Diploma Thesis

Pneumocystis jirovecii fungal load in the respiratory tract and comparison with serum beta-D-glucan level in immunocompromised patients

submitted by

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Pasching, 09.09.2021

I declare that I have written this diploma thesis independently, that I have not used other than the sources/ resources cited, and that I have explicitly marked all material, which has been quoted either literally or by content from the sources used.

Pasching, am 09.09.2021 Sarah Niessner eh

Aknowledgements:

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Abstract:

Background: The diagnosis of *Pneumocystis jirovecii* pneumonia (PJP) has relied on microscopy with immunofluorescence. Nowadays, alternative diagnostic tests including detection and quantitation of *P. jirovecii* DNA based on real-time quantitative PCR and determination of serum beta-D-glucan (BDG) are increasingly used.

Objectives: The aim of this pilot study was to evaluate the analytic performance of a new assay for detection and quantitation of *P. jirovecii* DNA. Furthermore, the fungal load of immunocompromised patients with suspected PJP was determined with two different methods and results were compared.

Materials and Methods: Detection and quantitation of *P. jirovecii* DNA was done with the RIDA®GENE Pneumocystis jirovecii kit. DNA was extracted on the automated EMAG® nucleic acid extraction platform followed by amplification and detection on the LightCycler® 480 II instrument. For BDG testing, the Fungitell® Assay was run using a modified protocol employing the BCS XP System. The accuracy of the RIDA®GENE Pneumocystis jirovecii kit was determined utilizing an international reference panel. Furthermore, 8 anonymized left-over specimens that had been obtained from female and male patients with immunosuppression and suspected pneumocystis pneumonia were studied and results compared.

Results: When the accuracy of the RIDA®GENE Pneumocystis jirovecii kit was evaluated, all vials containing (any dilution of) *P. jirovecii* were correctly identifed as positive and the vial without *P. jirovecii* was correctly identified as negative. When the clinical performance was evaluated, all samples tested positive with the molecular test system, while BDG testing resulted in 4 positives only.

Conclusion: Detection and quantitation of *P. jirovecii* DNA based on real-time quantitative PCR appears to be the superior method when compared to serum BDG determination. Besides increased sensitivity, it is easier to handle and shows faster time-to-result allowing an earlier start of antifungal therapy. To underline these findings, further testing with a larger sample size is recommended.

Kurzfassung:

Hintergrund: Die *Pneumocytis jirovecii* Pneumonie (PJP) wird üblicherweise mittels Mikroskopie kombiniert mit Immunfluoreszenz diagnostiziert. Heutzutage nehmen diagnostische Verfahren, die auf der quantitativen Real-Time PCR bzw. auf dem Nachweis von Beta-D-Glucan (BDG) im Serum beruhen, eine immer wichtigere Rolle in der Diagnostik der PJP ein.

Ziele: In dieser Pilotstudie wurde die analytische Leistung eines neuen Tests zum quantitativen Nachweis von *Pneumocysis* (*P.*) *jirovecii* DNS beurteilt. Darüber hinaus wurde die Pilzbelastung mit Candida bei immunsupprimierten PatientInnen mit zwei unterschiedlichen Methoden ermittelt und die Ergebnisse verglichen.

Materialien und Methoden: Für die Quantifizierung der *P. jirovecii* DNS mittels RIDA®GENE Pneumocystis jirovecii Test wurde auf der EMAG® Plattform die DNS extrahiert und am LightCycler® 480 II nachfolgend amplifiziert und quantifiziert. Die analytische Leistung des RIDA®GENE Pneumocystis jirovecii Tests wurde mit einem internationalen Ringversuchspanel beurteilt. Die Beta-D-Glucan (BDG) Testungen wurden mit dem CE gekennzeichneten Fungitell® Assay auf dem BCS XP System durchgeführt, wobei ein eigens dafür modifiziertes Programm verwendet wurde. Die klinische Leistung beider Testsysteme wurde mit 8 anonymisierten klinischen Restproben untersucht.

Ergebnisse: Die Ergebnisse des Ringversuchs zeigten, dass alle Proben, welche *P. jirovecii* in verschiedenen Verdünnungen enthielten, mit dem molekularen Testsystem korrekt als positiv und die negativen Proben korrekt als negativ erkannt wurden. Bei den klinischen Proben wurden mit der PCR-basierten Diagnostik 8 positive Proben gefunden, während mit dem Beta-D-Glucan Test nur 4 Proben positiv waren.

Fazit: Der RIDA®GENE Pneumocystis jirovecii Test weist im Vergleich mit dem Fungitell® Test eine höhere Sensitivität auf. Ein weiterer Vorteil des RIDA®GENE Pneumocystis jirovecii Tests ist die einfachere Handhabung, die einen schnelleren Nachweis und daher eine schnellere

Antimykotikatherapie ermöglicht. Die Ergebnisse dieser Studie müssen jedoch mit einer größeren Probenanzahl bestätigt werden.

1. Introduction

1.1 History and etiology

Pneumocystis was first described in its cyst form in 1909 by Chagas and a year later in 1910 by Carinii after whom it was also initially named. The organism was first thought to be a protozoa and in 1952 primarily associated with human disease causing "plasma cell interstitial pneumonitis" in malnourished children and neonates [1].

As the number of patients with PJP increased substantially in patients receiving corticosteroids and chemotherapeutic drugs, the Centers for Disease Control (CDC) started conducting studies in the 1970s. However *P.carinii* first gained global recognition in the 1980s after being recognized as the first AIDS-defining illness causing over one-fourth of community-acquired pneumonias in HIV-infected persons and over 200.000 cases of PJP since 1979. Furthermore, it was discovered that the species was host-specific, and the human pathogen was renamed *P. jirovecii* after the Czech parasitologist who made the discovery [2]. *P. carinii* is nowadays known as a fungus found in rodents.

P. jirovecii was later also reclassified to the taxonomic kingdom of fungi based on conserved mRNA sequences. In humans as well as animals the organism has been found in three forms: trophozoite, cyst, and sporozoite. The trophozoite is smaller than the cyst with only 2-5 μm in diameter and has a round or sickle shape. The cyst is up to 6 μm in diameter with a three-layered cell wall and its cytoplasm contains up to eight of the oval sporozoites. In the alveolus, Pneumocystis is found covered with surface glycoproteins which are produced by both the host and the organism itself. The organism has the ability to adapt to the host by sharing its antigenic epitopes in addition to its own.

