THE MOLECULAR EPIDEMIOLOGY OF PNEUMOCYSTIS JIROVECII IN CAPE TOWN, SOUTH AFRICA"

Derrick Banda, Andrew C Whitelaw and Kim Hoek

ABSTRACT: Pneumocystis jirovecii is an opportunistic fungal pathogen that causes Pneumocystis pneumonia (PCP) in immunocompromized hosts. PCP is associated with substantial morbidity, and mortality rates range from 10% to 40%. The diagnosis of PCP relies on the microscopic detection of P. jirovecii in stained clinical samples. Polymerase chain reaction (PCR) may provide better sensitivity than microscopy; therefore, evaluation and implementation of PCR assays are required for the detection of Pneumocystis infection.

P. jirovecii is not cultivatable, therefore molecular tools are used for characterizing P. jirovecii genotypes; common targets are the dihydropteroate synthase (DHPS) and mitochondrial large subunit rRNA (mtLSU rRNA) genes. DHPS is a therapeutic target; mutations may be associated with co-trimoxazole prophylaxis and treatment failure. Polymorphisms in mtLSUrRNA have been used for phylogenetic studies.

Aims:

- to evaluate a real time PCR (rtPCR) assay for diagnosis of PCP by comparing the performance to immunofluorescence (IF) and
- to describe the molecular epidemiology of P. jirovecii isolates from Tygerberg Hospital by analyzing DHPS and mtLSU rRNA genes.

Methods:

Clinical samples from 305 children and adult patients at Tygerberg Hospital were collected, after testing using IF. DNA was extracted using the NucliSens easyMAG platform (Biomérieux). The rtPCR assay targeting the major surface glycoprotein (MSG) gene was evaluated to detect P. jirovecii DNA. The DHPS and mtLSU rRNA genes were amplified by nested PCR and analyzed by DNA sequencing.

Results:

The SYBR Green rtPCR detected P.jirovecii in 57% of samples (175/305) compared to the 7% (21/305) detected by IF. Our rtPCR had a sensitivity of 100% and specificity of 46%, although this increased if the detection threshold increased. Of the 50 negative control samples used in this study, none tested positive for P.jirovecii. There were 237 lower respiratory tract (LRT) and 58 upper respiratory tract (URT) samples. The yield of PCR in LRT samples was 55.3% (131/237) compared to 70.6% (41/58) in URT samples (p=0.03). In contrast, none of the URT samples were positive using IF, and 8.9% (21/237) of LRT samples were positive on IF.

DHPS was successfully amplified in 123 (70.3%) samples; and mtLSU in 126 (72%) samples. Genotype 1 (wild type) was the predominant DHPS genotype, and a mutation rate of 42.3% was recorded for this gene. The mtLSU genotype 3 was present in 50.8% of samples, genotype 1 (42%) was the next most common genotype. Mixed genotypes were detected in 2.4% of the samples analyzed for each gene. There was no clear association between DHPS polymorphisms and mtLSU genotype.

Conclusions:

Upon The SYBR Green rtPCR was more sensitive than IF for detection of P. jirovecii; especially in URT samples, which is comparable to previous studies. The DHPS mutation rate increased to 42% from 27% recorded in 2013 from our division. The increase in DHPS mutation rate may be a result of on-going co-trimoxazole use, for prophylaxis or treatment of PCP or other infections. Our findings need to be linked to clinical data to better understand transmission dynamics and potential impact of strain variation on clinical outcome, and further studies are required to better describe the local strain diversity.

Introduction

RPneumocystis jirovecii (formerly Pneumocystis carinii) is a common, atypical opportunistic fungal pathogen, causing a severe, life-threatening disease called Pneumocystis pneumonia (PCP) in patient's immunosuppressed by HIV infection, malignancy, transplantation, or therapeutic immunosuppression (Saric et al., 1994; Aderaye et al., 2003) The Pneumocystis organism was first discovered in 1909 in Brazil by Carlos Chagas in the lungs of guinea pigs, and later in the lungs of a patient who died

of trypanosomiasis. Chagas misidentified the organism as a new schizogonic state of Trypasonoma cruzi and proposed a name of Schizotrypanum (Kovacs et al., 2009). In 1910, at the Pasteur Institute of Sao Paolo, Antonio Carinii found Pneumocystis in rat lungs and considered it a new type of trypanosome (Jang-Jih Lu et al., 2008.). In 1912, Delanoë & Delanoë recognized that the organism identified by both Chagas and Carinii was a new organism (Delanoë & Delanoë, 1912) and named the organism Pneumocystis carinii to highlight its lung tropism, cyst-like morphology and to give credit to Antonio Carinii, who provided the tissue samples (Delanoë & Delanoë, 1912).

