Acanthamoeba polyphaga mimivirus (APMV) belongs to the amoebae-associated microorganisms. Antibodies to APMV have been found in patients with pneumonia suggesting a potential role as a respiratory pathogen. In addition, positive serology for APMV was associated with an increased duration of mechanical ventilation and intensive care unit stay in patients with ventilator-associated pneumonia. The aim of the present study was to assess the presence of APMV in bronchoalveolar lavage fluid samples of critically ill patients suspected of ventilator-associated pneumonia. The study was conducted in the intensive care unit of the Maastricht University Medical Centre. All consecutive bronchoalveolar lavage fluid samples obtained between January 2005 and October 2009 from patients suspected of ventilator-associated pneumonia were eligible for inclusion. All samples were analyzed by real-time PCR targeting the APMV. A total of 260 bronchoalveolar lavage fluid samples from 214 patients (139 male, 75 female) were included. Bacterial ventilator-associated pneumonia was confirmed microbiologically in 105 out of 260 (40%) suspected episodes of ventilator-associated pneumonia (86 patients). The presence of APMV DNA could not be demonstrated in the bacterial ventilator-associated pneumonia positive or in the bacterial ventilator-associated pneumonia negative bronchoalveolar lavage fluid samples. Although suspected, APMV appeared not to be present in critically ill patients suspected of ventilator-associated pneumonia, and APMV does not seem to be a frequent cause of ventilator-associated pneumonia. *J. Med. Virol.* **85:**1836–1841, 2013. © 2013 Wiley Periodicals, Inc.
aeruginosa and Staphylococcus aureus [Chastre and Fagon, 2002]. However, using criteria described previously [Baselski, 1993; Allaouchiche et al., 1996], approximately 30–40% of patients suspected of ventilator-associated pneumonia have a bacterial ventilator-associated pneumonia which is confirmed microbiologically. Often, the cause of infection is not found in the remaining 60–70% of patients suspected of ventilator-associated pneumonia. In recent years, viruses have been suggested as the cause of respiratory infections in non-immunocompromised critically ill patients.

Acanthamoeba polyphaga mimivirus (APMV) is a giant virus, discovered in 2003, which belongs to the amoeba-associated microorganisms [Raoult et al., 2007]. Other amoeba-associated microorganisms, such as Legionella pneumophila, are known to cause pneumonia in different patient groups. Various studies have suggested the possible pathogenicity of APMV. Seroconversion to APMV was shown in patients with community-acquired pneumonia [La Scola et al., 2005] or nosocomial pneumonia [Berger et al., 2006]. In these studies, antibodies against APMV were found in respectively 9.7% and 7.1% of the patients included. In addition, in patients with ventilator-associated pneumonia, positive serology for APMV was associated with an increased duration of mechanical ventilation and stay in the intensive care unit [Vincent et al., 2009]. The virus itself was found only in one patient in one particular study [La Scola et al., 2005]. Because of the potential role of APMV as a respiratory pathogen and the significant proportion of patients suspected of ventilator-associated pneumonia without a causative microorganism found, the aim of the present study was to assess the presence of APMV in bronchoalveolar lavage fluid samples of critically ill patients suspected of ventilator-associated pneumonia by real-time PCR (RT-PCR).

MATERIALS AND METHODS

Patients

This retrospective study was conducted in the intensive care unit of the Maastricht University Medical Centre, Maastricht, the Netherlands, a 750-bed hospital. All consecutive bronchoalveolar lavage fluid samples obtained between January 2005 and October 2009 from patients suspected of ventilator-associated pneumonia were eligible for inclusion.