The cell wall of *P. jirovecii* contains only cholesterol and no ergosterol and does not synthesize sterols de novo which explains the lack of susceptibility to

the azole and polyene anti-fungal antibiotics. Its surface is rich of the carbohydrates glucose, mannose, and β-1,3-glucan which are believed to be part of the attachment-process to epithelial or surfactant layers in the host. Furthermore, they play a role in diagnostics as a diagnostic target.

It is not possible to cultivate human-derived organisms *in vitro* as they grow inconsistently; as a result, the full life cycle of *P. spp.* is not yet completely understood [3,4]. What is known about the life cycle has mostly been observed in the microscope as well as extrapolation from the life cycle of other ascomycetous fungi. As shown in Fig. 1 the proposed life cycle consists of sexual an asexual reproduction and the above mentioned three forms: trophozoite, cyst, and sporozoite. The sporozoites are released from the ruptured mature cyst.

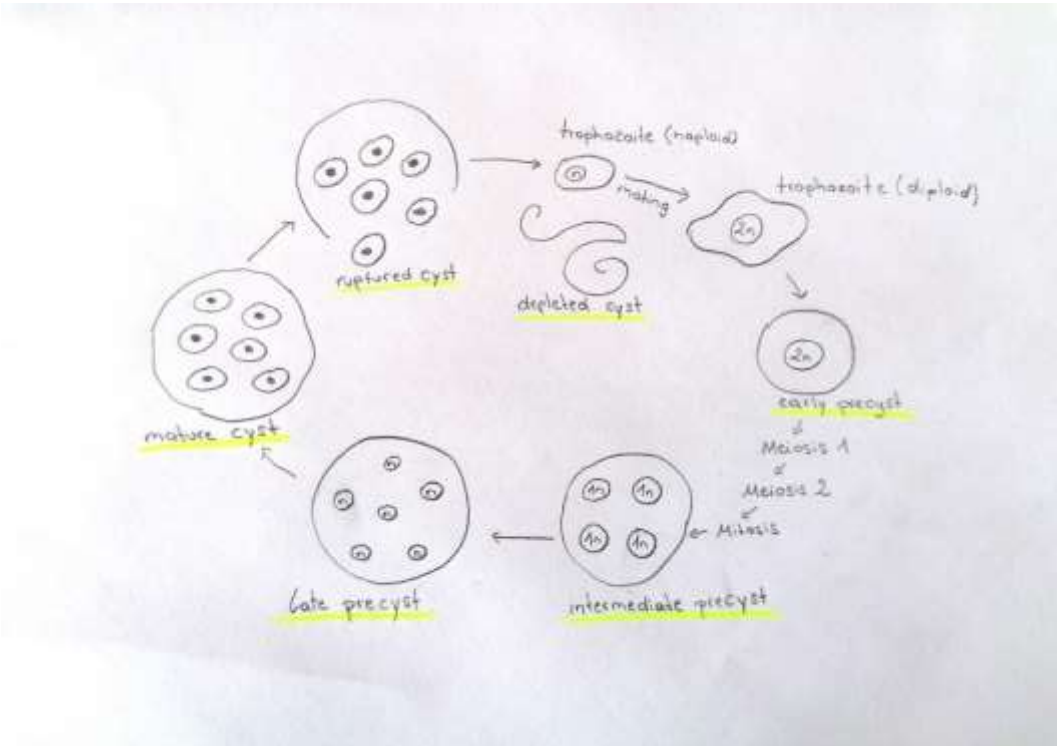

Fig. 1. Proposed life cycle of *P. jirovecii*. (image source: Sarah Niessner)

1.2 Epidemiology and risk factors

Studies of Pneumocystis in humans as well as in animal models suggest that the disease could arise de novo via airborne transmission or from reactivation of untreated infection [5-8]. Moreover, some studies even suggest that *P. jirovecii* may exist in the environment even though no definitive environmental reservoir has yet been identified for human disease [9].

When looking at interhuman transmission in the hospital environment, studies have shown an incubation period of up to 150 days. Studies conducted before the broad implementation of prophylaxis suggest that the risk of developing PJP among immunocompromised patients due to solid organ transplantation is 5%-15% and believed to be similar in patients receiving equivalent immunosuppressive therapy. Nowadays in patients receiving prophylaxis PJP should not occur [10,11].

Serological studies have shown that exposure to *P.jirovecii* occurs commonly in childhood but the immunocompetent children show only very mild to no symptoms. Symptomatic disease in children as well as adults is limited to individuals who are immunocompromised [12].

The patients at highest risk of PJP are those being treated for acute lymphoblastic leukemia (ALL) (up to 16%) [13] and for allogeneic haemopoietic stem cell transplant (HSCT) (up to 15%) [14]. Patients receiving high-dose corticosteroid treatment daily over a course of 4 weeks and more are also at a high risk for PJP regardless of the underlying type or stage of malignancy [15]. Receiving chemotherapy or monoclonal antibodies also puts patients at high risk as well as diseases causing prolonged lymphopenia [16,17].

In general risk factors include:

1) Immunosuppressive therapies
    a. Corticosteroids
    b. Chemotherapy
    c. Purine analogs
    d. Antibody therapies
    e. Mycophenolate
    f. Calcineurin inhibitors
    g. Sirolimus
2) CMV disease
3) Allograft rejection
4) GVHD patients
5) Low CD4+ T-cell counts
6) Neutropenia

Whether a patient is at risk for PJP is influenced by multiple factors and the individual risk should be continually assessed throughout the course of treatment.

1.3 Clinical manifestations

The symptoms of patients with PJP can be quite variable but infection in immunosuppressed HIV-negative individuals is classically more acute and has a shorter duration of onset [18]. Furthermore, bronchoalveolar lavage fluid from immunosuppressed non-HIV-infected patients contains a lower count of organisms but a higher inflammatory score [19].

In general, the severity of the disease correlates to the length of hospitalization and the need for mechanical ventilation. Studies show that the

mortality rates observed in non-HIV-infected individuals (34–39%) is scientifically higher than in patients with HIV (6-7%) [18, 20-22].

The typical signs and symptoms of PJP are depicted in Table 1 and include most commonly fever combined with dyspnea and a nonproductive cough. In general, the more rapid the onset the more severe the resulting pneumonia and lung involvement with lower arterial-oxygen tension and more frequent respiratory failure. It is possible for corticosteroids, calcineurin inhibitors, and sirolimus to mask the symptoms of PJP [23,24].

Table 1
Incidence of PJP symptoms.

