (AMs, Figure 1.2, 1.3) are mononuclear cells with a diameter of 10 µm to 40 µm. They have a large amount of light colored cytoplasm which is equally divided round the nucleus. The nucleus is round to oval. The cytoplasm can contain all sorts of phagotized material such as hemosiderine, carbon, micro-organisms and debris. In some diseases (for instance drug-induced pneumonitis) macrophages contain many small vacuoles in their cytoplasm, they are called “foamy” alveolar macrophages.

Figure 1.2 May-Grünwald Giemsa stain. Three alveolar macrophages (center) surrounded by polymorphonuclear neutrophils. One lymphocyte is present in the upper left corner. Magnification 1000×.

Figure 1.3 May-Grünwald Giemsa stain. Five eosinophils are present in the center. Eosinophils have a nucleus that usually consists of two lobes and their cytoplasm contains orange granulae. In the top left and right corner, lymphocytes are present. In the lower left corner part of an alveolar macrophage is visible. Magnification 1000×.

3.4.2 Lymphocytes

Mature lymphocytes (Lyms, Figure 1.2, 1.3) are the smallest nucleated cells present in BALF. They contain a relatively large nucleus and little cytoplasm. The nucleus is round, sometimes with some indentations and has a dark purple color. Often only a small rim of light blue cytoplasm is visible. Activated Lyms are larger than mature Lyms and contain more cytoplasm which varies from

pale blue (near the nucleus) to dark bleu (near the cell border). Often the cell border has a somewhat irregular appearance.

3.4.3 Polymorphonuclear neutrophils

Polymorphonuclear neutrophils (PMNs, Figure 1.2) have an average diameter of 12 to 15 µm. Their nucleus has an irregular shape and consists of two to five lobes (average 3). The cytoplasm is slightly pink colored and contains numerous pink granules. An influx of PMNs can been seen in the lungs of patients after traumatic insertion of a bronchoscope, in ventilated patients an patients with infectious disorders.
Necrobiotic neutrophils are smaller than PMNs, with only one, compact dark colored nucleus. They are degenerated PMNs which can be seen in case of longstanding inflammation.

3.4.4 Eosinophils

Eosinophils (Eos, Figure 1.3) have approximately the same size as PMNs, but their nucleus usually consists of two, round, lobes. Their cytoplasm is colorless and contains orange granules which are larger than those seen in the PMNs. An increased number of Eos can be seen in patients with *Pneumocystis jiroveci* pneumonia, asthma, drug-induced pulmonary disease, eosinophilic pneumonia, extrinsic allergic alveolitis and idiopathic pulmonary fibrosis[24].

3.4.5 Mast cells

Mast cells (MCs) are large cells with blue or purple-red granules in their cytoplasm which cover the nucleus completely. They are uncommon cells to be found in BALF. Increased number of MCs can be encountered in patients with asthma, bronchiolitis obliterans or farmer's lung disease[25,26].

3.4.6 Bronchial epithelial cells

The presence of bronchial epithelial cells indicates contamination from the higher bronchial tract. They can be divided into:
Ciliated epithelial cells (Figure 1.4):
The ciliated epithelial cells are easily noted columnar cells with a basal nucleus, a pale cytoplasm and a distinctive endplate with a bunch of cilia. Loose cilia can sometimes be confused with slim Gram-negative rods.

Figure 1.4 May-Grünwald Giemsa stain. A group of ciliated epithelial cells. These are columnar cells with a basely located nucleus and cilia at the apical end. Magnification Magnification 1000×.

Goblet cells:
The mucus secreting Goblet cells are more difficult to recognise. They are also pale, elongated cells with a nucleus at the base. The cytoplasm extends above the nucleus in a shape like that of a wine goblet.

3.4.7 Squamous epithelial cells

Presence of squamous epithelial cells in BALF points to oropharyngeal contamination of the BALF. They are large, square, flat, angular cells with a dark colored, relatively small nucleus. Often they are covered with bacteria and can therefore influence the specificity of the quantitative culture in a negative way.

3.4.8 Reactive type II pneumocytes

Type II pneumocytes are surfactant producing cells present at the inside of the alveoli (alveolar lining cells). In normal circumstances these cells are not seen in BALF, or are indistinguishable from alveolar macrophages. However, in case of serious pulmonary damage, they can be found in BALF as reactive type II pneumocytes (RPII, Figure 1.5.). RPII cells are large cells which can be easily spotted, even at a low magnification. They have a small, dark colored nucleus. Their cytoplasm has a dark color and contains large vacuoles. Often they cluster together giving them a glandlike appearance. Reactive type II pneumocytes resemble malignant cells. These cells can be seen in patients with, for instance, acute respiratory distress syndrome (ARDS)[27] or pneumocystis pneumonia[28].