Clinical Variables

Clinical suspicion of ventilator-associated pneumonia was defined as described by Bonten et al. [1997]. Ventilator-associated pneumonia confirmed microbiologically was defined as admission to the intensive care unit at least 48 hr prior to BAL collection, a total quantitative culture result of ≥10^4 cfu/ml and/or ≥2% cells with intracellular organisms [Baselski, 1993; Allaouchiche et al., 1996]. Collected data included patients’ demographic characteristics such as: age, gender and clinical data such as: APACHE II score, reason for performing bronchoalveolar lavage, diagnosis upon admittance, admittance to intensive care unit ward, smoking, length of intensive care unit stay before the performance of bronchoalveolar lavage, total length of stay at intensive care unit, total length of hospital stay, mortality, diagnosis at post-mortem examination, diagnosis of pulmonary disease after bronchoalveolar lavage and laboratory data concerning bronchoalveolar lavage fluid: total cell count, differential cell count, presence of ciliated epithelial cells, squamous epithelial cells, reactive pneumocytes type II [Linssen et al., 2004] and quantitative culture results.

Rejection Criteria

Bronchoalveolar lavage fluid samples were rejected if the recovered volume was less than 20 ml, if the total cell count was less than 60,000 cells/ml, if preparations showed excessive amounts of intercellular debris or damaged nucleated cells or more than 1% squamous epithelial cells.

Sampling Technique

Bronchoscopy with subsequent lavage was performed as described previously [Linssen et al., 2008]. Bronchoalveolar lavage fluid samples were transported to the laboratory within 15 min after collection and processed immediately upon arrival at the microbiology laboratory.

Laboratory Processing

The first fraction of bronchoalveolar lavage fluid, representing the bronchial fraction, was used only for Mycobacterium spp. culture and/or PCR. The remaining three fractions (alveolar fractions) were pooled. A total cell count was performed using a Fuchs-Rosenthal haemocytometer chamber. Quantitative bacterial culture was performed as described previously [Jacobs et al., 2000]. Cytocentrifuged preparations [De Brauwer et al., 2000] were stained with May-Grünwald Giemsa stain and Gram stain. A differential cell count [De Brauwer et al., 2002] was performed including the number of cells, intracellular organisms and Reactive type II pneumocytes [Linssen et al., 2004]. From each sample, 6 ml was centrifuged (250g, 10 min), dividing the sample into cells and supernatant. The supernatant was stored in tubes of 1 ml at −80°C. The cells were resuspended in 6 ml of a mixture of Eagle’s Minimal Essential Medium with 2% Dimethyl Sulfoxide and stored in tubes of 1 ml at −80°C for future use.

Additional Samples

To identify possible infectious sources of APMV in the hospital, a total of 20 water samples (1 L each)
were collected. These samples included 10 water samples obtained from taps located at the intensive care unit and 10 samples from cooling towers supplying the water to the hospital. Samples were filtered through 0.2 μm polycarbonate filters (Sartorius Stedim biotech, Goettingen, Germany). Afterwards the filters were placed in 5 ml sterile water and sonicated for 5 min in a sonication bath (Bandelin electronic GmbH & Co. KG, Berlin, Germany) at 40 kHz.

APMV Real-Time PCR

A total of 500 μl of the stored bronchoalveolar lavage cell fractions and 5 ml of the filtered water samples were used for DNA isolation using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Purified DNA was resuspended in a final volume of 120 μl. Each sample was spiked with a control plasmid (murine cytomegalovirus glycoprotein B gene [mCMV-gb]) [Vliegen et al., 2004] before DNA isolation as an isolation and amplification control. An in-house PCR was performed using primers and probes, designed with the Primer3 software, which target the Mimi L396 VV A18 helicase gene (Table I). Assays were performed in a 96-well Optical Reaction plate (Applied Biosystems, Madison, WI). Purified DNA was resuspended in a final volume of 120 μl. Each sample was spiked with a control plasmid (murine cytomegalovirus glycoprotein B gene [mCMV-gb]) [Vliegen et al., 2004] before DNA isolation as an isolation and amplification control. An in-house PCR was performed using primers and probes, designed with the Primer3 software, which target the Mimi L396 VV A18 helicase gene (Table I). Assays were performed in a 96-well Optical Reaction plate (Applied Biosystems, Foster City, CA) in a total volume of 50 μl which contained 0.3 μM forward primer, 0.3 μM reverse primer, 0.1 μM probe, 25 μl ABsolute QPCR Mastermix (ABgene, Epsom, UK), and 10 μl DNA. The PCR reactions for the isolation and amplification control (mCMV-gb) were carried out using primers and a probe described previously [Vliegen et al., 2004]. The PCR thermal profile for both APMV and mCMV-ie consisted of an initial incubation of 15 min at 95°C followed by a total of 43 cycles of amplification (15 sec at 95°C and 1 min at 60°C). Amplification was performed using the ABI Prism 7000 Sequence Detection System and data were interpreted using the ABI Prism SDS software. A positive control containing APMV was supplied by professor D. Raoult [Raoult et al., 2007]. This positive control was included during each PCR run.