Symptoms of PJP	Incidence (%)
Abnormal chest radiography	92-96
Hypoxemia	78-91
Fever	81-87
Cough	71-81
Dyspnea	66-68
Abnormal lung auscultation	30-34
Chest pain	23-24

1.3 Diagnostics

1.3.1 Radiography

In general, there is no pathognomonic pattern for Pneumocystis and the radiographic image ultimately depends on the patient's duration of infection, state of immunosuppression and whether there are underlying and/or accompanying diseases. However as can be seen in table 1 92%-96% of patients show abnormal chest radiography. In early PJP the manifestation often shows fine, bilateral, perihilar, diffuse infiltrates that spread to the bases or apices as the disease progresses. Despite treatment in the early stages this pattern consolidates over 3-5 days [25].

Chest computed tomography (CT) often shows abnormalities as well and are part of the routine diagnostic process as a normal chest CT excludes PJP with a high probability. Notably normal radiographic findings do not exclude an abnormal CT. The findings most common for PJP are bilateral, ground-glass changes with apical predominance and peripheral sparing. In HIV patients, cystic changes are much more common than in immunocompromised HIV negative patients. Abnormal PET scans can be observed in the early stages of the disease [26,27,28].

1.3.2 Microbiological testing

Various staining methods are used to visualize and identify respiratory specimens of *P. jirovecii*. Toluidine blue and methenamine preparations only

stain the wall of the cyst and thereby not allow the detection of trophozoites (Fig. 2, Fig. 3). Therefore, Giemsa staining is the preferred preparation as it allows the detection of all life stages (Fig. 4). Every life stage of *P. jirovecii* has a specific direct and indirect immunofluorescent assay (DFA, IFA) depending on the antibody used. In general, the accuracy of the different staining methods is highly dependent on the experience of the person diagnosing the specimen, the sample processing, the reaction of the individual specimen to the staining method chosen and the quality of the specimen itself. Furthermore, the lower respiratory burden in non-HIV patients makes it more difficult to correctly diagnose them as well as the patients already on prophylaxis [29].

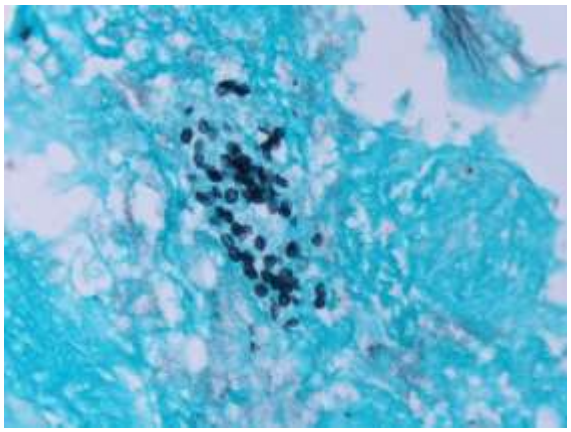

Fig. 2. Cysts of *P. jirovecii* in toluidine blue stain [30].

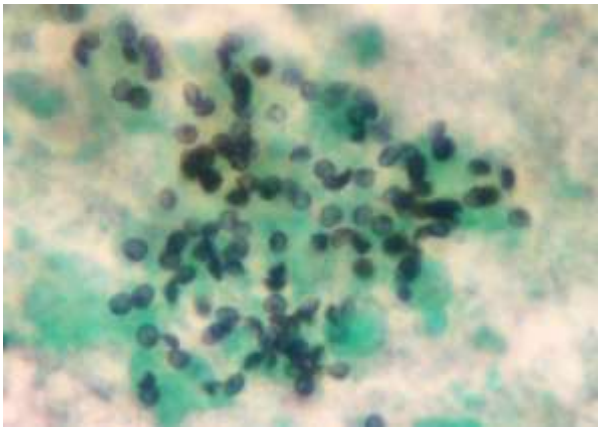

Fig. 3. Cysts of *P. jirovecii* in smear from BAL (Methenamine silver stain) [31].

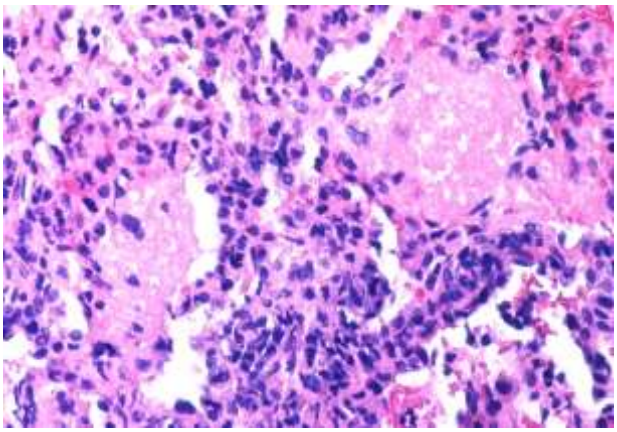

Fig. 4. Pneumocystis jirovecii infection, Giemsa staining [32].

Overall, as staining methods are rather prone to errors they have largely been replaced by highly sensitive molecular techniques, using semi- or fully quantitative polymerase chain reaction (PCR). They directly target the *P. jirovecii* specific genes and a meta-analysis has shown a pooled sensitivity of 99% and specificity of 92% in the non-HIV patient population. Quantitative PCR (qPCR), with defined upper- and lower-quantitation thresholds of P. *jirovecii* is nowadays the most often used and most accurate method [33, 34].

Furthermore, due to the MIQE guidelines for validation of qPCR assays, results are much more comparable and homogenous when using qPCR [35]. Nevertheless, PCR is limited as it is not possible to distinguish between asymptomatic colonization and infection.

BAL fluid is the preferred specimen for qPCR as it is the most studied with the most reliable specimen load and the one which can best be standardized. Moreover, the sensitivity of induced sputum is only 30%-55%, compared with 80%-95% with BAL. However, as BAL is semi-invasive and requires trained personnel, it is not always possible to carry out the procedure. Nevertheless, the viral load in non-HIV patients is commonly rather low, so the sputum often does not contain enough specimen fur diagnostic purposes, which makes BAL necessary to obtain meaningful results. If BAL is not possible or the use of the method is refused by the patients, upper respiratory specimens as induced sputum, oral washings, nasal swabs and nasopharyngeal aspirate are possible alternatives. However, as stated before, they have lower sensitivity [36].