Figure 1.5 May-Grünwald Giemsa stain. Reactive type II pneumocytes (center) are large cells which can be easily spotted, even at low magnification, they have dark cytoplasm which contains vacuoles. Three macrophages are present (one top right corner, two low left corner) together with four polymorphonuclear neutrophils Magnification 1000×.

3.4.9 Intracellular micro-organisms

Infected cells or intracellular organisms (Infected cells, IC, Figure 1.6), are cells which have phagotized micro-organisms. The majority of these cells are PMNs, however in a small percentage of cases alveolar macrophages are also involved. Different stains can be used for the evaluation of the percentage of IC in BALF, the May-Grünwald Giemsa stain was found superior over the Gram-stain and the acridine-orange stain, resulting in the highest reliability[29]. An additional advantage of the MGG stain is the fact that the percentage IC can be enumerated at the same time as performing the differential cell count. The presence of ≥2% IC per 500 nucleated cells in the MGG stain points to the diagnosis ventilator-associated pneumonia (VAP) in a patient on ventilation[30].

3.4.10 Plasma cells

Plasma cells (PCs) are cells with a diameter of 8 to 20 µm, they have a similar appearance as Lyms, but with a round to oval nucleus which lies eccentric. Their cytoplasm is stained intense blue with a clear zone in the vicinity of the nucleus. Plasma cells can be seen in BALF of patients with pneumocystis pneumonia, drug-induced pulmonary disease, extrinsic allergic alveolitis and malignant lymphomas.

Figure 1.6 May-Grünwald Giemsa stain. In this figure infected cells are shown. Infected cells are cells that have phagotised micro-organisms. Magnification 1000×.

3.5 The differential cell count

The differential cell count is performed on MGG stained preparations. When screening at low magnification (magnification: 100×), the presence of squamous epithelial cells, clusters of RPII cells, large clusters of *P. jiroveci* and mucus plugs can be noted. The differential cell count is performed using a magnification of 1000× and a standardised protocol[31,32]. A total of 500 nucleated cells are counted, including the IC, and reported as a percentage of 500 cells. Specific morphological phenomena, such as foamy alveolar macrophages, activated Lyms, necrobiotic neutrophils and RPII cells, are reported separately. The differential cell count can be used as an instrument to limit the differential diagnosis in pulmonary diseases (Table 1.3)

3.6 The quantitative culture

The quantitative culture of BALF is the current “gold standard” for the diagnosis VAP. Since the alveoli are rinsed with approximately 200 ml of fluid, the actual BALF sample is an approximately 10-100 times diluted representation of the situation in the alveoli. In case of a bacterial infection, the bacterial concentration in the alveoli is 10^5 tot 10^6 colony forming units (cfu)/ml. For the quantitative culture, this results in a cut-of value of 10^4 cfu/ml[33].

Table 1.3 The direction in which the elevation or the presence of certain cells in the differential cell count in BALF can point[a]

Associated diseases	AMs	Lyms	PMNs	Eos	PCs	MCs	RPII
Non-infectious diseases							
Sarcoidosis		↑	=	=/↑	-	=/↑	-
Extrinsic allergic alveolitis	FAM	↑↑	↑	=/↑	+/-	↑↑	-
Drug-induced pneumonitis	FAM	↑↑	↑	↑	+/-	↑↑	-
Idiopathic pulmonary fibrosis		↑	↑/↑↑	↑	-	↑	+/-
BOOP*	FAM	↑	↑	↑	+/-	=/↑	
Eosinophilic pneumonia		↑	=	↑↑	+/-	=/↑	
Alveolar proteinosis	FAM	↑	=	=	-	=	
Diffuse alveolar hemorrhage		=/↑	↑	=/↑	-	=	+/-
ARDS**		↑	↑↑	↑	-	=/↑	+/-
Hematologic malignancies		↑	↑	=/↑	-	=/↑	
Astma		=	=	↑	-	=	
Infectious diseases							
Ventilator-associated pneumonia		=	↑↑	=	-	=	+/-
*P*neumocystis pneumonia		=/↑	↑	=/↑	+/-	=	+/-
Viral pneumonia		=	↑↑	=	-	=	+/-
Aspiration pneumonia	FAM	=	↑↑	=	-	=	+/-

[a] Adapted from Drent *et al.*[8]
AMs: alveolar macrophages, Lyms: lymphocytes, PMNs: polymorphonuclear neutrophils, Eos: eosinophils, PCs: plasma cells, MCs: mast cells, RPII: reactive type II pneumocytes, FAM: foamy alveolar macrophages, +: present, -: not present, +/-: can be present
* BOOP: bronchiolitis obliterans with organising pneumonia
** Acute respiratory distress syndrome.

