ORIGINAL ARTICLE

# BAL fluid analysis in the identification of infectious agents in patients with hematological malignancies and pulmonary infiltrates

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## Abstract

The present study aims to evaluate the diagnostic yield of bronchoalveolar lavage (BAL) fluid in patients with hematological malignancies and describe the most common pathogens detected in BAL fluid (BALF.) An analysis of 480 BALF samples was performed in patients with hematological malignancies over a period of 7 years. The results of culture methods, PCR, and immunoenzymatic sandwich microplate assays for *Aspergillus* galactomannan (GM) in BALF were analyzed. Further, the diagnostic thresholds for *Aspergillus* GM and *Pneumocystis jiroveci* were also calculated. Microbiological findings were present in 87% of BALF samples. Possible infectious pathogens were detected in 55% of cases; 32% were classified as colonizing. No significant difference in diagnostic yield or pathogen spectrum was found between non-neutropenic and neutropenic patients. There was one significant difference in BALF findings among intensive care units (ICU) versus non-ICU patients for *Aspergillus* spp. (22% versus 9%, $p = 0.03$). The most common pathogens were *Aspergillus* spp. ($n = 86$, 33% of BAL with causative pathogens) and *Streptococcus pneumoniae* ($n = 46$, 18%); polymicrobial etiology was documented in 20% of cases. A quantitative PCR value of $> 1860$ cp/mL for *Pneumocystis jirovecii* was set as a diagnostic threshold for pneumocystis pneumonia. The absorbance index of GM in BALF of 0.5 was set as a diagnostic threshold for aspergillosis. The examination of BAL fluid revealed the presence of pathogen in more than 50% of cases and is, therefore, highly useful in this regard when concerning pulmonary infiltrates.

## Introduction

Lower respiratory tract infections have a high morbidity and mortality in immunocompromised patients (Rano et al. 2001; Boersma et al. 2007; Hummel et al. 2008). The identification of the causative agents may influence previously introduced empirical antimicrobial therapy and enable the administration of a specific therapy, if at all

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available (Oren et al. 2016; Hohenadel et al. 2001; Hummel et al. 2008).

The patients with hematological malignancies have an increased risk of opportunistic infections, such as invasive mycoses, *Pneumocystis jirovecii* pneumonia (PCP), or *cytomegalovirus* (CMV) pneumonia (Rano et al. 2001; Joos et al. 2007). Common viral infections of the upper respiratory tract have a high tendency to progress towards severe pneumonia, resulting in a high mortality rate estimated in 50–70% of these patients. These infections are frequently associated with bacterial or fungal co-infections and/or super-infections (Chemaly et al. 2006).

The bronchoalveolar lavage (BAL) is a valuable and a uniformly accepted safe procedure (Chemaly et al. 2006; Joos et al. 2007; Kuehnhardt et al. 2009) that may identify the etiological agent of pulmonary infections. The aim of our study was to evaluate the diagnostic yield of microbiological BAL fluid (BALF) analysis in patients with malignant hematological disorders using diagnostic methods that included PCR. Furthermore, we evaluated clinically relevant cutoffs of the two most important opportunistic pathogens: *Pneumocystis jirovecii* DNA quantity for the diagnosis of PCP and galactomannan (GM) aspergillus index in BALF for the diagnosis of invasive aspergillosis (IA).

## Patients and methods

Our retrospective study analyzed the microbiological agents detected in BALF from patients with hematological diseases and pulmonary infiltrates and whom were being treated at the 4th Department of Internal Medicine – Hematology of the University Hospital and Faculty of Medicine of Charles University at Hradec Kralove, Czech Republic, between January 2007 and December 2014. This study was reviewed and approved by the Ethics Committee of the University Hospital Hradec Kralove.

Patients with pulmonary infiltrates, confirmed by chest X-ray scan and/or high-resolution computed tomography (HRCT), were indicated for bronchoscopy examination with BAL according to their clinical status. These deeply immunosuppressed patients were treated with wide spectrum antibiotics, antimycotics, and antiviral therapy according to current standards and guidelines. These included upfront combination of piperacillin/tazobactam plus levofloxacin in stable patients, meropenem instead of piperacillin/tazobactam in cases of instability or known previous colonization with bacteria resistant to piperacillin/tazobactam.

The following criteria were also included for further analysis: demographic data, underlying diseases, previous transplantation of allogeneic hematopoietic stem cells, concurrent neutropenia (neutrophil count $> 1000/\mu L$), intensive care

(ICU), and/or mechanical ventilation necessity, as well as death caused by an underlying pulmonary pathology < 30 days after BAL.

BAL sampling was routinely performed by experienced physicians using a fiberoptic bronchoscope (FOB, Olympus, Japan) within 1 or 2 days after HRCT (or, occasionally, chest X-ray when HRCT was not available). FOB procedures were performed at the bedside within the intensive care unit in cases of mechanically ventilated subjects (invasive or non-invasive) and under local anesthesia (lidocaine) and mild sedation (midazolam i.v.); further, platelet transfusions were administered ($> 20,000/\mu L$) before FOB. All other patients underwent FOB at the bronchoscopy unit. BALFs were obtained by gently wedging the tip of the bronchoscope into the area of greatest radiographic abnormality. BAL was performed in the most severely impaired pulmonary segment/sub-segment when multiple consolidation areas were present. Each BALF aliquot consisted of 50 mL of sterile physiological saline pre-warmed to body temperature; the aspiration of BAL aliquots used very low pressure to avoid airway collapse. The BALF samples were stored in transparent plastic containers with non-adherent surfaces and immediately ($\leq 1$ h) processed in the laboratory.