Another contributing factor for diagnosing PJP is the beta-D-glucan (BDG) test. BDG is a major cell wall polysaccharide in fungi of medical importance including *P. jirovecii*. Its antigen can be found in the blood of patients suffering from PJP and detected by several tests developed for the purpose. However, the Fungitell® Assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA) is the only one FDA-approved (2004) and the one most frequently used in Europe

and America [37]. Serum beta-D-glucan assays have a high sensitivity (>90%) with lower specificity (<80%). The high negative predictive value makes it a good test for excluding the disease. However, immunocompromised patients tend to have inadequate antibody production, which may cause false negatives [37].

1.4.3 Laboratory evaluation

Patients with PJP have a PO2 < 60 mm Hg and a respiratory alkalosis. In addition, their serum lactic dehydrogenase (LDH) enzyme is elevated to over 300 IU/ml in most PJP patients. Their PAO2-PaO2 gradient rises, and studies show that gradients over 30 mm Hg at the start of the therapy are associated with a high mortality rate. However, if the treatment for *P. jirovecii* is successful, the arterial oxygenation gradient and LDH will return to normal [38]

1.5 Treatment

1.5.1 First line agent

Trimethoprim and sulfamethoxazole (TMP-SMX) is the first-line prophylactic agent for PJP with no other agent showing better results. TMP-SMX should be administered daily with adults/adolescents receiving 15-20 mg/kg/day of the TMP component given IV every 6-8h. In mild cases lower doses are sufficient and the dose should be adapted individually. TMP-SMX is also the recommended drug when treating PJP in children adapting both the TMP as well as the SMX component to the age and weight of the children. Complications of the drug include myelosuppression, gastrointestinal disturbance and drug interactions. Furthermore, renal impairment is a possible side effect, which is why the kidney function of patients receiving TMP-SMX

has to be monitored as well as the hydration status. Contraindications for TMP-SMX include planned methotrexate chemotherapy in adults as well as very limited kidney function.

1.5.2 Second-line agents

Pentamidine is the recommended second-line agent if TMP-SMX cannot be given. It is an isethionate is an aromatic diamidine derivative and the preferred mode of administration is aerosolization as this reduces the likelihood of side effects. Side effects include pancreatitis, hypoglycemia, hyperglycemia, bone marrow suppression, renal failure, and electrolyte disturbances. A relative contraindication is pancreas transplantation; however, necessary studies still need to be conducted to show the correlation [39].

Dapson is an alternative second-line agent; it is a synthetic sulfone that acts against *P. jirovecii* by inhibiting the synthesis of dihydrofolic acid. The drug should be administered orally with a bioavailability of 70-80%. Side effects include agranulocytosis, aplastic anemia, rash, nausea and sulfone syndrome (rash, fever, hepatitis, lymphadenopathy and methemoglobinemia). Contraindications are a G6PD deficiency or patients who have experienced severe side effects with TMP-SMX [40].

Atovaquone is usually a second-line agent used in mild cases of PJP. It is administered as an oral suspension and must be taken twice a day best accompanied by a fatty meal to increase bioavailability. Side effects include rash, nausea, diarrhea, elevated transaminases and headache. Up to date there are no contraindications, only indicators where it has to be used carefully as for example a combination with antiviral drugs can cause Steven-Johnson syndrome [41].

1.5.3 Adjunctive corticosteroid therapy

Adjunctive corticosteroid administration in PJP patients is recommended with every drug as it prevents the early decline in oxygenation, which can be seen after the initial administration of the PJP treatment. Studies show a reduced respiratory failure and a reduced mortality when corticosteroids are given in severe PJP cases. Furthermore, a shorter duration of mechanical ventilation as well as lower supplement oxygen has been documented. However, corticosteroids have not changed the rate for the need for intubation. In milder cases, there has not yet been a demonstrated benefit of corticosteroids [42].

1.6 Prophylaxis

Routine anti-Pneumocystis prophylaxis is generally recommended as soon as a patient is at risk for infection with PJP. For those with risk factors such as intensive immunosuppression, infection with CMV, higher dose corticosteroid therapy, prolonged neutropenia or flares of autoimmune disease prophylaxis should be given throughout the period of increased susceptibility. After solid organ transplantation prophylaxis should be given for 6-12 months.

TMP-SMX is also the medication of choice when it comes to PJP prophylaxis as studies show that all second-line agents have inferior results when comparing efficacy, side effects and cost. Furthermore, TMP-SMX also prevents other opportunistic pathogens as for example *Toxoplasma gondii*. If TMP-SMX is contradicted for any of the reasons mentioned above all the second-line agents mentioned can be used as prophylaxis as well and should be individually chosen.

The patient must be closely monitored throughout the prophylaxis as side effects of TMP-SMX may occur such as bone marrow suppression, rash up to

Stevens-Johnson syndrome, hepatitis and nephritis. If another second line agent is given their specific side effects must be monitored as well [43].

Patients with PJP must be separated in a hospital setting due to the risk of infection of other immunocompromised patients. Moreover, facemask filtering is recommended to prevent transmission among infected individuals. Nosocomial infections are believed to be both by environmental contamination and direct person-to-person [44].

2 Objectives

The diagnosis of PJP has relied on microscopy with immunofluorescence. Nowadays, alternative diagnostic tests including detection and quantitation of *P. jirovecii* DNA based on real-time quantitative PCR and determination of serum BDG are increasingly used.

The aim of this pilot study was to evaluate the analytic performance of a new assay for detection and quantitation of *P. jirovecii* DNA. Furthermore, the fungal load of immunocompromised patients with suspected PJP was determined with two different methods and results were compared.

3 Study Design

3.1 Methods

3.1.1 Quantitation of *P. jirovecii* DNA

Quantitation of *P. jirovecii* DNA was performed at the Molecular Diagnostics Laboratory, Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz.

Extraction of *P. jirovecii* DNA was performed on the EMAG® nucleic acid extraction platform (Fig. 5). Immediately after thawing, 500 µl of BAL fluid were pipetted into the extraction vessel of the NucliSENS® easyMAG® instrument. For extraction of nucleic acids, the specific B protocol was used. The elution volume was 50 µl.