4 Detection of soluble factors in BALF

Unlike the different cellular components of BALF, soluble components are effected by the dilution of BALF since quantitative measurements per milliliter are mostly use to express measurements[34]. Four main problems prevent accurate quantification of soluble factors in BALF, namely: i) there is a unknown amount of dilution during lavage, ii) contamination from the epithelial lining fluid (ELF) sample with bronchial material can occur during BAL, iii) the sample can be inadequate by inadequate mixing and iv) permeability of the lung varies, this may lead to loss of introduced fluid into the tissues and increase leakage of soluble factors from the blood and tissues into the ELF[34]. Using a standardized protocol, like the one we use, helps reduce the above mentioned problems. As recommended by the ERS task force, a standard volume of NaCl is introduced (200 ml) in four aliquots (50 ml each). In case of diffuse pulmonary disease, a standard lobe (lingula) is used for lavage. However, even if a standardized protocol for BAL is used, the volume of fluid recovered can not be controlled. Two possible solutions can be used to, largely, overcome this problem. The first is expressing the concentration of a

soluble factor as proportion of another factor, similar to the percentage of cells in the differential cell count. For example, expressing individual proteins as a proportion of the total protein[34]. The second possible solution is the use of the urea concentrations in BALF and serum. Since urea is a very small molecule, it can freely pass the barrier between blood and ELF. Therefore, at least in theory, the concentration of urea in ELF and serum should be the same. Wiedermann and co-workers described an easy to use formula for this purpose[35]:

$$[X]ELF = ([X]BALF \times \text{urea serum})/\text{urea BALF concentrations}$$

To assess permeability of the blood/ELF barrier, the concentration of albumin can be of use. Normally albumin is too large a molecule to pass the barrier. In case of leakage, high concentrations of albumin can be found in ELF and therefore in BALF[36].
Several non-cellular components can be detected in BALF, among these are: immunoglobulins[37], proteases and antiproteases[38], angiotensin-converting enzyme[39], antioxidants, oxidants and oxidation products[40], lipid mediators[41], cytokines[42], soluble adhesion molecules[43], markers of fibrosis[44], granulocyte derived markers[45], tumour markers[46], markers of cell death and other components.

4.1 Procalcitonin

Procalcitonin (PCT) is a 116 amino acid protein with a molecular weight of approximately 13 kDalton, which undergoes posttranslational proteolysis to become the hormone calcitonin[47]. In healthy patients calcitonin is produced and released by the C-cells of the thyroid gland after specific intracellular proteolysis of the prohormone. However, in severe bacterial infections and sepsis, procalcitonin is found in the blood and is produced extra thyroidal. Until now, it is not clear which cells are responsible for the production of PCT during infection. Several cells have been proposed: liver[48,49], neuroendocrine cells of lungs or intestine, and peripheral blood-monocytes[50]. The indication for PCT lies primarily as a, supporting, diagnostic parameter for bacterial infections such as sepsis and septic shock[49,51-54], which can be explained by the fact that bacterial lipopolysaccharid (LPS) is (*in vitro*) a major stimulus for the production of PCT. Furthermore, PCT could also be useful in other infections like yeast infection[55] and parasitic infection[56]. Even though the clinical relevance of PCT has been shown in patients with sepsis, PCT can also be elevated in patients with nonseptic systemic inflammatory response syndrome (SIRS)[57,58], trauma patients[59] and immediately after surgery[58] or trauma, without obvious infection. PCT concentration in both serum and BALF have been investigated as a

diagnostic method in patients with VAP. Serum PCT, but not PCT determined in BALF, was indicated as a helpful parameter in early VAP diagnosis[60]. However, PCT in BALF was determined by a test designed for serum, with a detection-limit that may be to high for the detection of PCT in BALF[60]. PCT kinetics have been used to predict the outcome in patients with severe community-acquired pneumonia admitted to the intensive care unit (ICU). It appears that the increase of the PCT level in serum over the first three days of admittance to the ICU, is correlated with a poor prognosis[61].