APMV Real-Time PCR Validation

DNA was isolated from cultured APMV and evaluated using a spectrophotometer (Nanodrop Spectrophotometer ND-1000, Isogen Life Science, De Meern, The Netherlands) to determine concentration and purity of the sample. Standard curves were generated using 10-fold serial dilutions (10^5, 10^4, 10^3, 10^2, 10, and 1 copies/ml). Tests were performed on cultured APMV resuspended in NaCl 0.9%, on both bronchoalveolar lavage fluid and tap water spiked with APMV. Amplification efficiency was calculated using ABI Prism SDS software showing an efficiency of 98%. To determine the detection limit, serial dilutions were tested, the lower limit of detection was established at 300 copies/ml (12 copies/PCR). Specificity of our assay was tested using the most common respiratory viruses (both RNA and DNA viruses). These viruses included: influenza A and B, para-influenza 1–4, RSV, hMPV, coronavirus, rhinovirus, HSV-1, and CMV. All tested negative in the PCR. To determine repeatability serial dilutions in bronchoalveolar lavage fluid (10^5, 10^4, 10^3, 10^2, 10, and 1 copy/ml) were tested by the same technician in 10 independent experiments. APMV dilutions were detected with a variation of 1–2 Ct values. A further five serial dilutions in bronchoalveolar lavage fluid were tested four times by two different technicians showing the in-house repeatability to be high.

RESULTS

Patient Group

Between January 2005 and October 2009, 363 bronchoalveolar lavage fluid samples were eligible for inclusion. A total of 103 of the 363 samples were excluded from the study. Insufficient material was available in 75 of the 103 samples and the other 28 excluded samples met the exclusion criteria. Finally a total of 260 samples from 214 patients were included. Table II shows the demographic data of the patients included.

Causative Microorganisms of Ventilator-Associated Pneumonia

Out of the 260 samples included with clinically suspected episodes of ventilator-associated pneumonia, 106 episodes in 101 patients were confirmed microbiologically. Table III shows the causative microorganisms in these episodes. When multiple microorganisms were found in the bronchoalveolar lavage fluid, only the microorganisms present in quantities ≥10^4 cfu/ml [Baselski, 1993; Allauochiche et al., 1996] were considered as causative microorganisms. The microorganisms isolated most frequently were enterobactericeae and non-fermenters (Table III). All samples included were tested retrospectively for the presence of cytomegalovirus, which yielded 12 positive samples.

APMV RT-PCR

APMV DNA was not detected in any of the 260 bronchoalveolar lavage fluid samples tested. APMV DNA was also not detected in any of the water samples collected from the hospitals cooling towers and the water taps of the intensive care unit.

TABLE I. Primers and Probe Targeting the Mimi L396 VV A18 Helicase Gene, Designed With the Primer3 Software

| Primer          | Sequence                             |
|-----------------|--------------------------------------|
| Forward primer  | TTTCTAGCAACCCATGGTGA                 |
| Reverse primer  | TCTTTTGTAGTTTCAATGGTTCA              |
| Probe           | ATCTCGTGCTTTGAATTTA                  |
| Amplicon length | 114 bp                               |

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DISCUSSION

In the present study, no APMV DNA was detected in bronchoalveolar lavage fluid samples of critically ill patients with suspected ventilator-associated pneumonia.