The BALF samples were tested using classic microbiological cultures designed for bacteria, fungi, and mycobacteria on conventional agar media (blood agar, chocolate agar, MacConkey, and anaerobic blood agar) at 37 °C under aerobic and anaerobic conditions, as required. All the species found in the culture isolates were identified by standard methods. The detection of *Legionella pneumophila* was achieved using BCYE (buffered charcoal yeast extract) medium. One of the collected BALF aliquots was processed for fungal culture (incubated on Sabouraud's agar under aerobic conditions at 30 °C for 7 days). *Mycobacterium tuberculosis*, and other mycobacteria, was detected through the microscopic examination of Ziehl-Neelsen-stained smears cultured under aerobic conditions at 37 °C for 6 weeks (Ogawa, Löwenstein-Jensen) and in mycobacterial growth indicator tubes (MGIT). *Pneumocystis jirovecii* was detected by microscopic examination with methanol-Giemsa staining.

DNA was isolated using the QIAamp DNA Mini Kit; Tissue protocol was used for bacterial or fungi DNA, QIAamp Viral RNA Mini Kit for RNA viruses (all Qiagen, Hilden, Germany). PCR tests were partly designed and validated in-house until 2013; these included *Varicella zoster virus* (VZV), *adenoviruses*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophila*, *Pneumocystis jirovecii*, *Aspergillus* spp., and respiratory DNA viruses (sequences are presented in Supplementary Table 1). Since 2013, commercial kits for the detection of these respiratory viruses have been available using the PCR Anyplex™ II RV 16 kit (Seegene, Inc., South Korea):

*influenza* (INF) *A, B, parainfluenza* (PIV) *1–4, respiratory syncytial virus* (RSV) *A, B, human metapneumovirus* (hMPV), *human rhinovirus* (HRV) *A/B/C, human adenovirus, human coronavirus 229E, NL63, OC 43, human enterovirus, human bocavirus 1/2/3/4.* The Platelia® *Aspergillus* EIA (Bio-Rad Laboratories, USA) immunoenzymatic sandwich microplate assay was used for detection of *Aspergillus* GM. The absorbance is determined with a spectrophotometer set at 450- and 620-nm wavelength. The presence of *herpes simplex* virus (HSV) and human *cytomegalovirus* (CMV) was tested using a Gene Proof system, whereas *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria were detected using the Anyplex MTB/NTM Real-time PCR kit (Seegene Inc., South Korea). All real-time PCR assays were performed according to the manufacturer's instructions using Rotor Gene (Qiagen, Hilden, Germany) and Light Cycler real-time PCR machines (Roche, AG, Switzerland). While the sensitivity of both techniques is comparable, the commercial methods offer a quicker analysis and reduced workload. All described PCR methods were validated annually and verified and controlled by an international system of quality control, QCMD (Quality Control for Molecular Diagnostics, Glasgow, UK). The quantitative tests express the pathogen load as number of DNA copies per milliliter of BALF (cp/mL). The limit of detection for each test was HSV type 1 = 213 cp/mL; HSV type 2 = 275 cp/mL; CMV = 100 cp/mL (equivalent of 79.4 IU/mL), *Pneumocystis jirovecii* = 100 cp/mL; *Aspergillus* spp. = 50 cp/mL. Other tests were only qualitative, and the results were simply reported as positive/negative.

The criteria used to define the pathogenic and colonizing agents in this study were based on an expert panel discussion and previous studies and are summarized in Tables 1 and 2. The categorization method used in this study is in agreement with the updated guidelines of the German Society of Hematology and Medical Oncology (Maschmeyer et al. 2015). The clinical diagnosis of proven and probable IA was based on current European Organization for Research and Treatment of Cancer (EORTC) criteria (De Pauw et al. 2008). Each suspected case was independently reviewed by two participating physicians (JR and AZ). The threshold for GM index was determined by receiver operator curve (ROC) curve. The clinical diagnosis of PCP pneumonia was independently reviewed by two participating physicians (JR and AZ). The threshold for *Pneumocystis jirovecii* qPCR was determined with a ROC curve.

## Statistical analysis

Descriptive statistics for demographic and baseline characteristics were summarized for all patients. The number and percentage of patients in each category were summarized for categorical variables. Categorical data was analyzed using

**Table 1** Agents considered as causative pathogens

- Cultivated: *Streptococcus pneumoniae, Staphylococcus aureus, Enterobacteriaceae (Escherichia coli, Enterobacter* spp., *Proteus* spp., *Klebsiella pneumoniae, Klebsiella oxytoca),* non-fermenters (*Pseudomonas aeruginosa, Burkholderia cepacia* group, *Stenotrophomonas maltophilia, Acinetobacter baumanii), Haemophilus influenzae, Legionella pneumophila, Moraxella catarrhalis (Branhamella catarrhalis), Bordetella bronchiseptica, Nocardia* spp., *Mycobacterium tuberculosis, Pneumocystis jirovecii, Aspergillus* spp., *Zygomycetes, Geotrichum clavatum*
- Detection by PCR: *Zygomycetes, Mycobacterium tuberculosis, Legionella pneumophila, Mycoplasma pneumoniae, Chlamydia pneumoniae, Nocardia* spp., *Aspergillus* spp., *Zygomycetes,* INF, RSV, PIV, Adenoviruses, hMPV, HRV, VZV, Coronaviruses
- DNA quantity of *Pneumocystis jirovecii* ≥ 1450 cp/mL, CMV DNA ≥ 10,000 cp/mL, HSV DNA ≥ 100,000 cp/mL in BALF.
- *Aspergillus* GM ≥ 0.5 index in BAL
- "Potentially relevant" non-tuberculous mycobacteria

*PCR* polymerase chain reaction, *INF* influenza, *RSV* respiratory syncytial virus, *PIV* parainfluenza virus, *hMPV* human metapneumovirus, *HRV* human rhinovirus, *VZV* varicella zoster virus, *CMV* cytomegalovirus, *HSV* herpes simplex virus, *DNA* deoxyribonucleic acid, *GM* galactomannan

the chi-square test or Fisher exact test using a 2 × 2 contingency table. The unpaired *t* test or non-parametric Mann-Whitney rank sum test was used for the analysis of the independent groups. *P* values < 0.05 were considered statistically significant. ROC curves were calculated for *Pneumocystis jirovecii* qPCR and GM index. The statistical analyses were performed using the software SigmaPlot for Windows, version 11.0. (Systat Software) and MedCalc v 9.5.2 (MedCalc, Belgium).