4.2 C-reactive protein

C-reactive protein was discovered in 1929 by Oswald Avery whilst studying patients with a *Streptococcus pneumoniae* infection. CRP is an inflammatory marker (protein), produced in the lever in response to interleukine-6 produced by macrophages and monocytes[62]. Healthy humans have peripheral blood CRP below 3 mg/l. In case of infection or inflammation (caused by physical trauma) blood CRP levels can increase 1,000-fold or more. CRP is used as a marker in the diagnosis of infections and to monitor the efficiency of treatment. Recently, CRP has been discovered as a marker of atherosclerosis, probably because low-grade chronic inflammation plays a part in atherosclerosis[63]. CRP can be influenced by different factors, such as: age, hormones, diabetes, smoking, obesity, hypertension and some medication (e.g. aspirin)[64]. Several studies have been performed to determine the value of CRP levels in serum of patients with VAP. Measurement of CRP levels (and PCT levels) upon admittance and after four days can predict the survival of patients with VAP. A decrease in either CRP or PCT is correlated with an increased survival of patients with VAP[65].

5 Value of BAL in non-infectious pulmonary diseases mimicking ventilator-associated pneumonia

Several non-infectious pulmonary diseases, such as ARDS, alveolar hemorrhage and drug-induced pneumonia, can mimic VAP[66]. Bronchoalveolar lavage with differential cell count can be a tool in differentiating between infection and non-infectious pulmonary disease (Table 1.1). For instance, a BALF with a high total cell count, a high percentage of PMNs, but no IC and a quantitative culture below 10^4 cfu/ml, suggests the presence of ARDS rather than VAP.

6 Value of bronchoalveolar lavage in infectious pulmonary diseases

BAL can be useful in many infectious pulmonary diseases (Table 1.1).

6.1 Ventilator-associated pneumonia

Ventilator-associated pneumonia (VAP) is defined as a pneumonia in patients receiving mechanical ventilation, occurring more than 48 hours after intubation. VAP can be divided in VAP occurring within the first five days of mechanical ventilation (early-onset VAP) and after five days (late-onset VAP)[67]. VAP is the most frequent hospital acquired infection in ICU patients, occurring in 9-27% of intubated patients[68,69] VAP is associated with a high mortality, varying from 20-70%, depending on the micro-organism involved[68]. Besides the high mortality associated with VAP, it has been demonstrated that VAP prolongs both the duration of mechanical ventilation and ICU stay[68,69].

The highest incidence of VAP is seen in patients after trauma or thermal injuries[70].

Usually the upper airways in humans are colonised with bacteria, whilst the lower airways are sterile. In patients receiving mechanical ventilation, a series of factors facilitate bacteria to colonise and finally infect the lower airways[71]. First of all, due to the presence of a tube, the cough reflex is prohibited, the clearance by means of mucus transport by ciliated cells is reduced and the tracheal epithelium is injured. Secondly, underlying illness and malnutrition have a negative influence on the immune system, which in turn responds to the infection in a less than adequate way. Table 1.4 shows the different bacteria associated with VAP. Since the “normal” commensal flora changes during the course of admittance to the ICU, the causative organisms in early- and late-onset VAP differ[67]. The main causative organisms in early-onset VAP include *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Staphylococcus aureus* whilst the organisms involved in late-onset VAP include *Pseudomonas aeruginosa*, Enterobacteriaceae and *Staphylococcus aureus*[72].

Table 1.4 Etiology of ventilator-associated pneumonia as documented by bronchoscopic techniques in 24 studies for a total of 1,689 episodes and 2,490 pathogens.

Pathogen	Frequency (%)
Pseudomonas aeruginosa	24.4
Acinetobacter spp.	7.9
Stenotrophomonas malthophilia	1.7
Enterobacteriaceae*	14.1
Haemophilus spp.	9.8
Staphylococcus aureus†	20.4
Streptococcus spp.	8.0
Streptococcus pneumoniae	4.1
Coagulase-negative staphylococci	1.4
Neisseria spp	2.6
Anaerobic bacteria	0.9
Fungi	0.9
Others (<1% each)‡	3.8

Adapted from Chastre *et al*.[68]
* Distribution when specified: Klebsiella spp., 15.6%; *Escherichia coli*, 24.1%; Proteus spp., 22.3%; Enterobacter spp., 18.8%; Serratia spp., 12.1%; Citrobacter spp., 5.0%; *Hafnia alvei*, 2.1%.
† Distribution when specified : Methicillin-resistant *S. aureus*, 55.7%, methicillin-sensitive *S. aureus*, 44.3%.
‡ Including Corynebacterium spp., Moraxella spp., and Enterococcus spp.