Fig. 5. The EMAG® platform (bioMérieux, Marcy-l´Etoile, France) (image source: Sarah Niessner)

After nucleic acid extraction, a gene fragment specific for *P. jirovecii* (if present in the eluate) was amplified and detected using the RIDA®GENE Pneumocystis jirovecii (R-Biopharm AG, Darmstadt, Germany) kit. Amplification is performed using the real-time PCR technology employing hydrolysis probes for detection of amplification products. Probes are labeled at one end with a quencher and at the other end with a fluorescent reporter dye (fluorophore). In the presence of the target, the probes hybridize to the amplification products. During the extension step, the Taq-polymerase breaks the reporter-quencher proximity. The reporter emits a fluorescent signal, which is detected by the optical unit of a real-time PCR instrument. The fluorescence signal increases with the number of formed amplification products. With the standards, Standard A, Standard B and Standard C, included in the kit, it is possible to generate quantitative results. Additionally, the RIDA®GENE Pneumocystis jirovecii kit contains an Internal Control DNA (ICD) that detects PCR inhibition, monitors reagent integrity and confirms that nucleic acid extraction was sufficient [45]. Amplification and detection was performed on the Light Cycler® 480 II CE/IVD (Roche Diagnostics, Penzberg, Germany) instrument (Fig. 6).

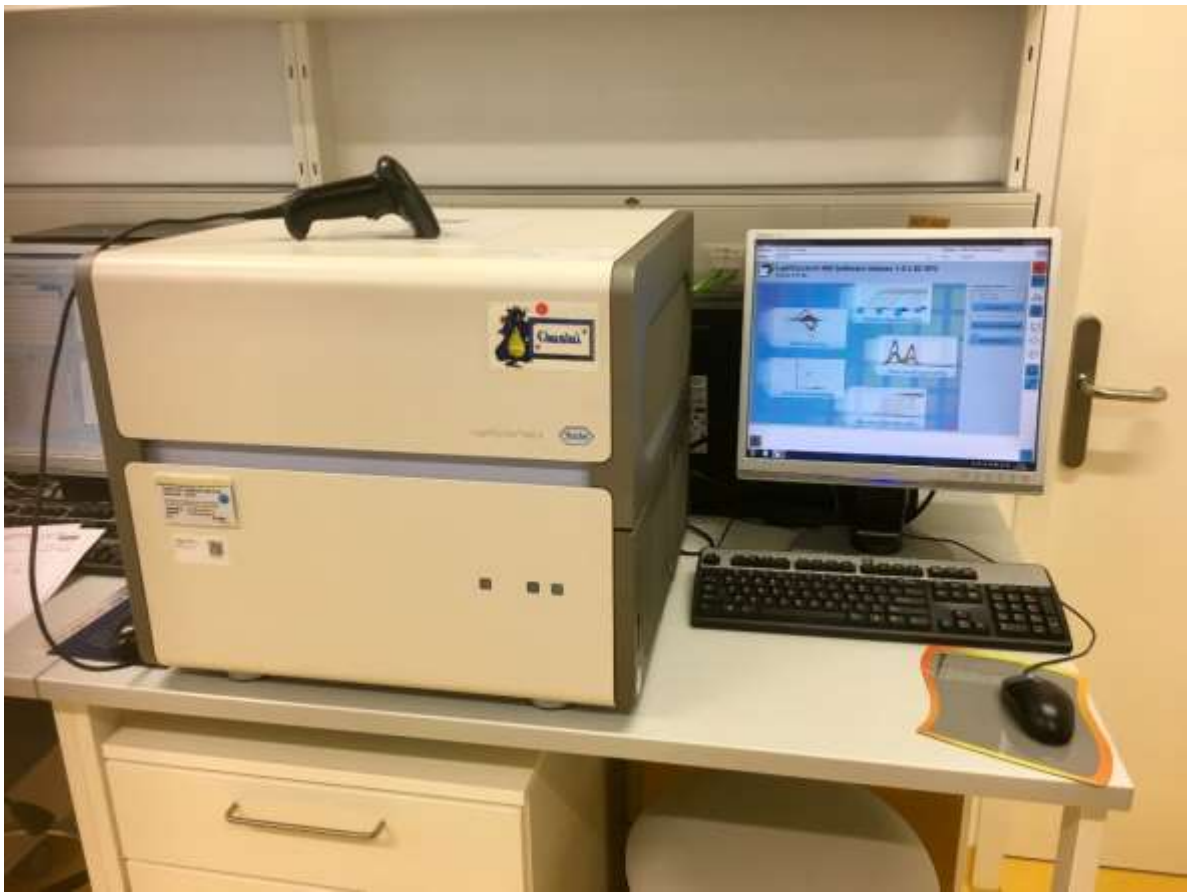

Fig. 6. The Light Cycler® 480 II CE/IVD instrument (Roche Diagnostics, Penzberg, Germany) (image source: Sarah Niessner).

3.1.2 Beta-D-glucan testing

Beta-D-glucan testing was performed at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz. Serum samples were tested with the CE marked Fungitell® Assay (Associates of Cape Cod, Inc., East Falmouth, MA, USA) using a modified protocol employing the BCS XP System (Siemens Healthcare Laboratory Diagnostics, Tarrytown, NY, USA) as published recently [46]. Figure 7 shows the platform used for beta-D-glucan testing.

Fig. 7. BCS XP coagulation analyzer (Siemens Healthcare Laboratory Diagnostics, Tarrytown, NY, USA) (image source: Sarah Niessner)

The Fungitell® Assay is a (1→3)-β-D-Glucan specific Limulus amebocyte lysate reagent containing a chromogenic peptide substrate. (1→3)-β-D-Glucan in the sample causes activation of serine proteases. An activated protease cleaves p-nitroaniline (pNA) from the peptide substrate and the free pNA is measured.

The Fungitell® assay for detecting BDG in serum samples was performed according to the manufacturer's instruction. 5µl of serum was pretreated with 20µl of an alkaline reagent (0.25 M potassium hydroxide and 1.2 M potassium chloride, mixed 1:1) for10 min at 37◦C in order to convert triple-helix glucan to single-helix glucan and inactivate serine proteases and serine–protease

inhibitors in the serum sample. After this step, 25µl of each of the five standards with corresponding concentrations (500, 250, 125, 62.5, and 31.25 pg/ml) and 25µl of the blank included in the assay were transferred to the microplate, followed by addition of 100µl Fungitell® reagent to each well. Then, the microplate was inserted into an incubating (37◦C) plate reader. Every serum sample was tested in duplicate. The assay was monitored at 405 nm kinetically for 40 min. According to the manufacturer, a BDG concentration of ≥80 pg/ml is considered to be positive, while a concentration of <60 pg/ml is considered to be negative [45, 47].