Materials and methods

Three hundred and five residual respiratory samples from adult and pediatric patients with clinical suspicion of PCP were included in the study. These samples had been sent to the NHLS Immunology laboratory at Tygerberg Hospital for routine diagnostic PCP investigations during the time period of March 2014 to January 2015. The Tygerberg hospital serves a drainage area of approximately half of Cape Town. The hospital acts as a referral centre for 4 regional hospitals (Karl Bremer, Paarl, Worcester and Helderberg Hospitals), 17 district hospitals and over 120 primary health care clinics. The population served is approximately 2.6 million, representing just under half the population of the Western Cape. Residual clinical samples were stored in a -20°C freezer following routine IF examination and included tracheal aspirates (TA), induced sputum (IS), bronchoalveolar lavage fluid (BAL),

Correspondence: Kevin Derrick Banda, Andrew C, THE MOLECULAR EPIDEMIOLOGY OF PNEUMOCYSTIS JIROVECII IN CAPE TOWN, SOUTH AFRICA,

Received: June 20, 2020, Accepted: July 02, 2020, Published: July 27, 2020

bronchial washes, and nasopharyngeal aspirates. Limited demographic data including age and gender were stored on a secure Microsoft Excel patient database. The patient demographics for both PCP suspects and the negative controls were included, to compare the age distribution of our populations.

Sample Preparation and DNA Extraction

Residual clinical samples (DTT treated and concentrated specimen following IF investigations) were thawed, followed by vortexing and centrifuging at 3 000 rpm for 5 minutes. The supernatant was discarded and the concentrated pellet was resuspended in 10 ml of 10x PBS (Diagnostic Media Products). Samples were then transferred to a 15 ml sterile tube and once again stored at -20°C until further use. DNA was extracted in batches of 12 specimens using the automated NucliSENS easyMAG extraction kit- and platform (bioMérieux, Boxtel, Netherlands). This procedure uses patented BOOM

technology which is based on the ability of positively charged magnetic silica beads to bind negatively charged nucleic acids in the presence of salts. The use of magnets allows the capture of these beads and heating them elutes the bound nucleic acids. 50 µL of each respiratory sample (IS, TA, BAL or NPA) was added to NucliSENS lysis buffer and incubated at room temperature for 10 minutes prior to the extraction process (Figure 2.3). Resultant DNA was stored at -20°C until further use.

Probe vs intercalating dye based detection of P. jirovicii DNA

Two real-time PCR technologies, SYBR green intercalating dye and a hydrolysis probe, were compared on a dilution range of P. jirovecii DNA to determine the best performance for detection of P. jirovecii DNA. Both methods employed a species specific primer set targeting a 250 bp region of the highly conserved MSG gene.

Primers and hydrolysis probe used for real-time PCR assays

SYBR Green I intercalating dye method

The qualitative SYBR rtPCR was adopted from previous study conducted in our division. Two microliters of each dilution range of the positive control was added to a reaction mixture containing 1x Rotor-Gene SYBR Green PCR Master Mix (Qiagen, Whitehead Scientific, Cape Town, South Africa). Briefly, 12.5 µl Rotor-Gene PCR Mix, 0.5 µl (50µM) of each forward and reverse primer (final concentration of each primer in our reaction mix was 1µM) and 9 µl of nuclease-free water. The PCR reaction tube final volume of 25 µl was amplified in a Qiagen Rotogene Q analyzer with cycling parameters of 50 sec at 95°C, followed by 40 cycles at 95°C for 5 sec and 60°C for 60 sec. Resultant amplicons were subjected to high-resolution thermal melt analysis (HRM) (Figure 2.3). The thermal denaturation profile was measured over the temperature range from 80°C to 90°C, and fluorometric readings were taken every 1°C. Rotorgene software was used to

calculate the derivative of the intensity of fluorescence at different temperatures (dF/dT), thereby generating a plot where the derivative peak represented the Tm value of the MSG DNA fragment. The presence/absence of MSG DNA (thereby presence or absence of P. jirovicii) was automatically determined by the software according to the presence of the derivative peak located within a defined temperature bin (width ± 2°C). Optimal temperature bins were determined during optimization of the PCR and specificity of the bin for MSG DNA was confirmed by sequencing the resultant amplicon. A software defined threshold of fluorescence was set (see section 2.2.6.4) to define a positive result. Melting curves with peaks within the pre-defined temperature bins and, above the set threshold were deemed positive.

