Pneumocystis jirovecii Can Be Productively Cultured in Differentiated CuFi-8 Airway Cells

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ABSTRACT **Although *Pneumocystis jirovecii* is a well-known and serious pathogen, all previous attempts to isolate, cultivate, and propagate this fungus have failed. This serious challenge in microbiology was addressed in the present study. We examined whether *P. jirovecii* could be cultured in a permanent three-dimensional air-liquid interface culture system formed by CuFi-8 cells, a differentiated pseudostratified airway epithelial cell line. Cultured pseudostratified cells were inoculated with bronchoalveolar fluid that had been confirmed to be positive for *P. jirovecii* using PCR. Five days later, the cells and basal medium were harvested and tested for *P. jirovecii* using quantitative PCR (qPCR), commercially available immunofluorescence detection assays, and Grocott staining of formalin-fixed, paraffin-embedded thin sections of infected-cell cultures. We successfully productively cultivated and propagated *P. jirovecii* from these *P. jirovecii*-positive bronchoalveolar lavage fluid (BALF) samples. Furthermore, we provide evidence that *P. jirovecii* induced cytopathic effects on lung epithelial cells and was even invasive in cell culture. To the best of our knowledge, the cell culture system developed herein represents the first methodology to enable molecular analyses of this pathogen's life cycle and further *in vitro* studies of *P. jirovecii*, such as assessments of drug sensitivity and resistance as well as investigations of the pathogen's stability against environmental factors and disinfectants.**

IMPORTANCE **This is the first report of the successful productive cultivation and propagation of *Pneumocystis jirovecii*, a human-pathogenic fungus of major clinical significance. These findings are groundbreaking because they will influence the field of diagnostic microbiology, facilitate the testing of antibiotics against *P. jirovecii*, and enable stability studies of this pathogen when exposed to the environmental factors and chemicals that hospitals are required to use for disinfection. Because productively culturing *P. jirovecii* has been attempted unsuccessfully for several decades, this study represents a breakthrough in this field.**

Received 10 April 2014 **Accepted** 14 April 2014 **Published** 13 May 2014

Citation Schildgen V, Mai S, Khalfaoui S, Lüsebrink J, Pieper M, Tillmann RL, Brockmann M, Schildgen O. 2014. *Pneumocystis jirovecii* can be productively cultured in differentiated CuFi-8 airway cells. mBio 5(3):e01186-14. doi:10.1128/mBio.01186-14.

Editor Louis Weiss, Albert Einstein College of Medicine

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The fungus *Pneumocystis jirovecii* is a well-known pathogen that causes severe medical and economic burdens worldwide. This pathogen was discovered in 1909 (1), detected in rats in 1910 (2), and classified as *Pneumocystis carinii* in 1912 (3). The association between *P. jirovecii* and pneumonia has been known since World War II (4–6), and this pathogen became a focus of clinicians with the onset of the HIV/AIDS pandemic (7). In the first decade of the 20th century (8), the human-pathogenic form of *Pneumocystis* was classified as a separate species from the rodent form (*P. carinii*) and was renamed *P. jirovecii* based on molecular analyses. Approximately 95% of the worldwide population is believed to become infected with this fungus during the first 2 years of life, but healthy adults are asymptomatic carriers of the fungus (7).

Another *Pneumocystis* species, *P. orctolagii*, i.e., the rabbit version of this *Pneumocystis* species, can be investigated in an animal model. Therefore, most of the knowledge about the life cycle of *Pneumocystis* is derived from animal studies or microscopic analyses of clinical specimens. As stated by Chabe et al. in 2011 (9), "most morphological data on this species can be extended to other *Pneumocystis* species." As shown schematically in Fig. 1, the life cycle of *Pneumocystis* is characterized by morphologically distinct forms that can be identified through the microscopic analysis of *Pneumocystis*-positive clinical specimens by trained scientists. An infection is believed to be initiated by the ameboid, thin-walled, mononuclear vegetative or trophic form of the pathogen (9). This form reaches a thick-walled cystic stage, from which eight ascospores are eventually formed. These ascospores can leave the cystic structure and attach to alveolar epithelia, where they return to the cystic stage (9).

In human patients, the pathogen may cause severe life-threatening pneumonia, and it is associated with an increasing number of other chronic, severe, and sometimes life-threatening lung diseases, such as chronic obstructive pulmonary disease (COPD) (10–13). Although *P. jirovecii* is primarily found in patients with severe immunosuppression due to AIDS, immune disorders, or therapies that directly or indirectly modulate the immune system (i.e., immunosuppression after transplantation or cancer therapy), it is becoming increasingly evident that otherwise

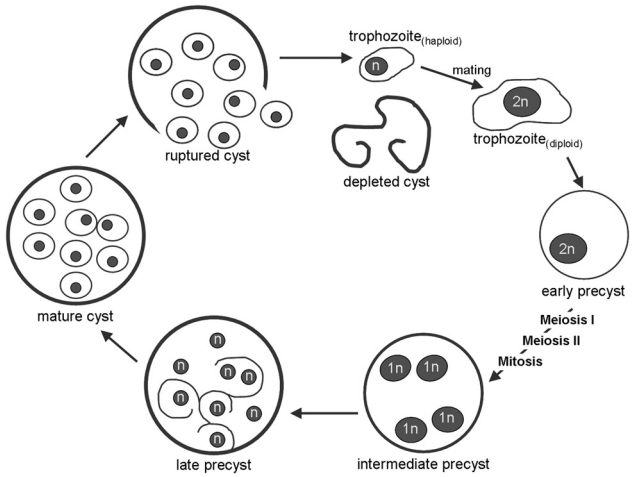

FIG 1 Schematic overview of the life cycle of *Pneumocystis*. The infection is believed to begin with an ameboid, thin-walled, mononuclear vegetative or trophic form. Through meiosis I and II, this form reaches a thick-walled cystic stage. Finally, after several maturation steps, eight ascospores are formed. These ascospores can detach from the cystic structure and attach to alveolar epithelia, where they return to the cystic stage.

healthy people can also be seriously infected with *P. jirovecii* (7). Early attempts to isolate and propagate *P. jirovecii*, which at that time was known as *P. carinii*, were moderately successful: coculturing human isolates with chicken embryonic lung cells (CELs) resulted in a maximum 10-fold increase in the parasite number (14), and a 10.8-fold increase was observed in cocultures with Vero cells (15). The rodent variant of *Pneumocystis* has been cocultivated with WI38 cells, with a human embryonic fibroblast cell line (16), and with human A549 cells (17); however, none of these models garnered sufficient recognition to become a standard method for the isolation of *P. jirovecii*.