## Results

A total of 480 BALF samples from 353 patients (145 females, 208 males) were analyzed. The median age at the time of BALF collection was of 61 years (range 18–81). There were 89 patients (25.2%) with repeated BAL. Median interval between procedures was 43 days (interquartile range (IQR) 15–177 days). The most common underlying diseases were non-Hodgkin lymphoma and acute myeloid leukemia. Twenty-nine percent (138 samples) of BALF samples were obtained from

**Table 2** Agents considered as capable of colonizing the airways

- Cultivated: viridans streptococci, gama-haemolytical streptococci, coagulase-negative staphylococci, *Enterococcus* spp., *Neiserria* spp., *Corynebacterium* spp., *Lactobacillus* spp., anaerobic bacteria (*Fusobacterium* spp., *Peptococcus* spp., *Peptostreptococcus* spp., *Prevotella* spp., *Propionibacterium* spp., *Veilonella* spp., *Eubacterium limosum, Actinomyces* spp.), *Candida* spp., and *Saccharomyces*
- Low quantities of *Pneumocystis jirovecii* DNA < 1450 cp/mL, CMV DNA < 10,000 cp/mL, HSV DNA < 100,000 cp/mL in BALF
- *Aspergillus* spp. GM < 0.5 index in BAL

*DNA* deoxyribonucleic acid, *CMV* cytomegalovirus, *GM* galactomannan

neutropenic patients, and 24% of examinations (116 BAL in 82 patients) were done in recipients of allogeneic hematopoietic stem cell transplantation (HSCT). 74.4% of patients were managed on ICU. The data concerning demographics, underlying diseases, and HSCT are summarized in Table 3. Overall pneumonia-related mortality was 25% in our cohort. Mortality related to ongoing pneumonia in our cohort was influenced by neither positive nor negative microbiological results of BAL ($p = 0.82$), nor type of agents ($p \geq 0.70$) nor agranulocytosis < 1000/μL ($p = 1$). The only significant mortality predictor was prior HSCT ($p = 0.023$), but it was independent on BAL results (positive versus negative microbiological results, $p = 0.68$).

Of 480 obtained BALF samples, microorganisms were found in 87% of them. Overall, 82% of patients in ICU and 54% of non-ICU patients received previous empiric ATB treatment according to current practice described above ($p < 0.0001$). The probable infectious pathogens were detected in 55% of samples; 32% were classified as colonizing (Fig. 1). The non-infectious causes of respiratory complications were diagnosed via 33 BAL procedures (7%). The diagnostic yield did not differ between non-neutropenic (56%) and neutropenic (52%) patients ($p = 0.84$); also, there was no significant difference in the pathogens detected between these two groups (bacterial $p = 0.24$, fungal $p = 0.40$, viral $p = 0.67$, monomicrobial/polymicrobial $p = 0.41$). There were no significant differences in BALF findings among ICU versus non-ICU patients except for *Aspergillus* spp. where ICU patients showed higher positivity (22% versus 9%, $p = 0.03$). FOB with BAL was generally well tolerated and safe, and

no serious adverse reactions were observed during the procedure, where no major bleeding was observed afterwards.

The assumed causative pathogens detected are shown in Table 4, whereas the detected colonizing agents are presented in Table 5. The etiological agents of pneumonia were considered to be polymicrobial in 20% of cases; viruses and fungi occurred as co-pathogens in almost one half of occurrence cases (54% resp. 46%), details in this regard are shown in Table 6 (list of specific co-pathogens is presented in Supplementary Table 2 The most common co-pathogens were *Aspergillus* spp. (42 cases of polymicrobial infections), *Streptococcus pneumoniae* ($n = 23$), *Klebsiella* spp. ($n = 20$), and *Pseudomonas aeruginosa* ($n = 16$). If multiple pathogens were found, all were treated accordingly. Four BALF samples proved positive for *Mycobacterium tuberculosis*, although only two were clinically relevant. Three cultures of *Mycobacterium avium* and 29 positive PCR results for *Mycobacterium* spp. (other than *M. tuberculosis*) were observed. Only one case met the American Thoracic Society Documents criteria of non-tuberculous mycobacterial pulmonary disease (Griffith et al. 2007). This diagnosis was confirmed through lung biopsy.

*Aspergillus* spp. was the most common agent detected in BAL samples of both non-neutropenic ($n = 52$) and neutropenic patients ($n = 34$) in our cohort. We detected 64 cases of probable/proven IA clinical diagnosis. We analyzed the GM significance in BALF samples as well. The median absorbance (A) index of GM in the probable/proven IA group was 1.24 (range 0.24–8.80) and was significantly higher ($p < 0.001$) than the median in the non-aspergillosis group

**Table 3** Characteristics of the patients

|  | No. of patients ($n = 353$) | % |
|---|---|---|
| Male | 208 | 59 |
| Female | 145 | 41 |
| Non-HSCT recipients | 271 | 77 |
| Allogeneic HSCT recipients | 82 | 23 |
| Age: median age 61 years; range 18–81 | | |
| Diagnosis | | |
| Acute myeloid leukemia | 77 | 22 |
| Acute lymphoblastic leukemia | 29 | 8 |
| Chronic myeloid leukemia | 7 | 2 |
| Chronic lymphocytic leukemia/small lymphocytic lymphoma | 53 | 15 |
| Hodgkin lymphoma | 21 | 6 |
| Non-Hodgkin lymphoma | 87 | 25 |
| Multiple myeloma (and plasma cell leukemia) | 42 | 12 |
| Other hematologic malignancies (hairy cell leukemia, prolymphocytic leukemia) | 6 | 2 |
| Myelodysplastic syndrome | 17 | 5 |
| Myeloproliferative disorder, myelodysplastic/myeloproliferative neoplasm | 9 | 2 |
| Aplastic anemia | 5 | 1 |