An animal study performed by Khan et al. [2007] has shown that APMV was able to induce histological evidence of pneumonia with the formation of diffuse alveolar damage in mice. Since the virus is able to induce pneumonia in mice, it was hypothesized that it would also be able to induce pneumonia in humans. Several studies have been conducted to detect APMV DNA in different patient groups [Larcher et al., 2006; Dare et al., 2008]. Until now, evidence of APMV as a respiratory pathogen has been based mainly on serologic studies. La Scola et al. [2005] performed a study in Canada showing the presence of antibodies to APMV in 9.7% of patients with community-acquired pneumonia. Berger et al. [2006] collected blood samples from all patients admitted to the intensive care unit suspected clinically of pneumonia. A total of 210 episodes of pneumonia were included. Antibodies against APMV were detected in 7.1% of these patients. Another study performed on an intensive care unit determined the APMV sero-status of the admitted patients [Vincent et al., 2009]. All patients with a suspicion of ventilator-associated pneumonia were sampled. Acute and convalescent-phase serum samples were tested for the presence of antibodies against APMV by an immunofluorescence assay. A total of 19.7% of the intensive care unit patients with a suspicion of ventilator-associated pneumonia were sero-positive for APMV. Furthermore, seropositivity for APMV was associated with an increased duration of both mechanical ventilation and stay at the intensive care unit. All these studies show a possible role for APMV in respiratory infections. However, the presence of antibodies against APMV was not confirmed in the present study.

### TABLE II. Demographic Data Included Patients (n = 214)

| Reason for intensive care unit admission (%) | Male:female ratio | Median age, years (range) | Days of intensive care unit admittance before lavage: mean (range) |
|---------------------------------------------|------------------|--------------------------|---------------------------------------------------------------|
| Pulmonary Community acquired pneumonia      | 2:1              | 64 (19–84)               | 10 (2–249)                                                   |
| Trauma                                      |                  |                          |                                                               |
| Surgery                                     |                  |                          |                                                               |
| Neurological/neurosurgical                  |                  |                          |                                                               |
| Other                                       |                  |                          |                                                               |
| Smoking (%)                                 |                  |                          |                                                               |
| Sepsis                                      |                  |                          |                                                               |
| Gastro-intestinal bleeding                  |                  |                          |                                                               |
| Kidney failure                              |                  |                          |                                                               |
| Other                                       |                  |                          |                                                               |
| APACHE II score, mean (range)               |                  |                          |                                                               |
| Outcome < 30 days                           |                  |                          |                                                               |
| developed after lavage (%)                  |                  |                          |                                                               |
| Died                                        |                  |                          |                                                               |
| Survived                                    |                  |                          |                                                               |
| Outcome < 30 days                           |                  |                          |                                                               |
| developed after intensive care unit admittance (%) |              |                          |                                                               |
| Died                                        |                  |                          |                                                               |
| Survived                                    |                  |                          |                                                               |

### TABLE III. Causative Micro-Organisms in the Microbiologically Confirmed Episodes of Ventilator-Associated Pneumonia

| Causative micro-organisms                       | Number (%) |
|------------------------------------------------|------------|
| Growth of one micro-organism                   |            |
| Enterobacteriaceae                             | 30 (28)    |
| Non-fermenters                                 | 21 (20)    |
| Staphylococcus aureus                          | 8 (8)      |
| Haemophilus spp.                               | 5 (4)      |
| Coagulase-negative staphylococci               | 2 (2)      |
| Streptococcus pneumonia                       | 2 (2)      |
| Other                                          | 4 (4)      |
| Growth of multiple micro-organisms             |            |
| Multiple types of Enterobacteriaceae           | 4 (4)      |
| S. aureus and Enterobacteriaceae               | 3 (3)      |
| S. aureus and non-fermenter                    | 2 (2)      |
| Enterobacteriaceae and non-fermenter           | 3 (3)      |
| Multiple types of non-fermenters               | 1 (1)      |
| S. pneumoniae and Haemophilus spp.             | 1 (1)      |
| S. pneumoniae and non-fermenter                | 1 (1)      |
| S. aureus and Haemophilus spp.                 | 1 (1)      |
| Other                                          | 3 (3)      |
| Commensal flora of oropharynx                  | 11 (10)    |
| Candida albicans                               | 2 (2)      |
| No growth                                      | 2 (2)      |