A major breakthrough was achieved in 2012 when Cisse and coworkers assembled the *P. jirovecii* genome from a single clinical specimen (18). However, those authors noted that, despite great efforts, it was impossible to productively propagate and cultivate *P. jirovecii* using any known methods. Thus, this issue has remained a major challenge in the field of diagnostic microbiology.

RESULTS

Development of a quantification method for *P. jirovecii*. Due to the lack of methods for productively amplifying and quantifying *P. jirovecii*, the first challenge of our study was to develop a reliable quantification protocol for measuring *P. jirovecii* replication and growth. Therefore, we utilized two established PCR protocols. The first PCR protocol was previously described by Botterel and coworkers (19) and is performed on the Roche LightCycler (LC) 2.0 platform. This reaction amplified the mitochondrial ribosomal large subunit (mtLSU). For the second quantitative PCR (qPCR), we employed the LightMix kit *Pneumocystis jirovecii* (TIB MOLBIOL, Berlin, Germany), which targets the multicopy surface glycoprotein (MSG) gene, which is present in 50 to 100 copies in the nuclear genome of *P. jirovecii*.

To compare these methods, we analyzed 10 clinical bronchoalveolar lavage fluid (BALF) samples from adults with *P. jirovecii* infections. As shown in Table 1, the mtLSU qPCR had a detection range of 1.7×10^7 to 5.5×10^{14} mitochondrial genome equivalents (*mt*geq), with a median detection of 1.10×10^{10} *mt*geq per ml of BALF. The detection range of the MSG qPCR was 2.99×10^3 to 1.54×10^8 target copy equivalents (*tc*eq), with a median of 7.68×10^4 *tc*eq per ml of BALF. As shown in Table 1, all the BALF samples with a high copy number of mtLSU *mt*geq clearly had a correspondingly high number of MSG *tc*eq. Therefore, the median number of mitochondrial genomes per MSG target copy was 6.09×10^4 *mt*geq/*tc*eq, with a range between 3.42×10^3 and 6.77×10^6 *mt*geq/*tc*eq. Thus, a high *mt*geq value does not precisely correspond to the number of infectious *P. jirovecii* particles, but it does indicate a large number of fungi, with a maximum of 10^8 particles per ml.

A novel system for culturing and propagating *P. jirovecii*. The results of this study represent a fundamental breakthrough in microbiology: we were able to productively culture *P. jirovecii* in human pseudostratified airway cells. Although this culture system contains human cells and is therefore not purely axenic, our approach enables remarkable growth of *P. jirovecii*.

On the basis of our previous research on human bocavirus, we implemented a cell culture system with human airway epithelial cells that can be differentiated into an air-liquid interface culture (Fig. 2A). This model utilized CuFi-8 cells, which were a kind gift from the group of John Engelhardt, Aloysius Klingelhutz, and Phil

TABLE 1 Overview of the qPCR results from 10 BALF samples positive for *P. jirovecii* according to both PCR methods[a]

BALF sample	qPCR result		
	mtLSU	TIB Molbiol MSG	No. of *mt*geq/no. of MSG *tc*eq
1	1.2×10^7	2.99×10^3	4.01×10^3
2	1.51×10^7	3.02×10^3	5×10^3
3	1.7×10^7	3.83×10^3	$4.43\ 10^3$
4	5.99×10^8	5.80×10^3	1.03×10^5
5	2.11×10^9	1.63×10^4	1.3×10^5
6	1.98×10^{10}	1.37×10^5	1.44×10^5
7	5.25×10^{11}	1.54×10^8	3.42×10^3
8	2.32×10^{12}	1.26×10^8	1.85×10^4
9	1.61×10^{14}	2.38×10^7	6.77×10^6
10	5.5×10^{14}	1.33×10^8	4.15×10^6
Range	$1.2 \times 10^7 - 5.5 \times 10^{14}$	$2.99 \times 10^3 - 1.54 \times 10^8$	$3.42 \times 10^3 - 6.77 \times 10^6$
Median	1.10×10^{10}	7.68×10^4	6.09×10^4

[a] Clearly, the BALF samples with a high copy number of *mt*geq also had a high copy number of MSG *tc*eq.