The diagnosis VAP is difficult since a world-wide accepted gold-standard is lacking. Clinical parameters, shown in Table 1.5[73], are highly sensitive but non-specific[73]. Most studies use quantitative culture of BALF as a "semi-gold"standard. The world-wide accepted cut-off value for the presence of VAP is established at 10^4 cfu/ml. Approximately 30-40% of patients clinically suspected of VAP, have microbiologically confirmed VAP using the quantitative culture method[33]. Primarily, the cut-off value for quantitative culture was chosen to avoid underdiagnosis of VAP, consequently this cut-off value may be to sensitive and result in treatment of patients without VAP. One of the disadvantages of quantitative culture of BALF is the delay of 24-48 hours until results are known. Microscopic techniques (e.g. differential cell count including IC) have proven to be reliable and fast method in the differentiation between VAP and non-VAP[30,74]. One of the major advantages of using BALF in the diagnosis VAP is the fact that clinicians can be directed towards a cause outside the pulmonary tract, in case of negative microscopy and/or culture. Since VAP is associated with a high mortality, an early diagnosis is essential as is the initial choice of antibiotics. Studies have shown that delay in administration of effective treatment against VAP is associated with higher mortality, morbidity and hospital-costs[75-77]. Up to 50% of the empirically started antibiotic treatment regimes need to be altered due to the presence of resistant bacteria (either unexpected isolates or multi-drug resistant bacteria)[75,78] Unfortunately, the latter can only partly be prevented by the use of microscopy of BALF in the diagnosis VAP.

Table 1.5 Clinical parameters indicating suspected VAP. Clinical suspicion of VAP is made when both criteria I and II are met[73].

Criteria
I. ≥ 3 positive of the following 4:
(a) rectal temperature > 38.0°C or <35.5°C
(b) blood leukocytosis (>10×10^3/mm^3) and/or left shift or blood leukopenia (< 3×10^3/mm^3)
(c) > 10 leukocytes per high-power field in Gram stain of tracheal aspirate
(d) positive culture from tracheal aspirate
II. new, persistent, or progressive infiltrate on chest radiograph

Adapted from Bonten *et al.*[73]

6.2 *Pneumocystis jiroveci*

The micro-organism was first described in 1909 by Chagas who discovered them in the lungs of guinea pigs. Thinking it was a type of trypanosome he called it, *Schizotrypanum cruzi*[79]. In 1912 Delanoë demonstrated these cysts to be unrelated to trypanosomes and renamed them *Pneumocystis carinii*[80]. In the period 1920-1960 an epidemic of pneumonia (interstitial plasma cell pneumonia) was identified in prematurely born infants, particularly in orphanages or foundling homes in Germany and nearby European countries[81]. In 1942, Pneumocystis was seen in the lungs of two Dutch infants who had died of interstitial pneumonia, however it was not attributed to the micro-organism at the time[81]. It was not until 1951 when Jirovec demonstrated the etiologic agent to be Pneumocystis[82]. Until the 1980s, Pneumocystis was thought to be a protozoan, due to its life cycle, sensitivity to co-trimoxazol and the fact that it did not react to antifungal therapy with amphotericin B. However in 1988, investigation of ribosomal RNA demonstrated it to be closely related to fungi[83]. It has generally been accepted that pneumocystis organisms are species specific[84]. Recently, the human form, *Pneumocystis carinii* f. sp. *hominis*, was renamed after Dr. Otto Jirovec: *Pneumocystis jiroveci*[85].

One of the major problems in researching *P. jiroveci* is that cultivation of the fungus in vitro is impossible. Some investigators were able to achieve replication of rat-derived *P. carinii* in different cell lines (limited to up to 10 cycli), cultivation of *P. jiroveci* has never succeeded yet. Studies dealing with the life cycle have been based on forms seen in microscopic images of limited cultures and infected pulmonary tissue.

Serologic studies have shown that *P. jiroveci* has a world-wide distribution and most children have already been into contact with the fungus at young age[86].