Hydrolysis probe method

The dilution series was amplified using the same primer set described in Table 2.2. The reaction mixture consisted of 12.5 µl Rotor-Gene PCR Mix, 0.5 µl (50µM) of each forward and reverse primer, 0.5 µl (5µM) of the probe, 2 µl of each dilution sample and 9 µl of nuclease-free water (final volume of 25µl). Cycling parameters included two initial holds, each for 2 min at 50°C and 95°C respectively, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The florescence was detected in the HEX channel at the end of the annealing step of each cycle. The assay and data analysis was performed in a Qiagen Rotor-Gene Q thermo-cycler. Separate rooms were used for each of the molecular procedures, including pre-PCR (DNA extraction), reagent preparation, addition of template DNA, thermo-cycling, and post-PCR steps (real-time PCR and gel electrophoresis). Non-template controls were included in PCR reactions to control and monitor reagent contamination.

Gel Electophoresis

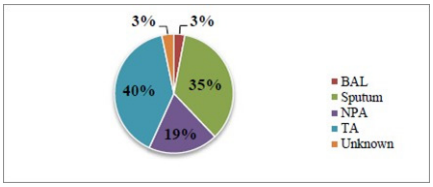
During optimization, resultant amplicons were confirmed by gel electrophoresis on a 2% TAE agarose gel run for 40 minutes at 120V. 10µl of PCR product was mixed with 2µl of a fluorescent loading dye (Novel juice, GeneDirex, Taiwan). A 100 bp plus DNA ladder (Thermo Scientific-GeneRuler) was included to aid in identification of the 250bp target size. Specificity of product was further confirmed by sequencing analysis (Inqaba Biotech, South Africa).

Data Analysis

The overall performance of the SYBR Green rtPCR assay on clinical specimen was assessed by calculating the sensitivity, specificity, positive predictive value, and negative predictive value using the standard formulae (Parikh et al., 2008). All the statistical analyses were performed using STATA for Windows (version 14). The Fisher exact test was used to assess significant associations among categorical variables. A p-value of less than 0.05 was considered significant and tests were two-tailed.

Results

During the study period, (April 2014- November 2015), 305 clinical specimens including 9 bronchoalveolar lavage (BAL) or bronchial aspirate, 106 sputa, 122 tracheal (TA) and

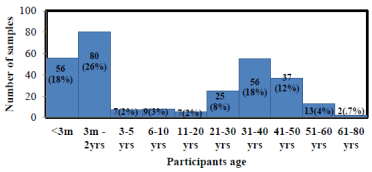


58 nasopharyngeal aspirates (NPA) were collected from the NHLS Immunology laboratory at Tygerberg Hospital, South Africa.

Patient demographics

The patients included children and adults. Clinical and demographic data were obtained from the clinical information system. There were 143 male and 155 female patients (sex ratio (M/F) of 0.93); in 7 patients the information on request forms did not specify sex. Age was not provided for 10 patients. Figure 2.5 shows the age distribution of the 295 study participants where age was available. The mean age of the patients was 18 years (range: 0.03–74 years), median age was 2.58 years; inter quartile range (3 months-3 years). For the negative control samples, the participant age ranged from 9 days to 77

years, the median was 0.58, the mean was 10.4, and the interquartile range was 5 months – 7 years.



Participants age distribution and number of samples

Our study population of PCP suspects consisted of 155 paediatric (age<13 years) and 140 adult patients. Real time PCR successfully amplified a portion of MSG from 95 of the paediatric and 75 of the adult samples (61.3% and 53.6% respectively).