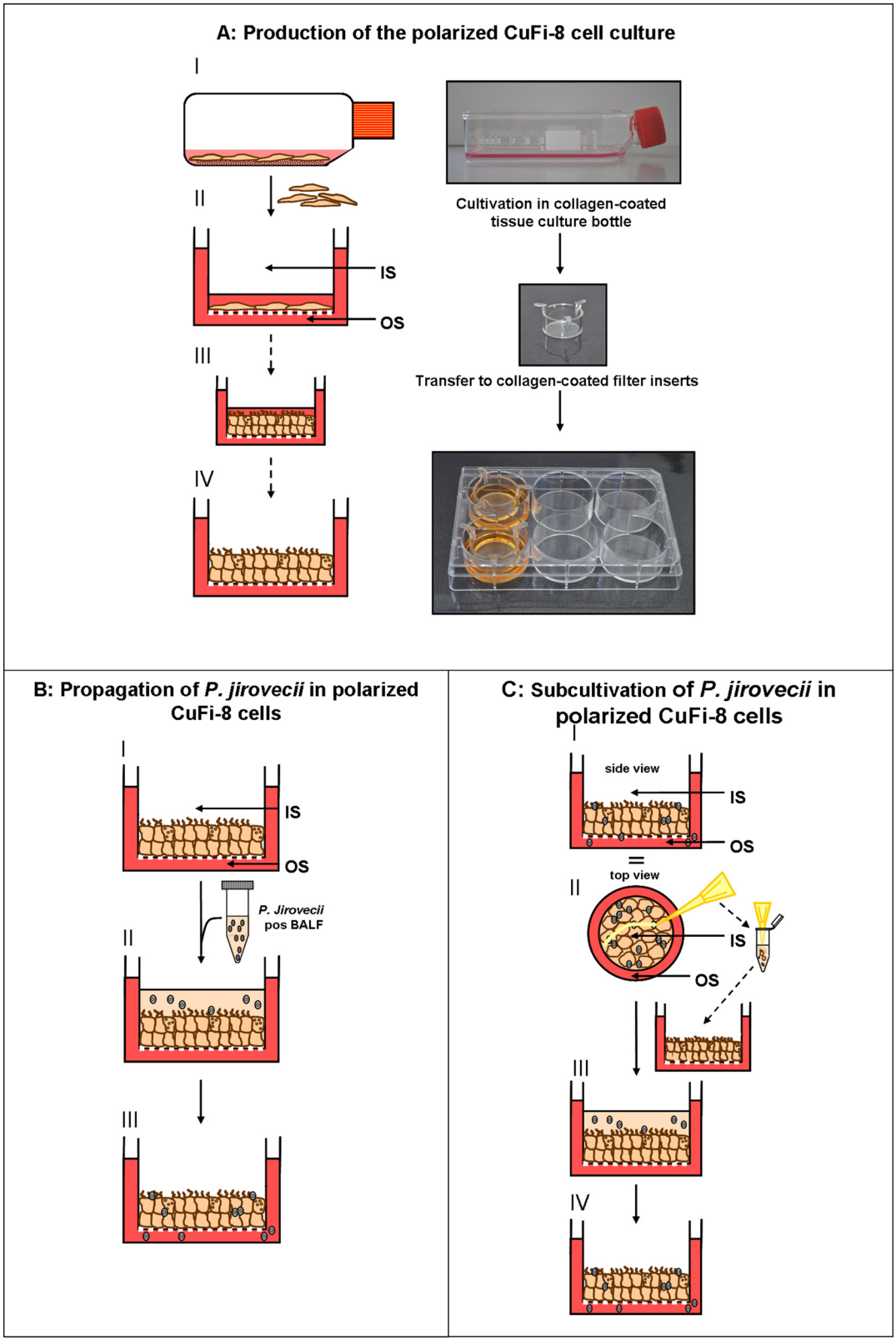

FIG 2 Schematic overview of the technical procedures performed using CuFi-8 and EpiAirway cells. Once confluence was reached and the cells began to form a pseudostratified epithelium, no basal medium reached the filter surface. Cells were fed from the basal side, and the cell surface appeared "slimy" due to the presence of a mucosal layer. Abbreviations: OS, outer space, i.e., the lumen between the insert and the six-well dish containing the basal medium; IS, inner space, which includes the surface of the collagen-coated membrane on which the CuFi-8 cells grew. (A) Production of the polarized CuFi-8 cell culture. (I) Cultivation of CuFi-8 cells in collagen-coated tissue culture bottles. (II to IV) Generation of polarized human airway epithelia by growing CuFi-8 cells on collagen-coated

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Karp at the University of Iowa and have been previously described (20). The CuFi-8 cells were derived from the bronchus of a patient with cystic fibrosis who had the ΔF508/ΔF508 genotype. The cells were transformed using Weinberg telomerase and the human papillomavirus type 16 genes E6 and E7, and they carry G418 and hygromycin resistance genes. It is noteworthy that several other CuFi cell lines are commercially available from ATCC.

We inoculated differentiated CuFi-8 cells with BALF samples from patients with respiratory problems who had tested positive for *P. jirovecii* via the real-time PCR amplification of the mtLSU gene of *P. jirovecii* (Fig. 2B) (19). To quantify the amount of pathogen present in the samples, we used a standard dilution series of the pCR2.1 TOPO TA vector (Invitrogen, Karlsruhe, Germany). This vector contains the *P. jirovecii* mtLSU sequence, which was derived from a sample determined to be positive via endpoint PCR during the first German round robin trial (INSTAND e.V., Düsseldorf, Germany) and was sequenced in triplicate (MWG Eurofins, Ebersberg, Germany).

Cultured CuFi-8 cells on the air side of the air-liquid interface were infected with 10 μl or 150 μl of BALF (Fig. 2B); after an overnight incubation at 37°C, the inoculum was removed. The cells were thoroughly washed and further incubated for up to 5 days postinoculation before being harvested from the filter membrane. The basal cell culture medium was collected and stored separately until DNA extraction was performed.

The subsequent qPCR, which targeted the mitochondrial genome, revealed an increase in *mt*geqs (from 10^2 to 10^7) in the examined infected cells, strongly indicating that *P. jirovecii* was able to efficiently grow in this culture system (Fig. 3A). Because it was possible that the mitochondria and their genomes could have increased in the absence of cellular replication, we confirmed the results of this quantification method by performing a second qPCR targeting the MSG gene of *P. jirovecii* (TIB MOLBIOL). As shown in Fig. 3B, this second PCR confirmed the increase in pathogen-derived components, strongly supporting the theory that the fungus had actively replicated and grown on the CuFi-8 cells.

In addition to finding that *P. jirovecii* was associated with the cell membrane, we were surprised to detect it in the basal cell culture medium, given that the BALF was added exclusively to the air side of the culture. Moreover, the filter membranes remained intact during the entire observation period; thus, the fungus must have actively passed through the filter membrane (Fig. 3). Figure 3A shows the results of 5 independent trials of the productive culturing of *P. jirovecii* in CuFi-8 cells. In all cases, *P. jirovecii* DNA was detected via qPCR (targeting the mtLSU gene) both in the basal cell culture fluid and bound to the cell membrane. Moreover, Fig. 3B shows three independent experiments in which another gene, namely, the nuclear encoded MSG gene complex, was targeted. This gene complex exists with 50 to 100 copies per cell. With this test, identical results, i.e., an increase of *P. jirovecii* genome copies of up to 3 $\log_{10}$ units were observed. Finally, Fig. 3C confirms the results obtained for CuFi-8 cells in a commercially available cell culture system named EpiAirway. The qPCR products were sequenced and confirmed to be derived from *P. jirovecii* using a BLAST analysis; the sequence CTGCGAATTGTTTTGGC AAATTGTTTATTCCTCTAAAAAATAGTAGGTATAGCACTG AATATCTCGAGGGAGTCGAGA exhibited 100% identity with the following GenBank accession numbers: JX499143.1, JF733757.1, EU979566.1, FJ357849.1, FJ357848.1, FJ357847.1, EF439817.1, EF439816.1, EF439814.1, EF439813.1, S77824.1, S42926.1, and AF337535.1. These results clearly show that part of the *P. jirovecii* mtLSU rRNA gene had been amplified.

Histological analyses of polarized CuFi-8 cells infected with *P. jirovecii*-positive BALF and labeled with Grocott silver staining revealed *P. jirovecii* particles on top of and between the cell layers and budding through the membrane (indicated by black arrows in Fig. 4B). The most prominent form of *P. jirovecii* observed in the CuFi-8 cells were particles budding through the membrane, as indicated by black arrows (Fig. 4B, panels II and III), which explains why *P. jirovecii* was also found in the basal medium without any disruption of the filter. The presence of *P. jirovecii* in the basal medium was further confirmed using the commercial antibody-based Merifluor immunofluorescence assay (Meridian Bioscience Inc., Cincinnati, OH, USA), which revealed antibody-positive structures with highly similar shapes to *P. jirovecii* previously isolated from PCR-positive lung tissues (21) (Fig. 5).