3.2 Analytical and clinical studies

The analytical performance of the RIDA®GENE Pneumocystis jirovecii was determined utilizing the Quality Control for Molecular Diagnostics (QCMD) 2018 Candida spp. EQA Programme. The panel consisted of 10 samples including 9 positive for *P. jirovecii* and one without *P. jirovecii*. The characteristics of the panel is shown in Table 2.

For the clinical study, 8 anonymized left-over specimens that had been obtained from female and male patients with immunosuppression treated at different departments of the University Hospital Graz and teaching hospitals of the Medical University of Graz were studied. BAL fluid specimens had been collected in 12-mL sterile screw cap tubes (GreinerBio-One) and transferred to the Molecular Diagnostics Laboratory for routine testing. The remaining sample materials were coded and studied anonymized. 1.5-mL aliquots containing BAL fluids were prepared in FALCON® tubes (Corning Science México S.A. de C.V., Tamaulipas, México) and frozen at −70 °C until further use. After thawing, samples were extracted and amplified according to the protocol as described above.

For the lab flow analysis, the turn-around time including hands-on time were estimated. Times required for the RIDA®GENE Pneumocystis jirovecii kit and the Fungitell® Assay were estimated and compared.

Table 2: Quality Control for Molecular Diagnostics (QCMD) 2018 Pneumocystis jirovecii pneumonia (PCP) DNA EQA Programme.

Sample no.	Sample code	Sample content	Matrix	Sample relationship *
1	PCPDNA 18S-01	*P. jirovecii*	Saline	D2
2	PCPDNA 18S-02	*P. jirovecii*	Saline	D1, DS2_2
3	PCPDNA 18S-03	*P. jirovecii*	Saline	DS2_1
4	PCPDNA 18S-04	*P. jirovecii*	Saline	DS1_1
5	PCPDNA 18S-05	*P. jirovecii*	Saline	D2
6	PCPDNA 18S-06	*P. jirovecii*	Saline	-
7	PCPDNA 18S-07	*P. jirovecii*	Saline	DS2_3
8	PCPDNA 18S-08	Negative	Saline	Negative
9	PCPDNA 18S-09	*P. jirovecii*	Saline	DS1_2
10	PCPDNA 18S-10	*P. jirovecii*	Saline	D1, DS2_2

*sample relationship indicates the relationships of the samples. The highest titer member of dilution DS1 is indicated by DS1<-1 and further members of the series as DS1_2, DS1_3, etc. in order of reducing titer. Additional dilutiion series are indicated by DS2 (DS2_1, DS2_2 etc.) DS3 (DS3_1, DS3_2 etc.). If one duplicate pair is present this is indicated by “D1”. Further duplicate pairs are indicated by “D2”and “D3”.

4. Results

4.1 Results of QCMD

When the analytical performance of the RIDA®GENE Pneumocystis jirovecii kit was evaluated using the QCMD panel, all samples containing (any dilution of) *P. jirovecii* were correctly recognized as positive and the vial without *P. jirovecii* was correctly identified as negative (Table 3).

Table 3: Results obtained from the Quality Control for Molecular Diagnostics (QCMD) 2018 Pneumocystis jirovecii pneumonia (PCP) DNA EQA Program.

QCMD18-No.	PCR [Ct value]	PCR [copies/ml]
1	27.85	437
2	27.24	766
3	25.96	2470
4	26.91	1030
5	28.13	339
6	27.49	401
7	28.37	271
8	0	0
9	29.24	122
10	27.18	803

4.2 Results of Beta-D-glucan testing and comparison to quantitation of *P. jirovecii* DNA

Eight anonymized left-over specimens that had been obtained from female and male patients with immunosuppression were tested on BDG and results were compared to those obtained with the RIDA®GENE Pneumocystis jirovecii kit. While all samples showed similar *P. jirovecii* concentrations when tested with the RIDA®GENE Pneumocystis jirovecii kit, the 4 samples giving positive results with BDG testing showed highly varying results (Table 4). Figures 8-10 show results of *P. jirovecii* DNA testing and comparison of *P. jirovecii* DNA results to those obtained with BDG testing.

Table 4: BDG testing and quantitation of *P. jirovecii* DNA using the RIDA®GENE Pneumocystis jirovecii kit.

Patient Nr.	BDG [pg/mL]	PCR [Cq]	PCR [copies/ml]
1	negative	26.58	1390
2	negative	28.92	164
3	861.41	25.02	5850
4	94.4	28.17	326
5	2569.46	22.22	75700
6	negative	28.36	275
7	negative	29.13	135
8	336.89	20.69	3090000