P. jiroveci is an opportunist, causing pneumocystis pneumonia (PCP) in immunocompromised patients. In the 1980s, it used to be a disease found mainly in patients infected with the human immunodeficiency virus (HIV), however, since the introduction of Highly Active Anti Retroviral Therapy (HAART) and chemoprofylaxis for PCP, there has been a shift towards HIV-

negative immunocompromised patients. The latter category includes patients using immunosuppressive medication or with an inherited or acquired immunodeficiency[87]. Clinical symptoms associated with PCP include: subtle onset of progressive dyspnea, non-productive cough and low-grade fever. Physical examination shows tachypneu and tachycardia whilst auscultation is often normal. Compared to HIV-negative patients, HIV-positive patients usually have a more subtle onset, symptoms develop over a longer period, *P. jiroveci* loads are higher but pulmonary damage is less severe. In HIV-positive patients, the influx of PMNs into the lung is decreased compared to HIV-negative patients, resulting in better oxygenation and survival in the HIV-positive group[88].

For years, the diagnostic method of choice has been BAL with specific stains. Different stains can be used, most diagnostic centres always use a combination of two stains: one suitable to detect trophozoit forms (ie. Giemsa, monoclonal antibodies) and one to detect cyst forms (Methenamine silver (Figure 1.7), Calcofluor white, Toluidine blue O). In the hands of an experienced microscopist each stain has a good sensitivity and specificity[89]. However, the microscopic evaluation of BALF samples for the presence of *P. jiroveci* is cumbersome and requires specialised microscopists. Especially in BALF samples containing a low burden of *P. jiroveci*, establishing the diagnosis is often difficult and time consuming. In this situation (i.e. HIV-negative patients) polymerase chain reaction (PCR) could prove its value.

Figure 1.7 Methenamine silver stain (Grocott). This stains the chitine present in the fungal cellwall black. This figure shows a cluster of *P. jiroveci* cysts. Magnification 1000×.

6.3 Herpes simplex virus

Herpes simplex virus (HSV) belongs to the human herpesvirus group. It causes a wide variety of infections. There are two serotypes that infect humans, herpes simplex virus 1 (HSV-1) and herpes simplex virus 2 (HSV-2). HSV-1 is normally associated with orofacial infections and meningitis whereas HSV-2 is associated with genital infections. Both viruses remain in the bodies sensory neurons as latent infection and can reactivate causing lesions near their entry point in the body.

HSV-1 has been described as cause of pneumonia in immunocompromised patients (e.g. AIDS)[90,91]. However, the prevalence of and the risk-factors for developing a HSV-1 pneumonia are still unknown. In literature the estimated mortality of a Herpes simplex pneumonia is high, varying between 20 till 50%[92,93]. HSV-1 is regularly cultured from respiratory samples, such as oropharyngeal swabs and BALF, from hospitalised patients[94,95] with and without suspected pneumonia. Due to the introduction of more sensitive diagnostic procedures for the detection of respiratory pathogens, like real-time polymerase chain reactions, the percentage HSV-1 positive respiratory samples will only increase in the near future[96]. Since HSV can be found in up to 5% of respiratory specimens of the general population at any given time, the clinical relevance of HSV presence in the respiratory tract in hospitalised patients remains unclear.

7 Nucleic acid amplification techniques in infectious diseases

In recent years, the introduction of molecular methods in the diagnostic work-up of respiratory infectious diseases has increased. Molecular techniques, such as polymerase chain reaction (PCR) offer some advantages over conventional techniques (culture, serology). First of all, the use of PCR leads to an increase in sensitivity, especially in micro-organisms that can not (easily) be cultured[97,98] and in case of a low burden of micro-organisms. Furthermore it is a universal technique making it possible to identify every micro-organism, using unique primers and probes, making it very specific. Finally, PCR is a relatively rapid method for identification compared to culture and serology[98]. Several PCR methods can be useful in the identification of causative organisms in pulmonary infectious diseases. In case of a conventional PCR method, post-PCR products are analysed by means of gel-electrophoresis, enzyme immunoassay detection or dot-blot hybridisation. In recent years, the conventional PCR methods are increasingly replaced by real-time PCR reactions (Figure 1.8) which do not require separate post-PCR product analysis. This has led to results being

available earlier and with less chance of contamination since there is no need to open PCR tubes after amplification. An additional advantage of real-time PCR methods is the fact that this method measures the amount of amplified PCR product during each cycle. This makes it possible to obtain quantitative results, which is essential when identifying micro-organisms which can lead to either carrier state or infection (e.g. *P. jiroveci*;[99]). Combining diagnostic PCRs in order to identify different micro-organisms at the same time may be the future since respiratory pathogens can cause the same clinical symptoms. Using PCR assays which only detect one micro-organism may prove to be too expensive and require too much material. Multiplex PCR reactions permit the amplification and identification of multiple pathogens simultaneously, however in general, they are less sensitive compared to mono-specific PCRs[100]. PCR can be used for the identification of many causative organisms of pneumonia. Table 1.6 sums up the most prevalent, causative micro-organisms of pulmonary infection for which a PCRs is described in literature with their references.