*HSCT* hematology stem cell transplantation

**Fig. 1** Diagnostic yield. Asterisk indicates other significant findings independent on microbiological results were identified in 33 BAL (7%): Diffuse alveolar hemorrhage (DAH, 18×), tumor (7×), bronchiolitis obliterans syndrome (BOS, 2×), and bronchiolitis obliterans organizing pneumonia (BOOP, 1×), all probably in association with graft-versus-host disease, usual interstitial pneumonia (UIP, 3×), chronic diffuse interstitial lung–idiopathic pulmonary fibrosis, sarcoidosis
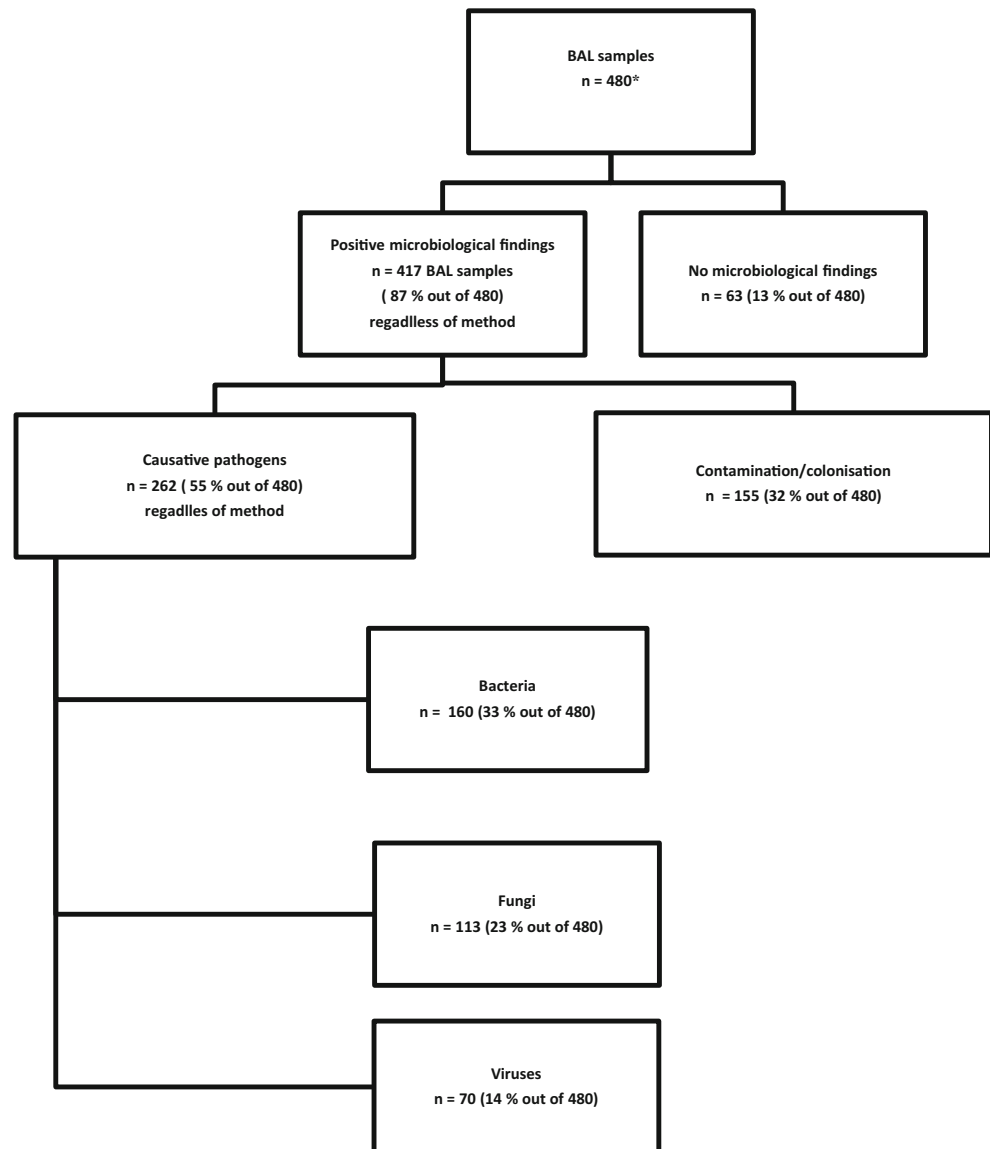


(0.28; range 0.14–0.6). The area under the ROC curve (AUC) for BAL GM ≥ 0.5 A index was 0.972 (95% CI 0.922–0.994), see Fig. 2.

There were a total of 42 *P. jirovecii* DNA positive BAL samples (median 6680 cp/mL, range 138–94,300,000 cp/mL). The clinical presentation, quantity of *P. jirovecii* DNA copies in BAL samples, and radiographic presentation were general triggers for specific PCP treatment in 26 patients (see Fig. 3). The median DNA quantity in BALF samples among the PCP patients' group was 12,700 cp/mL (range 600–94,300,000), which was significantly higher ($p < 0.001$) when compared to the non-PCP group (680 cp/mL, range 144–6680). Subsequently, 1860 cp/ml was calculated as a threshold associated with PCP diagnosis, showing an excellent sensitivity of 91% (95% confidence interval (CI) 76.3–98.0) and specificity 96% (95% CI 78.8–99.3). The area under the ROC curve

(AUC) for this threshold was 0.95 (95% CI 0.851 to 0.987), see Fig. 4.