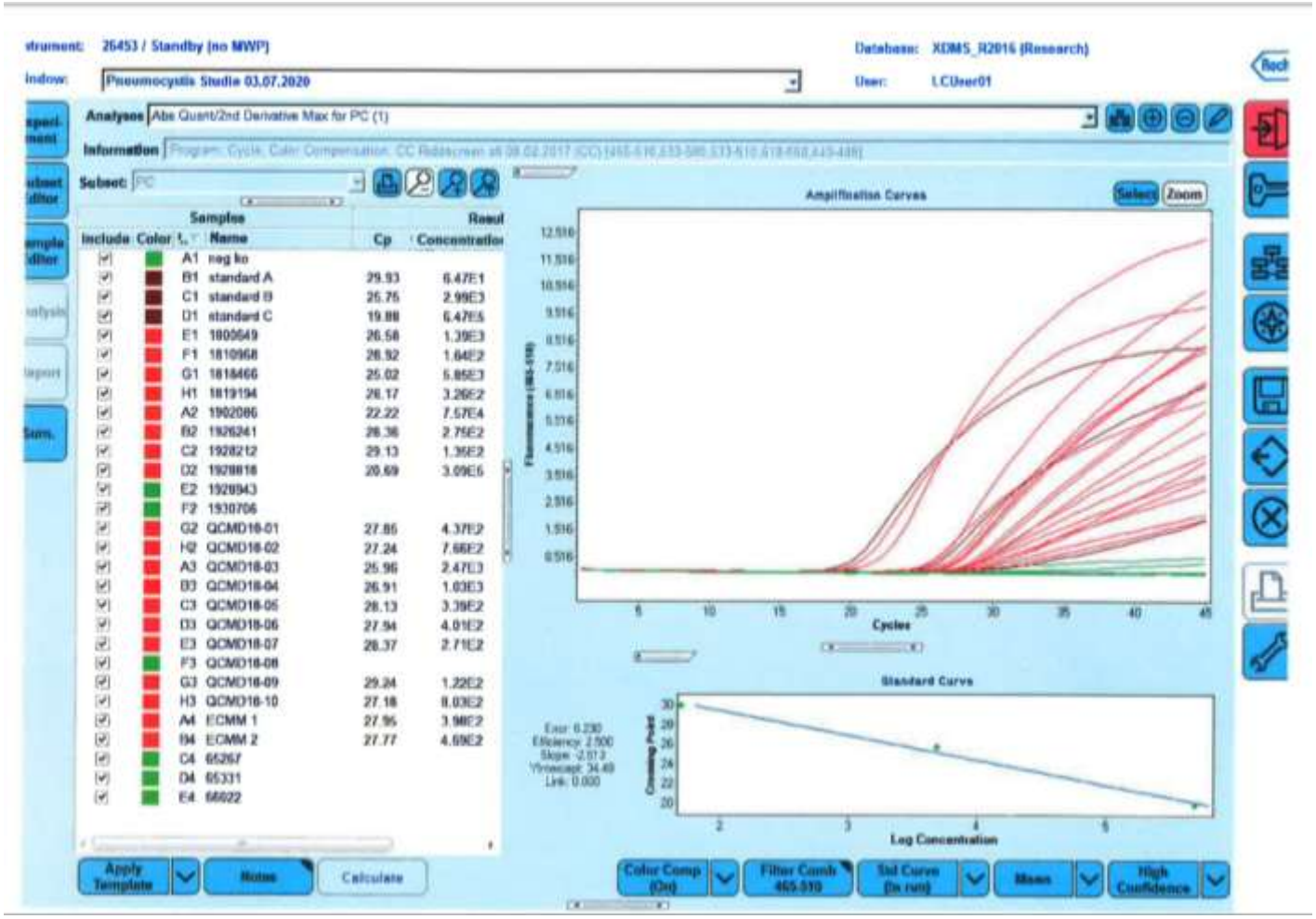

Fig. 8. Results for *P.jirovecii* DNA testing with the RIDA®GENE Pneumocystis jirovecii kit (image source: Sarah Niessner)