Table 1.6 PCR assays described in literature for different micro-organism causing pulmonary infection.

Micro-organisme	PCR described*
Bacteria:	
Streptococcus pneumoniae	(108, 109)
Haemophilus influenzae	(108, 109)
Mycoplasma pneumoniae	(100, 108)
Chlamydia pneumoniae	(100, 108)
Legionella pneumophila	(110, 111)
Fungi:	
Pneumocystis jiroveci	(112-114)
Aspergillus fumigatus	(115, 116)
Viruses:	
HSV-1,2	(117, 118)
RSV	(100, 119)
HMPV	(120, 121)
Influenza A,B	(100, 119)
Parainfluenza 1,2,3	(100, 119)

* for detection in respiratory samples.

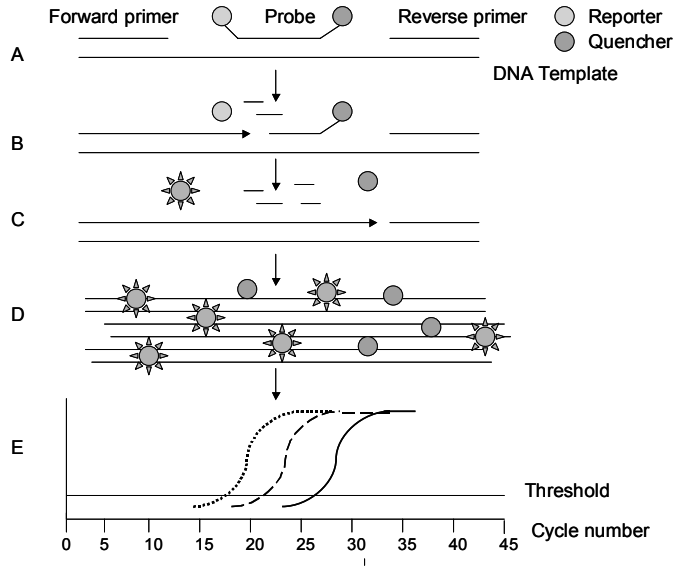

Figure 1.8 Theoretic background of real time PCR

A. After the DNA has been isolated from the sample, it is denaturated into two single strands. This single stranded DNA is used as a template. Forward and reverse primers are designed to bind to a conserved region of the target DNA. A non-extendable hybridization probe is designed to bind the target internal to the PCR product. The probe contains a reporter fluorescent dye on the 5'end and a quencher dye to the 3'end.
B. Taq polymerase extends the DNA from the forward primer until it encounters the probe. Since the probe is non-extendable the Taq polymerase removes the probe nucleotides
C. This results in the parting of the quencher dye and the reporter fluorescent dye, allowing the detection of the fluorescent dye.
D. At the end of the 40 cycles, the sample contains the extended DNA, and a concomitant amount of fluorescent dye.
E. Product analysis. The amount of fluorescence (y-axis) is measured at the end of each cycle (x-axis). Relative quantification is possible by means of the Ct value (threshold cycle), referring to the cycle at the end of which the fluorescent signal crosses the threshold.

PCR: Polymerase Chain Reaction, DNA: Desoxyribo Nucleic Acid

8 Aims of the study

The scope of the studies presented in this thesis was to improve the quality of BAL as a diagnostic tool in pulmonary infectious diseases and to shorten the time in which the diagnosis can be made. In combination with the patients history, physical examination, and imaging of the lungs, BALF cytology is a widely used additional tool in establishing or ruling out of pulmonary infections. Traditionally BALF work-up includes a total cell count, a differential cell count, additional stains, quantitative culture and additional cultures. The standardisation of BALF analysis is very important to ensure comparability between the different laboratories. In our laboratory BALF analysis (differential cell count as well as quantitative cultures) is well standardised using the protocol developed by de Brauwer and co-workers[21,29,31,32,101]. However microscopic analysis of BALF does not always reach the sensitivity required to make a definitive diagnosis, for instance due to the quality of the sample, use of antibiotics at the time of BAL or due to a low burden of the micro-organism in BALF. Furthermore, bacterial and viral cultures take time, up to 48 hours for bacterial and up to 7 days for viral culture and since an early diagnosis is essential in most infectious diseases, fast alternatives are investigated.