Immunofluorescence staining of formalin-fixed, paraffin-embedded sections of infected CuFi-8 cells using a monoclonal antibody against *P. jirovecii* (Dako, Hamburg, Germany) resulted in a strong fluorescent signal, providing additional support for the finding that *P. jirovecii* can be cultured in CuFi-8 cells (Fig. 5). However, it has to be noted that the collagen-coated membrane displays an autofluorescence (Fig. 5B), a phenomenon that is well-known in molecular pathology (22–24). During the examination of the sections, it became obvious that *P. jirovecii* had exerted a cytopathic effect that had damaged the integrity of the cell layers. In this context, it is important to note that during the productive infection with *P. jirovecii*, the epithelial cultures became thinner, a phenomenon previously observed by Huang and colleagues, who analyzed infection by human bocavirus (20). A clearly cytopathic effect was also observed in EpiAirway cells infected with *P. jirovecii*, as shown in Fig. 4C.

Finally, we examined whether *P. jirovecii* could be propagated in the CuFi-8 culture system, a prerequisite for further *in vitro* studies that had not been previously achieved. To this end, CuFi-8 cells were inoculated as described above, and a small portion was harvested with a pipette tip (Fig. 2C). This tip was placed into 150 μl of phosphate-buffered saline (PBS) and mixed, and 100 μl of this mixture was used to inoculate a new culture of differentiated CuFi-8 cells. These inoculated cells were treated and harvested as described above. The transfer of two different isolates resulted in an increase in *P. jirovecii* mitochondrial DNA of up to

Figure Legend Continued

filter inserts (4-μm pore size) at an air-liquid interface environment (3 to 4 weeks). The polarity of the culture was determined via transepithelial electrical resistance (TEER). (B) Propagation of *P. jirovecii* in polarized CuFi-8 cells. (I) Air-liquid interface culture of CuFi-8 cells. (II) Inoculation with 150 μl BALF that tested positive (pos) for *P. jirovecii* by PCR overnight. Cultures were inoculated with *P. jirovecii* if a value of >1,000 Ω cm^2 was achieved by TEER. Cells were washed with PBS 12 h postinfection (p.i.). PBS was removed after the cells were washed. (III) Cells were incubated for 5 days. Subsequently, infected cultures were tested by PCR, silver staining, and fluorescence staining. (C) Subcultivation of *P. jirovecii* in polarized CuFi-8 cells. (I) *P. jirovecii*-infected CuFi-8 culture. (III) Harvesting was done after 5 days of incubation. Cultures were tested subsequently by PCR.

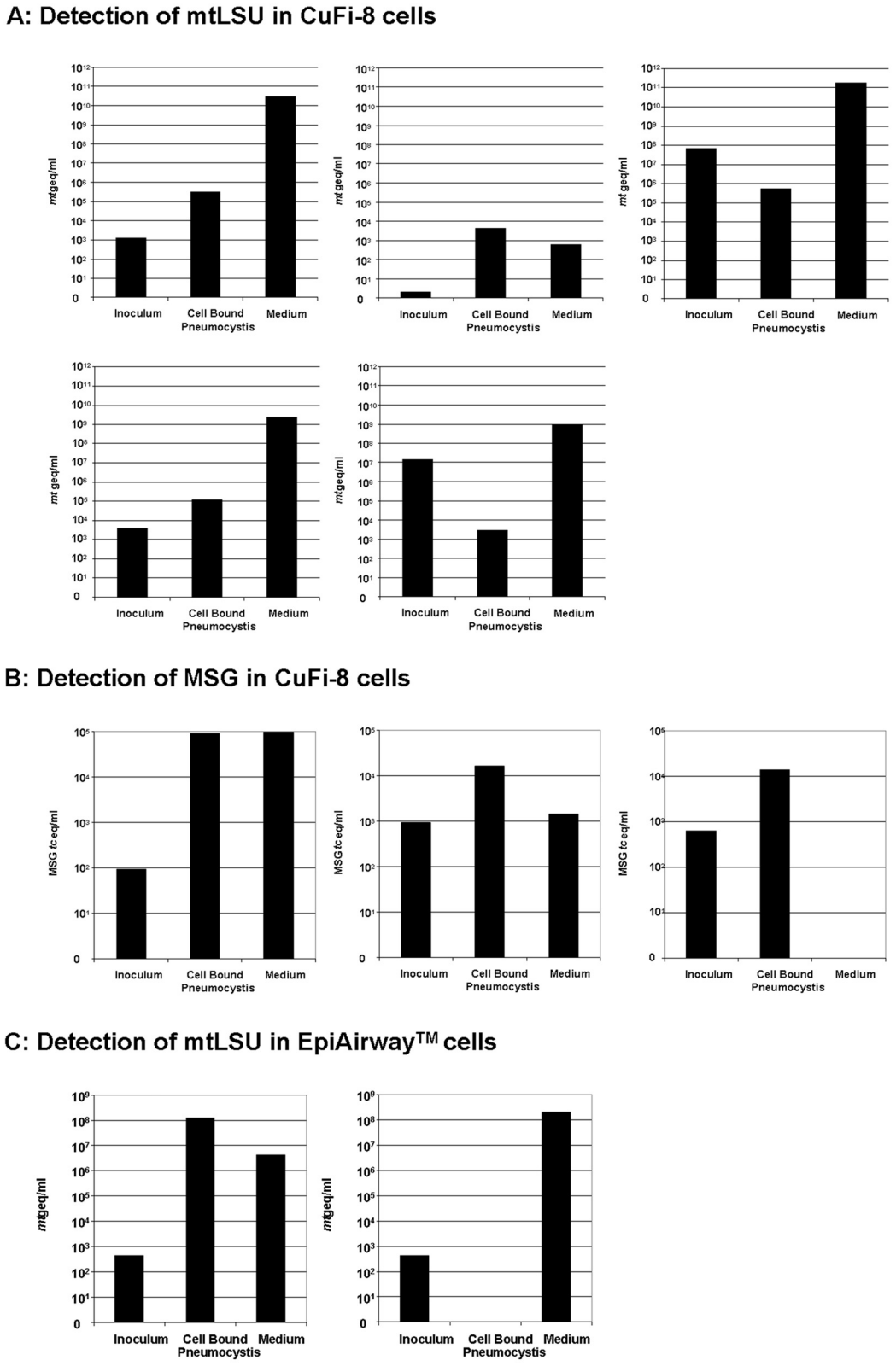

FIG 3 (A) Five independent trials of *P. jirovecii* productive culturing from BALF samples collected from patients who had tested positive for *P. jirovecii* via qPCR amplification of the ribosomal mtLSU gene (19). The numbers of genome equivalents amplified from the inoculum and from the cell membrane-bound *Pneumocystis* as well as the amount of *P. jirovecii* found in the basal cell culture medium are shown. The quantification was performed by measuring mitochondrial genome equivalents per ml (*mt*geq/ml). (B) Three independent trials of *P. jirovecii* productive culturing from BALF confirmed to be pathogen positive via qPCR (TIB MOLBIOL, Berlin, Germany) amplification of the nucleus-encoded multicopy surface glycoprotein (MSG) gene. Although this qPCR detects a

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10^7 *mt*geq/ml, clearly demonstrating that it is possible to propagate *P. jirovecii* in CuFi-8 cells (Fig. 6).