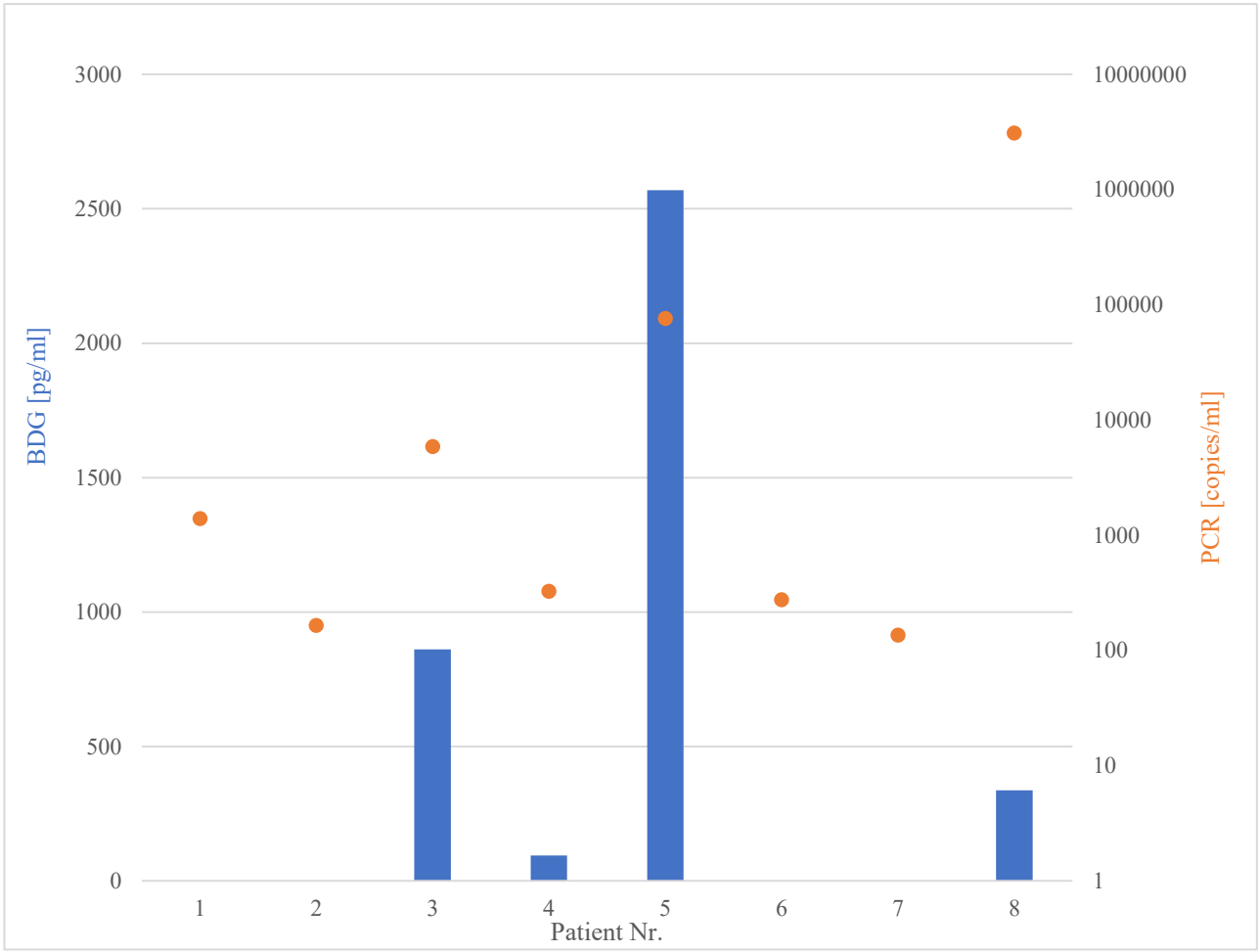

Fig. 9. Comparison of BDG and PCR test results

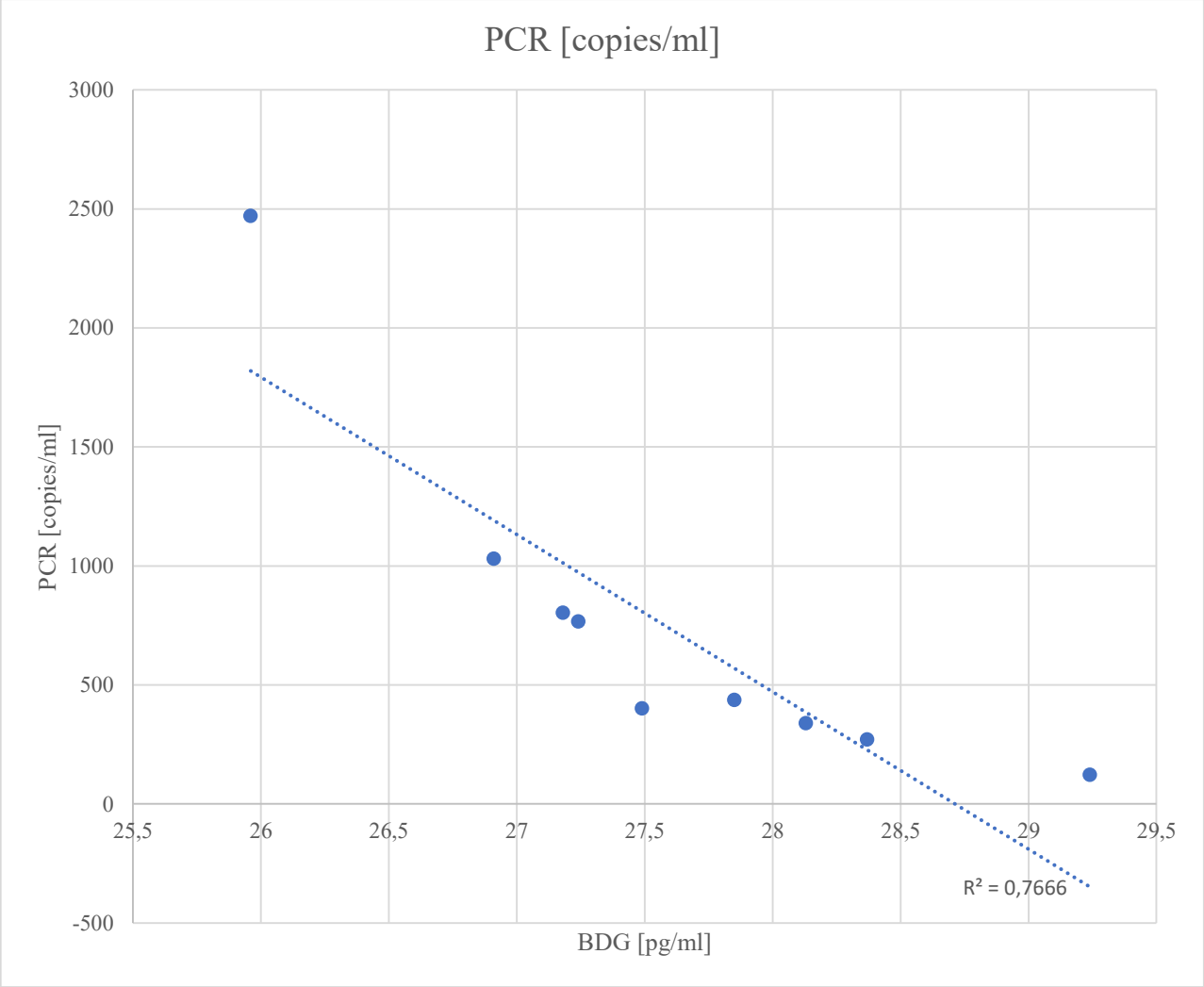

Fig. 10. Correlation between BDG and PCR test results

5. Discussion

Today, molecular assays are becoming more and more important for detection of *P. jirovecii* pneumonia. One of the advantages of molecular diagnostics is the short time-to-result. With the present gold standard, *P. jirovecii* culture, results are not available in less than 48 hours. A recent study has shown that early antifungal therapy can improve survival up to 80% [48]. Furthermore, molecular diagnostics helps to reduce cost, as unnecessary antifungal therapy is expensive.

The accuracy of the RIDA®GENE Pneumocystis jirovecii was determined utilizing the Quality Control for Molecular Diagnostics (QCMD) 2018 Candida spp. EQA Programme. The panel consisted of 10 vials including 9 positive for P. *jirovecii* and one without *P. jirovecii*. In this study, all vials containing (any dilution of) *P. jirovecii* were correctly recognized as positive including both of the samples designated as educational samples and thus harder to detect. The vial without *P. jirovecii* was correctly identified as negative Thus the RIDA®GENE Pneumocystis jirovecii was found to meet the standards of QCMD proficiency panel testing.

In the clinical study, BALs obtained from 8 patients with suspected pneumocystis pneumonia were included. With the molecular assay, all of the samples tested positive. In contrast, the BDG test revealed positive results in only 4 patients. When comparing quantitative results, values obtained by the molecular assay did not correlate very well with those obtained by the BDG test.

With the RIDA®GENE Pneumocystis jirovecii, results can be obtained within less than 3 hours, with the modified Fungitell® Assay, time-to-result was 45 min, including instrument setup [46]. While the RIDA®GENE Pneumocystis jirovecii allows single sample testing (however, might not be preferable due to economic reasons), this is not possible with the Fungitell

assay that is designed to test 21 samples in duplicate per plate. Introduction of better automated protocols (handling of reagents and samples in a closed system) would be preferable to minimize airborne contamination. Therefore, BDG testing is done using a modified protocol employing the BCS XP System at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz. However, this makes single sample testing impossible and increases the time-to-results [46].

The present study was limited by the low number of patients due to the criteria that only BAL fluids were used for detection of *P. jirovecii* DNA. In the real world, induced sputa as well as nasal swabs are commonly used. Moreover, the samples had been stored for up to a year, which may lead to false negative results. Furthermore, a very low pathogen load in the specimen (below the analytical sensitivity) can also lead to a negative result. An underquantitation of *P.jirovecii* DNA may also occur in presence of PCR inhibitors in the samples. Furthermore, a positive PCR result does not prove the presence of replicable pathogens.

In conclusion, the RIDA®GENE Pneumocystis jirovecii showed a high accuracy. It is able to detect *P.jirovecii* DNA reliably from BAL fluid. However, it must be taken into consideration that the presence of a very low DNA concentration may indicate colonization only but not necessarily mean pneumocystis jirovecii pneumonia. In comparison to culture, the major advantage of molecular detection and quantitation of *P.jirovecii* DNA is the faster time-to-result leading to faster anti-fungal treatment. In contrast, the Fungitell assay seems to have a reduced sensitivity, which is a significant limitation for *P.jirovecii* diagnostics.

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