## Discussion

Microorganisms were detected in 87% of the analyzed BALF samples. This is slightly higher than in previous recent reports which were able to detect them in only 50–73% of samples (Bissinger et al. 2005; Kim et al. 2015; Hardak et al. 2016). This difference could be due to the wider range of microorganisms included in our standard BALF detection. It is important to point out that the majority of BAL procedures were done after initiation of ATB treatment (82% of ICU patients and 54% of non-ICU patients), which is similar to situation in other reports and centers (Rano et al. 2001; Boersma et al.

**Table 4**　Causative pathogens

| | | | Positive hits in BALF (regardless of method) | Cultivated | PCR | Other |
|---|---|---|---|---|---|---|
| Bacteria | *Enterobacteriaceae* | *Klebsiella pneumoniae* | 27 | 27 | – | – |
| | | *Klebsiella oxytoca* | 6 | 6 | – | – |
| | | *Escherichia coli* | 24 | 24 | – | – |
| | | *Enterobacter* spp. | 11 | 11 | – | – |
| | | *Acinetobacter* spp. | 7 | 7 | – | – |
| | | *Stenotrophomonas maltophilia* | 6 | 6 | – | – |
| | | *Proteus* spp. | 5 | 5 | – | – |
| | Non-fermenters | *Pseudomonas aeruginosa* | 30 | 30 | – | – |
| | | *Burkholderia cepacia* group | 14 | 14 | – | – |
| | *Streptococcus pneumoniae* | | 46 | 0 | 45 | 2 (microscopy) |
| | *Staphylococcus aureus* | | 10 | 10 | – | – |
| | *Haemophilus influenza* | | 6 | 6 | – | – |
| | *Legionella pneumophila* | | 5 | 1 | 5 | – |
| | *Mycobacterium tuberculosis* | | 4 | 2 | 4 | – |
| | *Mycobacterium avium* | | 1 | 1 | 1 | – |
| | Other bacterial pathogens (*Moraxella catarrhalis*, *Bordetella bronchiseptica*, *Nocardia* species) | | 4 | 4 | 1 (*Nocardia* spp.) | – |
| Fungi | *Aspergillus* spp. | | 86[a] | 8 | 45[a] | 62[b] |
| | *Pneumocystis jirovecii* | | 28[a] | – | 28[a] | – |
| | *Zygomycetes* | | 6 | 2 | 6 | – |
| | *Geotrichum clavatum* | | 1 | 1 | – | – |
| Viral | Community respiratory viruses | *Respiratory syncytial virus* | 19 | – | 19 | – |
| | | *Influenza virus* | 13 | – | 13 | – |
| | | *Parainfluenza virus* | 5 | – | 5 | – |
| | | *Rhinovirus* | 12 | – | 12 | – |
| | | *Adenovirus* | 11 | – | 11 | – |
| | | *Human metapneumovirus* | 3 | – | 3 | – |
| | | *Coronavirus* | 1 | – | 1 | – |
| | Herpes viruses | *Herpes simplex* virus[a] | 1[a] | – | 1[a] | – |
| | | *Human cytomegalovirus*[a] | 9[a] | – | 9[a] | – |
| | | *Varicella zoster virus* | 3 | – | 1 | – |

[a] qPCR in "significant quantities": *Pneumocystis jirovecii* $\geq 1450$ cp/mL, *human cytomegalovirus* $\geq 10,000$ cp/mL, *herpes simplex* virus $\geq 100,000$ cp/mL

[b] Aspergillus GM BAL $\geq 0.5$ index

2007; Hummel et al. 2008; Kuehnhardt et al. 2009); therefore, our results could be negatively influenced by any ongoing antimicrobial treatment, resulting in lower diagnostic yield in BALF cultures (Kuehnhardt et al. 2009).

Potential infectious pathogens were detected in 55% of BAL samples. Other published studies report this value in 25–50%. An accurate comparison of various reports is somewhat complicated by the heterogeneity of laboratory methods (e.g., conventional culture versus molecular methods), different definitions of pathogens, patient cohorts, and prior ATB treatment (Hohenadel et al. 2001; Rano et al. 2001; Boersma et al. 2007; Maschmeyer et al. 2015). We assume that our slightly higher detection rate of potential pathogens is related to the combination of different diagnostic techniques in one sample, including molecular biology methods. Two recent studies (Tang et al. 2018; Oren et al. 2016) using molecular analysis proved infectious ethology of pneumonia in 49–63% of BAL procedures, which is similar to our study.

There was no statistical difference in pathogen detection between BAL samples from neutropenic (52%) and non-neutropenic (56%) cohorts; similar results were also reported by other authors (Cordonnier et al. 1994; Kuehnhardt et al. 2009). The relatively minor difference between both groups might be attributed to the fact that these non-neutropenic patients were immunocompromised; further, most of them had undergone chemotherapy and had previous episodes of

**Table 5** Colonizing agents

| Colonization | Cultivation | PCR | Other |
|---|---|---|---|
| *Streptococcus viridans* | 133 | – | – |
| Coagulase-negative *staphylococci* | 141 | – | – |
| *Enterococcus* spp. | 146 | – | – |
| *Neisseria* spp. | 10 | – | – |
| Other bacterial contamination | 16 | – | – |
| (other *streptococci, corynebacteria, lactobacilli*) | | | |
| Non-tuberculous mycobacteria | 2[a] | 28[a] | – |
| Anaerobic bacteria | 75 | – | – |
| *Candida albicans* | 29 | – | – |
| Non-albicans *Candida* | 42 | – | – |
| *Saccharomyces cerevisiae* | 8 | – | – |
| *Aspergillus* spp. | – | – | 61[c] |
| *Pneumocystis jirovecii* | – | 14[b] | – |
| *Human cytomegalovirus* | – | 42[b] | – |
| *Herpes simplex* virus | – | 67[b] | – |

[a] The criterion of non-tuberculous pulmonary disease was not fulfilled in any case

[b] qPCR in "non-significant quantities": *Pneumocystis jirovecii* < 1450 cp/mL, *human cytomegalovirus* < 10,000 cp/mL, *herpes simplex* virus < 100, 000 cp/mL

[c] *Aspergillus* GM BAL < 0.5 index

neutropenia, even if they were non-neutropenic at the time of BAL collection (Kuehnhardt et al. 2009). Detection of *Aspergillus* spp. (by any method) was more frequent in ICU patients. The fact that *Aspergillus* is a common finding in BALF of ICU patients has been previously reported (Zarrinfar et al. 2013; Khodavaisy et al. 2015) and might be the reflection of the overall severity of ICU patients.