DISCUSSION

Taken together, the data produced using several highly specific methods demonstrate that the fungus productively cultivated from BALF in our study is, in fact, *P. jirovecii*. These results were confirmed using two different antibody assays from different manufacturers; both methods have been previously published by others and are accepted for use in routine diagnostics. Moreover, the quantification of *P. jirovecii* was performed according to a published and accepted PCR protocol that makes use of primers highly specific for *P. jirovecii*. In concert with the results of the classical Grocott silver staining, which revealed typical morphological features, i.e., the parenthesis-like structures of *P. jirovecii* trophozoites, the successful propagation of *P. jirovecii* indicates that we have established the first productive system to cultivate, propagate, and study *P. jirovecii* infection *in vitro*.

The advantage of the CuFi-8 cell culture system is that although CuFi-8 cells are not yet commercially available, other CuFi cells have been deposited in ATCC. Furthermore, newly available commercial cell culture systems have features that are identical to the system we generated with CuFi-8 cells (25). In addition, once established in the laboratory, these cells are simple to cultivate as monolayers, and they can be differentiated easily by mounting them onto filter inserts and changing the culture medium. A promising approach that we used in a pilot experiment involved culturing the pathogen in commercially available EpiAirway cells. However, in addition to their high cost, one caveat of using these cells is that they are of heterogeneous origin; thus, not all infections may be productive.

Prior to this study, culturing the human-pathogenic fungus *P. jirovecii* was considered impossible. Despite the existence of animal models for the rodent variant *P. carinii* and some cocultivation approaches with low *P. jirovecii* replication rates, no animal model or any other method had been published that allowed for the isolation and propagation of *P. jirovecii* (reviewed in references 7 and 26). Using our model, both the cultivation and propagation of *P. jirovecii* are possible, which is a major improvement over earlier attempts (14–17).

In addition to the ability of this model to propagate the fungus, we have shown that *P. jirovecii* is able to pass through a multilayer cell culture and the basal filter membrane, which simulates tissue damage. The cytopathic effect was not expected but is not unusual for invasive pathogens, especially given that *P. jirovecii* can actively pass through pseudostratified epithelia. The filter membranes have a pore size of 4 μm and allow the basal medium to pass through, as long as the cell culture differentiation is not complete. Once a pseudostratified epithelium has formed, the membrane pores are blocked and the membrane is sealed by the cells. Although *P. jirovecii* trophozoites, which have an average diameter of 2 to 5 μm, may pass through the membrane, they must actively cross the epithelium before reaching the membrane and entering the basal medium. These findings are supported by occasional reports of serious and invasive *P. jirovecii* mycosis in immunocompromised and AIDS patients.

A possible source of bias in our study is that we did not count the total number of *P. jirovecii* particles in the basal cell culture medium using cytocentrifugation. The reason for this was that the majority of the cell culture fluid was used for DNA extraction for the subsequent qPCR. Because the only accepted method for quantifying *P. jirovecii* is qPCR, we would have preferred to demonstrate that *P. jirovecii* particles were also countable in the cytocentrifugation pellet. However, in the future, it will be possible to compare results from qPCR and immunofluorescence staining of cytocentrifugation preparations to correlate the two methods.

An additional possible criticism could be based on the finding of Botterel and colleagues that large numbers of genome equivalents were detected using qPCR (19). It is crucial to keep in mind that those high copy numbers were mitochondrial genome copies and did not correspond to nuclear genome copies or fungal particle numbers. As shown by our comparison of the Botterel qPCR (19) with a commercial PCR that targets a multicopy gene in the nuclear genome, the effective number of fungal particles clearly reached a maximum level at approximately 10^8 particles per ml. Thus, we can claim not only that the novel system described herein is able to support productive culturing of *P. jirovecii* but also that we can estimate the median number of mitochondria per target copy of *P. jirovecii* MSG, which is 6.09×10^4 *mt*geq/MSG *tc*eq. This value is within the range of other eukaryotic organisms. As early as 1974, it was shown that murine cells contain $1{,}100 \pm 250$ *mt*geq per cell, whereas human HeLa cells contain >8,000 *mt*geq per cell (27). The cell with the highest *mt*geq value appears to be the human oocyte, with a minimum of 10^5 *mt*geq per cell (28). Considering that different forms of the fungus are present in clinical material and in cell cultures, the finding of extremely large *mt*geq values in cell cultures is not unusual and supports the assumption that a productive culture was successful. Moreover, it must be considered that all previous attempts to quantify *P. jirovecii* using qPCR were performed using clinical materials, mainly BALF. Although this is a valid technique, the detection of *P. jirovecii* from BALF is not a fully quantitative method; positivity for *P. jirovecii* strongly depends on the locus from which the samples were taken, and it cannot be assumed that the entire tissue and lung lumen contain a homogeneous distribution of fungal particles. Unfortunately, this issue was not discussed in the earlier report by Botterel and colleagues, and the authors did not present the range of copy numbers, making it difficult to compare our copy number to the literature (19).

In summary, the CuFi-8 cell culture system and the EpiAirway cell culture system will allow further research on this highly pathogenic fungus and will facilitate the search for specific antifungal drugs to overcome the problem of emerging resistance.

Figure Legend Continued

multicopy gene, it can be assumed that the number of MSG copies remains constant within a single trial; thus, the results indicate the productive culturing of *P. jirovecii* in CuFi-8 cells. (C) In addition to CuFi-8 cells, a commercially available pseudostratified cell line, EpiAirway, was used in a pilot experiment (MatTek Corp., Ashland, MA, USA). Two independent experiments are shown. In one case, *P. jirovecii* was not detected in the cell-bound fraction due to technical difficulties. Due to budget limitations, no further experiments using EpiAirway cells were performed in this study.