| a Including: Escherichia coli, Proteus spp., Klebsiella spp., Enterobacter spp., and Citrobacter spp. |
| b Including: Pseudomonas aeruginosa, Acinetobacter spp., and Stenotrophomonas maltophilia. |
| c Including: one methicillin-resistant Staphylococcus aureus. |
| d Microscopic evaluation did show 2% intracellular micro-organisms thereby confirming the diagnosis ventilator-associated pneumonia; both patients had already received adequate antibiotic therapy before lavage. |
APMV does not prove an active infection caused by the virus. In order to establish a causal link between APMV and pulmonary infections the criteria described in an article by Robinson [1958] could be used. These specific criteria are based on Koch’s postulates but adapted to fit (the majority of) viruses. These criteria state that the virus should be present in respiratory specimens of a significant percentage of patients with a suspicion of the infection and should be absent in the majority of healthy people. The mere presence of antibodies shows that the individual has been into contact with the virus, but does not always imply disease. The results of the present study are in line with two previous studies. APMV DNA could not be detected in neither patients with community-acquired pneumonia nor in patients with hospital-acquired pneumonia in nasal and nasopharyngeal aspirates of nine different patient-groups consisting of 496 patients [Dare et al., 2008]. The patients included in the present study were all critically ill patients with a suspicion of ventilator-associated pneumonia opposed to the study performed by Dare et al. [2008] consequently, the patient-groups may not be fully comparable. The patient material on which the PCR was performed also differed; the present study only used bronchoalveolar lavage fluid samples, whilst the PCR in the study of Dare et al. [2008] was conducted on nasal and nasopharyngeal aspirates. In a study conducted by Larcher et al. [2006], a total of 214 nasopharyngeal aspirates from children, hospitalized for respiratory tract infections, were tested for the presence of different respiratory viruses, including APMV. APMV could not be detected in any of the respiratory samples tested by APMV-specific PCR. Only one study currently describes a patient in whom APMV DNA was detected in a bronchoalveolar lavage specimen. La Scola et al. [2005] performed a study in a Canadian population consisting of 376 patients diagnosed with community-acquired pneumonia and 511 healthy controls. The patient in whom APMV was detected was a 60-year-old critically ill patient who had two episodes of hospital-acquired pneumonia during hospitalization. APMV DNA was only detected in the sample from the second episode; unfortunately no serum was tested for the presence of antibodies against APMV in this patient. Since APMV was only detected during the second episode of suspected hospital-acquired pneumonia it suggests it was acquired during hospitalization, probably through water present on the intensive care unit.

Since APMV has only been isolated once in a single patients with a clinical respiratory infection [La Scola et al., 2005], APMV might not be a major cause of respiratory infections in intensive care patients.

However, a remark has to be made concerning the negative PCR results of the present study. Since the discovery and description of the first APMV strain, many other APMV-related viruses have been detected. The probe used in the present study matches with both AMPV and mamavirus, but not with other closely related viruses of the Mimivirus family. Therefore, the patient in the present study could be positive for other viruses of the Mimivirus family. Since the viruses of the Mimivirus family are prone to polymorphisms at the nucleotide level, designing a universal PCR model, with a high sensitivity, for the complete Mimivirus family is difficult. One of the limitations of the present study was the lacking of serology results of the patients. Therefore, the extent of exposure of the studied patients to APMV is yet to be unraveled. Likewise, to the authors’ best knowledge the seroprevalence of antibodies against APMV in the general Dutch population is also not known, making it impossible to predict the population at risk for a possible APMV infection. Another limitation might be the retrospective nature of the study. Due to the duration of the study, all bronchoalveolar lavage fluid samples were stored and frozen, some of them for a long time. This might have altered the composition of the bronchoalveolar lavage fluid samples. A third limitation of the study is the amount of excluded bronchoalveolar lavage fluid samples. Due to the amount of other test already performed on the bronchoalveolar lavage fluid samples for diagnostic and research purposes, a proportion of samples did not contain enough material to perform the DNA extraction for APMV correctly.

Although suspected, APMV appeared not to be present in the studied critically ill patients suspected of ventilator-associated pneumonia. Therefore, APMV does not seem to be a frequent cause of ventilator-associated pneumonia.