The etiological agents of pneumonia were considered polymicrobial in 20% of our cases. The incidence of pulmonary polymicrobial infections in patients with hematological malignancies and pulmonary infiltrates can vary from 20 to 60% (Rolston et al. 2007; Hardak et al. 2016). This wide range is likely due to different definitions for bacterial and viral pneumonia, as well as differences in laboratory methods. An Israel study (Oren et al. 2016) that included PCR methods reported the same proportion of polymicrobial pneumonia

(21%) in similar patients' cohort. A recent Beijing study (Tang et al. 2018) using various flexible bronchoscopy methods determined the polymicrobial etiology in 47% of cases. Contrasting to our work, this work also described viruses as the most common infectious etiology of pneumonia (70%). These results were influenced by their patients' cohort (allogeneic HSCT recipients only), different definition of the

**Table 6** Polymicrobial infections

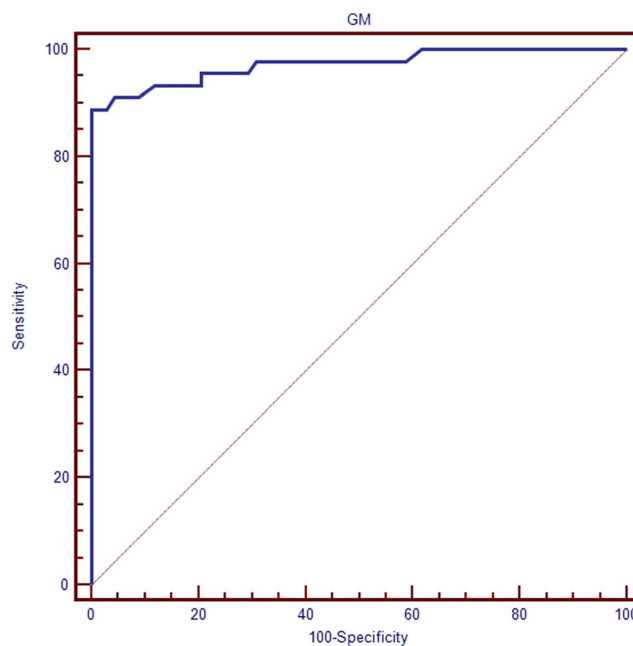| Polymicrobial infections | No. of BAL ($n = 480$) |
|---|---|
| Total | 98 (20%) |
| Polybacterial infections | 19 |
| Mixed fungal infections | 2 |
| Polyviral infections | 4 |
| Bacteria plus fungi | 35 |
| Bacteria plus virus | 21 |
| Fungi plus virus | 9 |
| Bacteria plus fungi plus virus | 8 |



**Fig. 2** ROC curve for *Aspergillus* galactomannan (GM) BAL $\geq 0.5$ index in probable/proven invasive aspergillosis
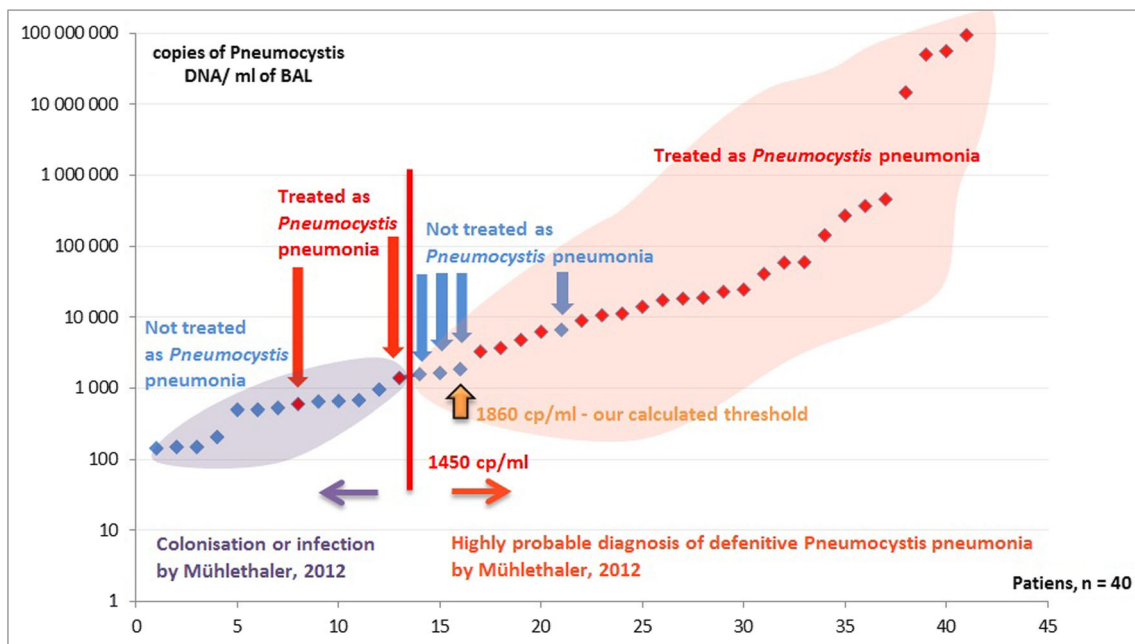
**Fig. 3** Pneumocystis pneumonia. The threshold value is set based on Maertens et al. (2007)