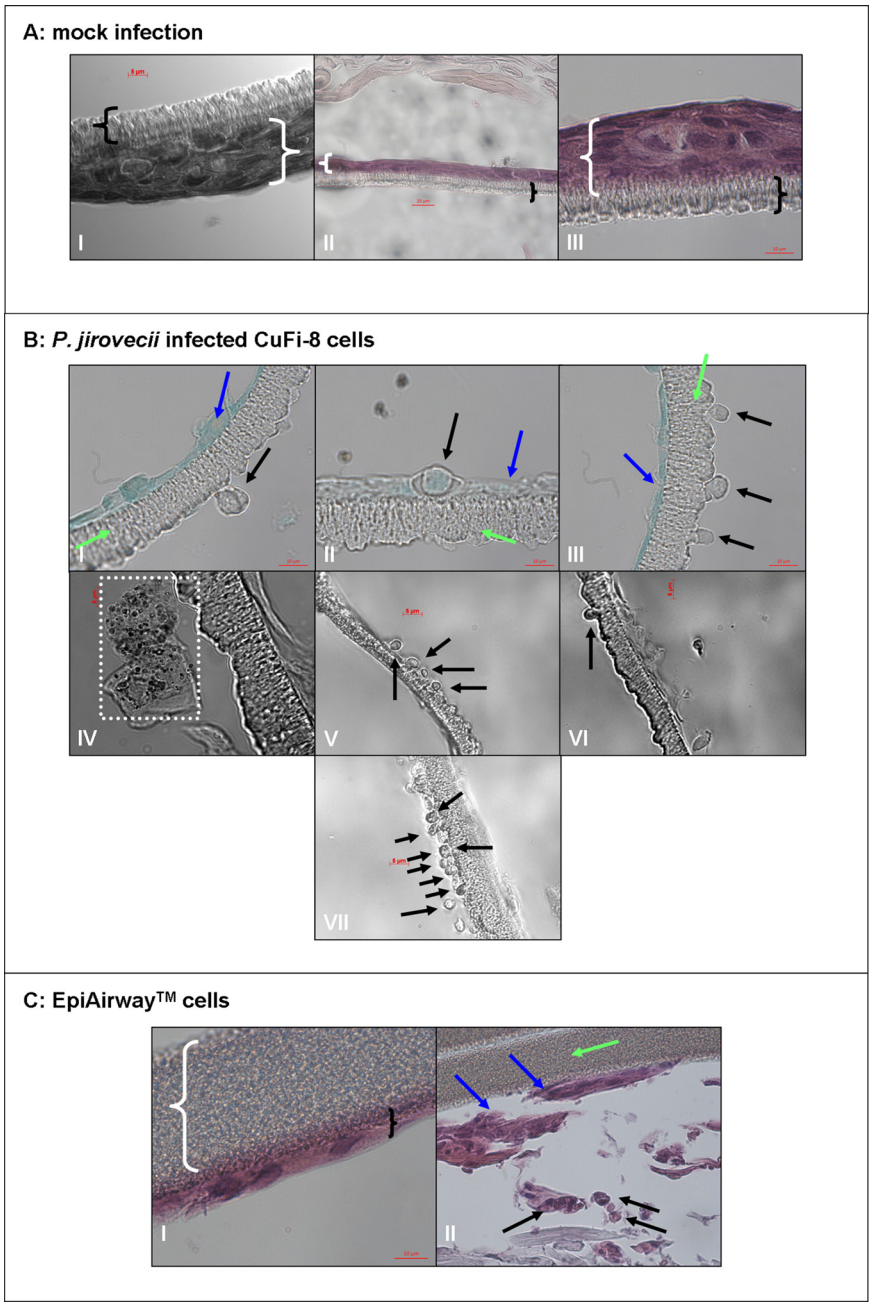

FIG 4 Bright-field and fluorescence microscopy were performed with a Zeiss Axioplan I microscope. Gray scale images were obtained with a Zeiss AxioCam Cm1, and color images were obtained with a Zeiss AxioCam Cc5 (Zeiss, Germany). The cells were mock inoculated with PBS or inoculated with *P. jirovecii*-positive BALF. Millicell inserts (Merck Millipore, Darmstadt, Germany) were deposited into 24-well cell culture plates and removed 5 days after inoculation. The membranes were cut out using a tapered scalpel, formalin fixed and paraffin embedded (FFPE), and sliced into thin, 3-μm sections before being stained with hematoxylin-and-eosin (HE) or Grocott silver stain. (A) Mock-infected cells with classical HE staining. Gray scale and color images of CuFi-8 cells that were differentiated for 3 weeks in air-liquid interface cultures are shown. Mock-infected and *P. jirovecii*-negative BALF-inoculated cells are shown, indicating that there was no cytopathic effect. The membrane is intact, and the tissue consists of multiple layers topped by a small ciliary border. (B) Grocott staining revealed typical *P. jirovecii* particles from CuFi-8 cells inoculated with *P. jirovecii*-positive BALF. Three independent trials are shown in color, and four additional pictures are shown in gray scale. It is noteworthy that an accumulation of *P. jirovecii* particles was observed in one case, as indicated by the box outlined by a white dotted line. (C) HE staining of the negative-control and *P. jirovecii*-infected EpiAirway cells. The *P. jirovecii* particles produced a serious cytopathic effect, as indicated by the arrows. (A) Negative controls. (I) Mock infection of CuFi-8 cells with PBS (HE staining; bar, 5 μm). (II) Infection of CuFi-8 cells with *P. jirovecii*-negative BALF (HE staining; bar, 20 μm). (III) Infection of CuFi-8 cells with *P. jirovecii*-negative BALF (HE staining; bar, 10 μm). Collagen-coated membrane (black bracket) and cell layers (white bracket) are indicated. (B) Infection of CuFi-8 cells with *P. jirovecii*-positive BALF (Grocott staining). Bars, 10 μm (I to III) and 5 μm (IV to VII). (C) Infection of EpiAirway cells. (I) Infection of EpiAirway cells with *P. jirovecii*-negative BALF (HE staining; bar, 10 μm). (II) Infection of EpiAirway cells with *P. jirovecii*-negative BALF (HE staining). Cell layers (blue arrows), collagen-coated membrane (green arrows), and *P. jirovecii* (black arrows) are indicated by different colored arrows. A cluster of *P. jirovecii* particles is indicated by the box outlined by a white dotted line.