REFERENCES

Allaouchiche B, Jaumain H, Dumontet C, Motin J. 1996. Early diagnosis of ventilator-associated pneumonia. Is it possible to define a cutoff value of infected cells in BAL fluid? Chest 110:1558–1565.

Baseslki V. 1993. Microbiologic diagnosis of ventilator-associated pneumonia. Infect Dis Clin North Am 7:331–357.

Berger P, Papazian L, Drancourt M, La Scola B, Auffray JP, Raoult D. 2006. Ameba-associated microorganisms and diagnosis of nosocomial pneumonia. Emerg Infect Dis 12:248–255.

Bonten MJ, Bergmans DC, Stobberingh EE, van der Geest S, De Leeuw PW, van Tiel FH, Gaillard CA. 1997. Implementation of bronchoscopic techniques in the diagnosis of ventilator-associated pneumonia to reduce antibiotic use. Am J Respir Crit Care Med 156:1820–1824.

Chastre J, Fagon JY. 2002. Ventilator-associated pneumonia. Am J Respir Crit Care Med 165:867–903.

Dare RK, Chittaganpitch M, Erdman DD. 2008. Screening pneumonia patients for mimivirus. Emerg Infect Dis 14:466–467.

De Brauwer EI, Jacobs JA, Nieman F, Bruggeman CA, Wagenaar SS, Drent M. 2000. Cyto centrifugation conditions affecting the differential cell count in bronchoalveolar lavage fluid. Anal Quant Cytol Histol 22:416–422.

De Brauwer EI, Jacobs JA, Nieman F, Bruggeman CA, Drent M. 2002. Bronchoalveolar lavage fluid differential cell count. How many cells should be counted? Anal Quant Cytol Histol 24:337–341.

Jacobs JA, De Brauwer EI, Cornelissen EI, Drent M. 2000. Accuracy and precision of quantitative calibrated loops in transfer of bronchoalveolar lavage fluid. J Clin Microbiol 38:2117–2121.
Mimivirus and Ventilator-Associated Pneumonia

Khan M, La Scola B, Lepidi H, Raoult D. 2007. Pneumonia in mice inoculated experimentally with Acanthamoeba polyphaga mimivirus. Microb Pathog 42:56–61.

La Scola B, Marrie TJ, Auffray JP, Raoult D. 2005. Mimivirus in pneumonia patients. Emerg Infect Dis 11:449–452.

Larcher C, Jeller V, Fischer H, Huemer HP. 2006. Prevalence of respiratory viruses, including newly identified viruses, in hospitalized children in Austria. Eur J Clin Microbiol Infect Dis 25:681–686.

Linssen KC, Jacobs JA, Poletti VE, van Mook W, Cornelissen EI, Drent M. 2004. Reactive type II pneumocytes in bronchoalveolar lavage fluid. Acta Cytol 48:497–504.

Linssen CF, Jacobs JA, Schouten JS, van Mook WN, Ramsay G, Drent M. 2008. Influence of antibiotic therapy on the cytological diagnosis of ventilator-associated pneumonia. Intensive Care Med 34:865–872.

Raoult D, La Scola B, Birtles R. 2007. The discovery and characterization of Mimivirus, the largest known virus and putative pneumonia agent. Clin Infect Dis 45:95–102.

Rello J, Ollendorf DA, Oster G, Vera-Llonch M, Bellm L, Redman R, Kollef MH. 2002. Epidemiology and outcomes of ventilator-associated pneumonia in a large US database. Chest 122:2115–2121.

Robinson CR. 1958. Koch’s postulates and the modern era in virus research. Can Med Assoc J 79:387–389.

Vincent A, La Scola B, Forel JM, Pauly V, Raoult D, Papazian L. 2009. Clinical significance of a positive serology for mimivirus in patients presenting a suspicion of ventilator-associated pneumonia. Crit Care Med 37:111–118.

Vliegen I, Duijvestijn A, Stassen F, Bruggeman C. 2004. Murine cytomegalovirus infection directs macrophage differentiation into a pro-inflammatory immune phenotype: Implications for atherogenesis. Microbes Infect 6:1056–1062.