infectious pneumonia as well as different PCR diagnostic criteria (much lower cutoff for significant PCR DNA CMV quantity in BALF and larger diagnostic spectrum including of EBV, polyomavirus, and others). Another work focusing on HSCT recipients admitted to the ICU described the polymicrobial findings in 30% of patients and worked with results from blood cultures, low PCR DNA CMV cutoff and serum GM. All these differences explain higher reported percentage of polymicrobial etiology of pneumonia.
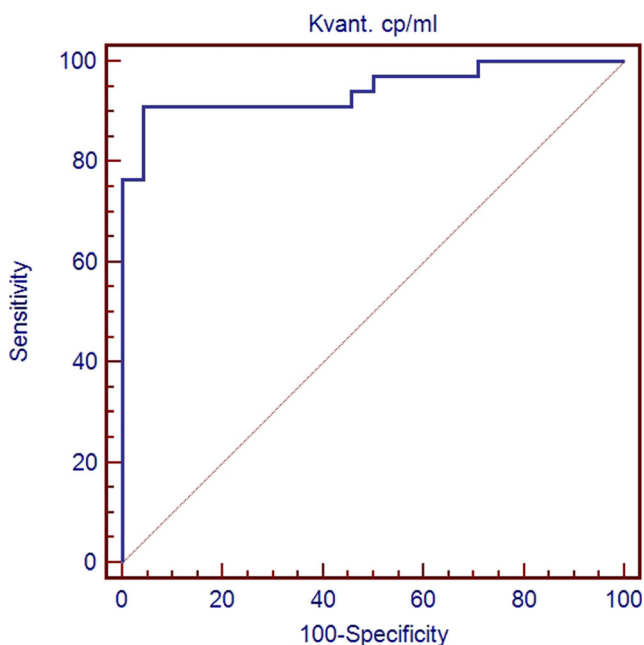


**Fig. 4** ROC curve showing *Pneumocystis jirovecii* DNA > 1860 cp/mL

Our categorization into pathogenic/colonizing agents is aligned with the updated guidelines of the German Society of Hematology and Medical Oncology (Maschmeyer et al. 2015). The microbiological agents isolated from BALF samples are not always relevant for etiology of pulmonary infiltrates and should therefore be interpreted critically (Maschmeyer et al. 2015). We report *coagulase-negative staphylococci*, *alpha-hemolytic streptococci*, and *enterococci* as the most common bacteria found in BALF samples, same as with other authors (Eriksson et al. 1996; Kuehnhardt et al. 2009). The significance of these organisms obtained from BAL samples remains unclear as most of them occur as commensals and rarely cause pneumonia (Kuehnhardt et al. 2009). However, some authors suggest that immunocompromised patients can develop pneumonia from these agents regardless (Kuehnhardt et al. 2009). All of these bacteria are abundant in mouth or oropharynx and can adhere to the bronchoscope during examination; therefore, we considered them as contaminating/colonizing agents. In similar manner, detected anaerobic bacteria were considered to be contamination from the oral flora since none of our patients had a lung abscess.

*Streptococcus pneumoniae* was the second most frequent pathogen after *Aspergillus* spp. based on PCR detection. *S. pneumoniae* is one of the most common causes of pneumonia in general population (Stralin et al. 2006; Murdoch et al. 2003; Falguera et al. 2002). Its prevalence in immunosuppressed patients is high (Kumar et al. 2008; Torda et al. 2014; Wieruszewski et al. 2018; Kumar et al. 2007). *S. pneumoniae* detection rate by conventional diagnostic methods is low, especially in cases when antibiotic therapy had been administered before examination (Strålin et al. 2006; Smith et al. 2003; Yu

et al. 2003). PCR detection methods may therefore be useful. However, these have relatively low specificity and positive predictive value for *S. pneumoniae* when used for non-sterile material such as BALF. This may have inflated the incidence rates in our cohort.

*Aspergillus* spp. was the most commonly detected agent in our study, which is consistent with the reports from other authors (Oren et al. 2016; Tang et al. 2018; Rano et al. 2001; Boersma et al. 2007; Hardak et al. 2016). We set the cutoff value to 0.5 for the BAL GM A index, obtaining excellent specificity and sensitivity; this is consistent with studies by Bergeron et al. (2010) and Maertens et al. (2007).

Zygomycosis, the second most frequently detected filamentous fungi, has recently been described as one of the most common type of mycosis reported in patients with hematological malignancies, particularly in acute leukemia patients or HSCT recipients (Caira et al. 2010; Cuenca-Estrella et al. 2009). We observed six positive qPCR hits for *Zygomycetes* in this study; all six patients were treated for zygomycosis, as they were high-risk patients with clinical signs consistent with invasive fungal disease. Two of these qPCR hits were also confirmed by BAL culture, meeting the criteria for "probable" fungal disease as per EORTC criteria (De Pauw et al. 2008). The remaining four were classified as "possible." The histopathological confirmation of invasive zygomycosis is required (Siwek et al. 2004; Stelzmueller et al. 2008); however, none was available in our patients. Despite our awareness of a potentially high false positivity rate, we firmly believe that the use of qPCR testing should be considered for *Zygomycetes* as one of the diagnostic criteria.

We calculated the threshold for qPCR detection of *P. jirovecii*, estimating a cutoff value of 1860 cp/mL. Our value obtained by retrospective analysis of patients with hematological malignancies corresponds to the more heterogeneous immunocompromised patient cohort reported by Mühlethaler et al. (2012).

It must be highlighted that these PCR values can vary significantly among centers due to the different procedures for sample collection, different qPCR kits, primers, and calibrations curves. Therefore, the value cannot be directly applied at other centers with different setups. This is one of the largest studies establishing the cutoffs of Aspergillus GM and PCR PCP dealing with large cohort of real world patients and therefore confirming the clinical values of cutoffs.