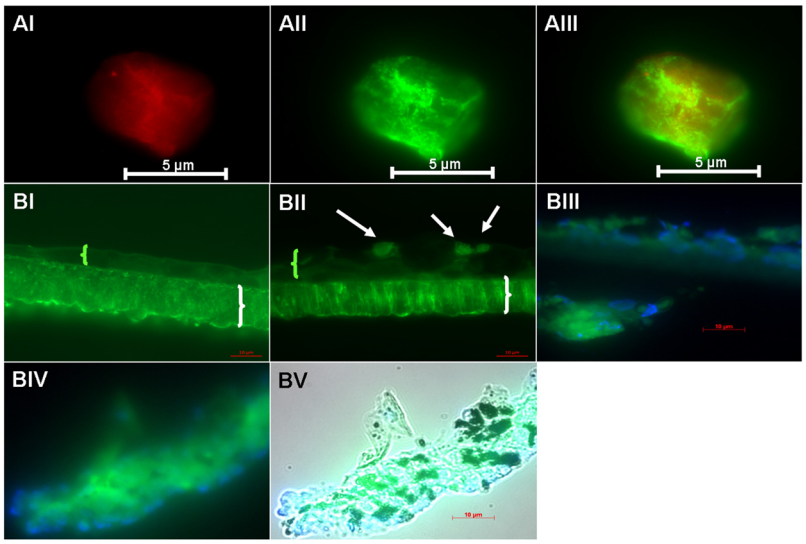

FIG 5 (A) A cytospin preparation from the basal cell culture medium that tested positive for *P. jirovecii* via qPCR. Staining was performed with the Merifluor Pneumocystis assay (Meridian Bioscience Inc., Cincinnati, OH, USA), which is specific for *P. jirovecii*. (AI to AIII) Representative staining results from one experiment at 1,000-fold magnification showing the red counterstaining (AI), shows the *P. jirovecii*-specific green fluorescence (AII), and merged images AI and AII (AIII). The stained form of *P. jirovecii* is most likely a late trophozoite or an early, thin-walled cyst. (B) Negative control (cells that were mock infected with *P. jirovecii*-negative BALF) and *P. jirovecii*-infected CuFi-8 cells stained with mouse monoclonal anti-*P. jirovecii* clone 3F6 (Dako, Hamburg, Germany). In the infected-cell cultures, clear green areas are visible, indicating the presence of *P. jirovecii*. Two additional independent experiments are shown as overlays of 4′,6′-diamidino-2-phenylindole (DAPI) staining and bright-field images. Neither the mouse monoclonal anti-*P. jirovecii* clone 3F6 nor the Merifluor assay was able to differentiate between the trophic and cystic forms of the pathogen. Unfortunately, collagen-coated membranes display autofluorescence, a phenomenon frequently observed in pathology stainings. (A) Cytospin photographs. (B) Infection of CuFi-8 cells (immunofluorescence [IF] staining with monoclonal mouse anti-*P. jirovecii* clone 3F6; bars, 10 μm). (BI) Infection of CuFi-8 cells with *P. jirovecii*-negative BALF (BII to BV). Infection of CuFi-8 cells with *P. jirovecii*-positive BALF costaining with DAPI (III) or costaining with DAPI (IV) and overlay with bright-field image (V). Collagen-coated membrane (white brackets), cell layers (green bracket), and *P. jirovecii* particles (white arrows) are indicated.

MATERIALS AND METHODS

Culturing of CuFi-8 cells. CuFi-8 cells were cultured and differentiated by the methods of Huang and colleagues (20). The detailed protocol was part of a material transfer agreement with the University of Iowa and is available upon request. Briefly, CuFi-8 cells were grown in cell culture flasks (75 cm^2; Corning, Munich, Germany) previously coated with human placental collagen (Sigma, Munich, Germany) using the BEBM (bronchial epithelial cell basal medium)/BEGM (bronchial epithelial cell growth medium) BulletKit medium (Lonza, Cologne, Germany). Cells were harvested and transferred to Millicell membranes (pore size, 4 μm; area, 0.6 cm^2; Merck Millipore, Darmstadt, Germany) coated with human placental collagen (Sigma). The medium was replaced with Ham's F-12 medium (Lonza), which was repeated daily until the cell layers had formed a mucous surface that hindered the passive diffusion of the basal medium through the membrane and cell layers. Subsequently, the transepithelial electric resistance (TEER) was measured, and the cell layer was inoculated with BALF positive for *P. jirovecii* at 1,000 Ω cm^2. A schematic of the protocol for the culture, inoculation, and passaging of CuFi-8 cells and *P. jirovecii* is shown in Fig. 2. Specifically, Fig. 2A shows how the polarized CuFi-8 cells were produced, and Fig. 2B indicates how the polarized cells were infected with 100 μl of *P. jirovecii*-positive BALF. The BALF was carefully removed from the cell surface after an overnight incubation. Figure 2C shows how *P. jirovecii* was transferred from a positive cell culture to a negative cell culture (passaging). Cells were harvested by scraping some cells from the top of an infected-cell membrane/polarized-cell layer with a pipette tip and resuspending them in 150 μl of Ham's F-12 medium. Then, 100 μl of this mixture was transferred to a new CuFi-8 membrane. Cells were cultured for 5 days postinfection before harvesting.

Commercially available differentiated airway epithelia. In addition to CuFi-8 cells, which are not commercially available, a pilot experiment was performed with commercially available EpiAirway cells (MatTek Corp., Ashland, MA, USA; http://www.mattek.com/EpiAirway/data-sheet). These cells were cultured as described by the manufacturer. The general procedure resembles the handling of CuFi-8 cells (Fig. 2), except that the cells can be purchased at the pseudostratified stage. EpiAirway cells were used for two independent trials of infection with BALF determined to be positive for *P. jirovecii* using quantitative PCR (qPCR) (Fig. 3C) and histochemistry (Fig. 4C).

***P. jirovecii*-specific qPCR.** Quantitative PCR was performed to detect *P. jirovecii* as previously described by Botterel and coworkers (19) using the Roche LightCycler (LC) 2.0 platform. This qPCR amplifies the mitochondrial ribosomal large subunit (mtLSU) gene. For quantification purposes, an in-house standard plasmid was cloned. Then, qPCR was performed with samples from the first German round robin trial for external quality control of diagnostic laboratories (organized by INSTAND e.V., Düsseldorf, Germany). The PCR product was cloned into the pCR2.1 TOPO TA vector (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Plasmid DNA was purified, confirmed by sequencing, and diluted to form a standard that was subsequently used for quantification. Randomly selected positive PCR products were repeatedly sequenced for quality control purposes.

To confirm the accuracy of this qPCR for quantifying *P. jirovecii*, we amplified a second genomic (nuclear) region using the LightMix kit *P. jirovecii* (TIB MOLBIOL, Berlin, Germany), according to the manufacturer's protocol, and the LightCycler Cobas 480 system (Roche, Mannheim, Germany). qPCR was performed with the LightCycler FastStart DNA Master HybProbe assay (Roche) according to the protocol of TIB MOLBIOL.