In the diagnostic approach of patients with diffuse interstitial lung diseases the differential cell count of BALF samples is helpful in guiding the clinician to a certain disease (Table 1.3). Therefore, the technical performance should be standardised and optimised. Previous reports[23] indicated that using cytocentrifuge slides may influence the recovery of lymphocytes in particular. This prompted us to improve our recovery rate of cells. Two previously performed studies by de Brauwer *et al.*[21,31] already showed that the differential cell count is influenced by the area counted[31] and the protocol used for cytocentrifugation[21]. We hypothesised that the use of slides with a positively charged cell surface may provide superior cell adhesion when used for cytocentrifugation of BALF. Previously, the same principal has been shown in the cytology of buccal smears[102], urine[103] and cerebrospinal fluid samples[104]. In **chapter 2**, we evaluated Polylysine-coated slides in the diagnostic work-up of clinical BALF samples.

In **chapter 3**, the presence of reactive type II pneumocytes in BALF were investigated. RPII cells had been described in patients with adult respiratory distress syndrome (ARDS)[27] and *Pneumocystis jiroveci* pneumonia[28]. It was hypothesised they may be present in specific pulmonary diseases and therefore could play a role in limiting the differential diagnosis in patients.

Antibiotic therapy at time of lavage has been shown to influence the quantitative culture results. Their influence on the percentage infected cells (%IC) has resulted in conflicting data. Some studies show no influence of antibiotic therapy on %IC[105] whilst others show a decline in both sensitivity and

specificity of %IC[106] when patient are on antibiotic therapy at the time of lavage. Since ventilator-associated pneumonia has a high mortality, an early diagnosis is important, however many patients admitted to the intensive care unit already receive antibiotic therapy at the time of the lavage. In **chapter 4**, therefore, the effect of antibiotic therapy on the reliability of cytological parameters in BALF in the prediction of VAP was assessed.
In most hospitals, facilities to perform BALF analysis on a 24-hour basis, are not present. Therefore a fast method for differentiating between VAP and non-VAP, present in all hospitals, was sought. PCT and CRP both are acute fase proteins associated with inflammation/infection and the since the diagnostic test is available in every hospital, they appeared ideal candidates. In **chapter 5** high-sensitivity-C-reactive protein and -procalcitonin concentrations in both serum and BALF were measured. The hypothesis was that local production of either or both of the proteins in the event of pneumonia would result in an elevation of the protein concentration in BALF, making it a possible to differentiate between VAP and non-VAP.
In the past, PCP was an indication for HIV. However, since the introduction of HAART and chemoprophylaxis the number of HIV-positive patients diagnosed with PCP has decreased. Patients at risk have shifted from HIV-positive patients to patients otherwise immunocompromised (e.g. malignancy, corticosteroid use). The main difference this makes for the laboratory diagnosis is the fact that the *P. jioveci* burden in these patients is often very low. Since microscopy of BALF still remains the “gold standard” for the diagnosis PCP, two problems have emerged. First of all, since the incidence of PCP has declined it has become more difficult to ascertain the required level of expertise. Secondly, since the majority of patients at risk have a low *P. jiroveci* burden, microscopy, even in the hands of experts, is extremely time-consuming and may be not sensitive enough. Real-time PCR may be a solution for this problem, however commercial kits and quality control panels are not available making it difficult to evaluate and implement the PCR for *P. jiroveci* in the diagnostic work-up of BALF. In **chapter 6**, the inter-laboratory comparison of three different real-time PCR assays for the detection of *P. jiroveci* in BALF is described, giving a starting point for the clinical validation of comparable PCRs.
In a large percentage of patients suspected of a pneumonia in whom BAL is performed, no causative organism is found. The hypothesis was that viral infections may play a role in these patients. HSV is relatively often isolated from BALF, however clinical relevance remains unclear. In **chapter 7**, therefore we evaluated the HSV viral load in BALF in relationship with outcome in intensive care patients. Finally, **chapter 8** contains a summary and the conclusions from the preceding chapters.

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