The prophylaxis of HSV infection with acyclovir has contributed to a currently decreased prevalence of HSV pneumonitis in high-risk patients (Azoulay 2011). Evidence of HSV qPCR hits was observed in only 14% of BAL samples, which is similar to the 19% reported by Gooskens et al. (2007). The diagnosis of HSV pneumonia needs to be confirmed by isolating the virus from respiratory secretions, either BALF samples or lung tissue, and by demonstrating viral cytopathic effects through histopathology (Azoulay 2011). Molecular detection techniques may be too sensitive and detect HSV in the lungs of asymptomatic individuals (Gooskens et al. 2007; Agusti and Torres 2009). Oral contamination following mucosal reactivation may also play a role in HSV detection in BALF samples (Gooskens et al. 2007). A clinically relevant DNA threshold in BAL samples has not been determined and differs according to the referred study (Gooskens et al. 2007; Linssen et al. 2008). We concluded that published quantities are comparable to ∼ 100,000 cp/mL in BALF samples; therefore, this value was used as our threshold to determine significance. Regardless, no conclusive data was found to prove that this is the best possible value; thus, more studies would be needed to determine specific qPCR thresholds for HSV in BAL samples.

The detection rate of CMV (any positivity in BAL) was of 10% in our study, which did not differ from Joos et al. (2007) or Kuehnhardt et al. (2009); however, our diagnostic methods were different. Evidence of CMV DNA in BAL samples has low positive predictive value (Boeckh 2011; Maschmeyer et al. 2015). In this regard, viral load in BAL samples and their association with CMV pneumonitis in solid organ transplant recipients were studied with contradictory results (Tan et al. 2016). We could not correlate viral load with CMV pneumonia itself (defined by Ljungman et al. (2002)), as the final diagnosis was not confirmed for any cases in our cohort. Based on an expert panel discussion, ≥ 10,000 cp CMV DNA/ mL per BALF sample was considered clinically significant. All patients with such CMV DNA quantity in their BALF samples had generally lower (100 cp/mL) or negative viral load in peripheral blood at the time of BAL collection.

Although pneumonia with detected human rhinoviruses (HRV) infection was reported in immunocompromised patients as well, it is still uncertain whether rhinoviruses are actually the primary cause of pneumonia or only lead to pulmonary injury and/or superinfection (Malcolm et al. 2001; Chemaly et al. 2006; Parody et al. 2007). Malcolm et al. (2001) and Parody et al. (2007) mentioned the common appearance of picornaviruses with other ongoing co-pathogens. Eight of our 12 HRV findings were associated with other co-pathogens as well: bacteria, viruses, or aspergilli. However, all the results in our study are based only on the qPCR method in BALF samples, so the real significance of this finding remains unclear.

The observed pneumonia mortality rate in our study was of 25%. Hardak et al. (2016) referred the same overall value in-hospital mortality rate as us; however, the mortality of patients with polymicrobial pneumonia was higher than of those with monomicrobial infection (49% versus 19%), which is contrary to our observation. Our patient cohorts were similar, but the strict criteria used in the definition of bacterial or viral pneumonia in Hardak's work may have played a crucial role in the observed difference. On the other hand, Kuehnhardt et al. (2009) reported only 16% mortality related to pneumonia. Their cohort of 58 examinations contained 2 allogenic HSCT recipients,

compared to our 82, which may explain the mortality difference. Multiple factors contribute to the high mortality and morbidity of HSCT recipients with pulmonary complications, e.g., T and B cell status and reconstitution after HSCT, immunosuppressive therapy, degree of neutropenia, presence and grade of graft versus host disease and its treatment, and status of the hematological disease at the time of HSCT.

The retrospective character of our work allowed a large patient cohort, but it is also a drawback. Some BALF results can be available as quickly as in a few hours (direct microscopy examination, some PCR methods), some can take up to days to weeks (e.g. 7 days for fungal culture, up to 6 weeks for mycobacteria cultivation). It is impossible in retrospective work to find out whether a change of antimicrobial treatment was based mainly on BAL results or on a change of patient's clinical status along with other examinations results. Hence, we analyzed the most important unbiased outcome: mortality. It remains undetermined whether acting on BALF results reduces mortality rates. Mortality related to ongoing pneumonia in our cohort was not influenced by positive nor negative microbiological results of BAL, nor type of agents or agranulocytosis $< 1000/\mu L$. The only significant mortality predictor was prior HSCT ($p = 0.023$), but it was independent on BAL results. A Beijing work (Tang et al. 2018) studying an impact of flexible bronchoscopy in patients after allogeneic HSCT showed no difference in 30-day mortality between patients in positive and negative FB groups. Rano et al. (2001) demonstrated a decreased mortality rate if treatment was adjusted as soon as possible based on the results from early diagnostics combining several methods, including BAL. Another study (Kuehnhardt et al. 2009) described the change in antibiotic treatment based on BAL results in a minority of patients with no difference in mortality rate.

Several limitations of our study should be noted. First, the retrospective design and collection of clinical data from a large cohort of patients did not allow us to accurately comment in detail on parameters such as antimicrobial treatment. Blood count was only available at the day of BAL collection, and we could not evaluate the impact of potentially prolonged neutropenia (Kuehnhardt et al. 2009). An important limitation is also the lack of histological evidence supporting the specific type of pneumonia (e.g., IA, PCP).

## Conclusion

In summary, BALF examination can reveal the infectious etiology of pneumonia in more than 50% of patients with hematologic malignancies, contributing key findings for individual cases (especially when a specific treatment may be administered). Up to one third of cases were classified as colonization, and 7% as a non-infectious disease. More studies are still needed to determine specific qPCR thresholds for opportunistic pathogens in BAL.

**Compliance with ethical standards** This study was reviewed and approved by the Ethics Committee of the University Hospital Hradec Kralove.

**Conflict of interest** The authors declare that they have no conflict of interest.

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