Formalin fixation and paraffin embedding of CuFi-8 cells. CuFi-8 cells were harvested at least 5 days postinfection. The membrane was cut from the plastic vessel using a sterile scalpel and was inserted into an embedding cassette. The cassette was transferred to a 4% formalin solution and then transferred twice to 75% ethanol for 45 s each time, twice to 96% ethanol for 45 s each time, three times to 100% ethanol for 1 min each

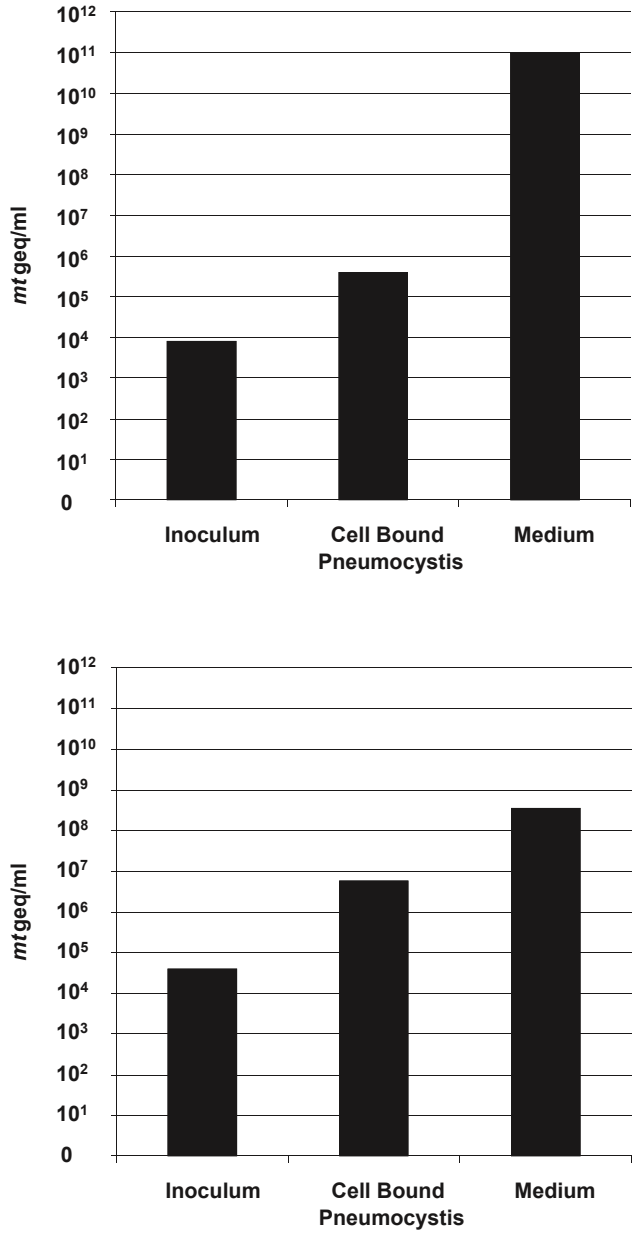

FIG 6 Two independent trials of *P. jirovecii* propagation. Cells containing *P. jirovecii* were carefully scraped from a membrane that had been incubated with *P. jirovecii*-positive BALF for 5 days using an inoculation loop and resuspended in 150 μl of PBS. Fresh CuFi-8 cells were inoculated with 100 μl of this suspension. After 24 h, the inoculum was removed, and the CuFi-8 cells were incubated for 5 days before harvesting. The inoculum, the cell-associated, membrane-bound *P. jirovecii*, and the amount of *P. jirovecii* found in the basal cell culture medium are shown. All the quantifications were performed by measuring mitochondrial genome equivalents per ml (*mt*geq/ml).

time, once to xylol for 1 min, once to xylol for 45 s, and finally four times to liquid paraffin for 40 s each time, before being embedded in paraffin.

Immunofluorescence staining of *P. jirovecii*. Immunofluorescence staining was performed as previously described. The cell culture medium from the Merifluor Pneumocystis assay (Meridian Bioscience Inc., Cincinnati, OH, USA) was used (21), and the mouse monoclonal anti-*P. jirovecii* antibody, clone 3F6 (Dako, Hamburg, Germany) was used for formalin fixation and paraffin embedding (FFPE) materials, as previously described (29).

Grocott silver staining. Silver staining for *Pneumocystis* was performed as previously described by Pintozzi and according to established standard procedures (30). Briefly, 3-μm FFPE sections were incubated in xylol, followed by a standard decreasing ethanol series before being stained with 5% chromic acid for 1 h at room temperature. The slides were washed with distilled water and incubated in 1% sodium thiosulfate solution for 60 s before being washed with distilled water. The slides were stained with methenamine silver nitrate solution for 30 to 60 s at 60°C until they appeared yellow to brown. The slides were washed with distilled water and stained with 0.1% gold chloride for several seconds before being washed again with distilled water. The slides were incubated in 1% sodium thiosulfate solution for 60 s, washed with distilled water, visualized with light green stain (approximately 30 s), subjected to an increasing ethanol series, and mounted with a coverslip. This procedure stains fungi nonspecifically and thus is an important stain for *P. jirovecii*, as shown in Fig. 4.

BALF samples from human patients. Clinical samples were collected for routine diagnostic procedures and archived. Double pseudonymized samples archived before September 2012 were permitted to be used for retrospective epidemiological analysis according to a vote from the Ethical Committee of the Private University of Witten/Herdecke (vote 73/2012) without the requirement of informed written consent.

ACKNOWLEDGMENTS

This work was supported by grants from the Else Kröner-Fresenius Stiftung, Germany (project 2013_A100), a grant from the Beatrix-Lichtken Stiftung (Cologne, Germany), and an internal grant from the Private University of Witten/Herdecke, Germany (project 11630).

We are grateful to John Engelhardt, Aloysius Klingelhutz, and Philip Karp from the University of Iowa Carver College of Medicine, Department of Anatomy and Cell Biology, for providing CuFi-8 cells and detailed cell culturing protocols. We also thank our clinical colleagues Wolfram Windisch, Christian Karagiannidis, and Alexander Prickartz for providing bronchoalveolar lavage fluid samples. Finally, we thank our colleagues Petra Rengel and Anne Schlimbach for technical assistance with the FFPE of CuFi-8 cells. The manuscript was edited for English language and grammar by American Journal Experts.

V.S. and O.S. designed the study, performed fluorescence and brightfield microscopy, and wrote the manuscript. M.B. analyzed the microscopy data and identified BALF samples that were positive for *P. jirovecii*. S.M., S.K., M.P., J.L., and R.L.T. performed experiments.

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