SYBR Green I PCR vs Hydrolysis probe based PCR

Two rtPCR detection chemistries were investigated for the detection of P. jirovecii DNA. Both methods were applied to a serial 10-fold dilution range (10-1 to 10-5) of a plasmid carrying the MSG target gene. The characteristic sigmoidal shaped curves obtained are shown in figure 2.6a and figure 2.6b,

and the post-PCR melting curve analysis for the SYBR green assay is shown in figure 2.6c.

The assays were compared using a relatively high concentration of plasmid DNA (10-5 corresponding to 4.9 x106 molecules /µ). The main objective behind this initial comparison was to assess the workflows associated with each method, the ease of use and analysis, and the costs. Both assays showed similar performance for detection of the limited dilution range (10-1 to 10-5). However, the SYBR Green I based assay offered a number of advantages: (1) The SYBR Green dye protocol is simpler and more cost effective than the probe based protocol, (2) SYBR Green dye protocol can more easily be added to current laboratory work- flow, (3) primer dimers and other non-specific interactions can be readily visualized by melt curve analysis (Figure 2.6c). We therefore decided to continue our study using the SYBR Green I rtPCR technology.

Detection of P. jirovecii DNA in clinical specimens

For detection of Pneumocystis, SYBR Green real-time PCR targeting the major surface glycoprotein of P. jirovecii was conducted on DNA extracted from all 305 specimens. Using 3 different thresholds, the SYBR Green PCR results are shown in table 2.3.

Table 2.3: Correlation of IF and PCR methods

		Positive	Negative	Total
Immunofluorescence (IF)		21 (7%)	284 (93%)	305
MSG SYBR-Green rtPCR	Threshold 1	175 (57%)	130 (43%)	305
	Threshold 1.5	150 (49%)	155 (51%)	305
	Threshold 3	65 (21%)	240 (79%)	305

Patients with suspected PCP

Of the 305 specimens tested for P. jirovecii by immunofluorescence (IF) microscopy and real time PCR methods, IF was positive in 7% (21/305), while rtPCR recorded positives of 57% (175/305), 49 % (150/305) and 21 % (65/305) at thresholds of 1, 1.5 and 3 respectively.

IF-microscopy was deemed equivocal (ie less than 5 cysts observed) for specimens from 6 patients, and all of these yielded positive results with PCR at all 3 thresholds. Of the 21 samples positive by IF microscopy, only 3 were male, while 18 were female patients. By IF technique, 8.4% (12/143) sample positivity rate was recorded from the age group of between 3 month and 4 years, and 7.7% (9/117) sample positivity rate was recorded from age group of between 20 and 50 years. Similar for the PCR method, at the threshold of 1, 72 male and 93 female patients were positive. A 63% (90/143) positivity rate was recorded from the age group of 3 months to 4 years, while 54% (63/117) positivity rate was recorded in the age group 20 to 50 years. Correlating the PCR result with microscopy, P. jirovecii DNA was detected in all of the IF-positive specimens using rtPCR analysed using all 3 thresholds. No false negatives were observer ("PCR−ve/IF+ve"). Table 2.3 provides the positivity correlation of the IF method and rtPCR using the 3 different thresholds. There were 237 LRT samples and 58 URT samples. The yield of PCR in LRT samples was 55.3% (131/237) compared to 70.6% (41/58) in URT samples (p=0.03) In contrast, none of the URT samples were positive using IF, and 8.9% (21/237) of LRT samples were positive by IF. There was a

significantly higher yield using PCR compared to IF for both URT and LRT samples (p<0.000001 in each case).

Comparing SYBR green I rtPCR results to the IF test (gold standard), the sensitivity, specificity, positive and negative predictive values of the SYBR Green I rtPCR assay were calculated for different thresholds as shown in Table 2.4.

Table 2.4: SYBR Green PCR sensitivity, specificity and predictive values and 95% Confidence Intervals at 3 different thresholds

	PCR(1)	PCR(1.5)	PCR(3)
Sensitivity (95% Confidence Interval [CI])	100% (80.8-100%)	100% (80.8-100%)	100% (80.8-100%)
Specificity (95% CI)	46% (40-51.8%)	54.5% (48.5- 60.4%)	84.5% (79.6-88.4%)
Positive Predicitve Value (PPV) (95% CI)	12% (7.8-18%)	14% (9.1-20.8%)	32% (21.5-45.2%)
Negative Predicitve Value (NPV) (95% CI)	100% (96.4-100%)	100% (97-100%)	100% (98-100%)

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As the PCR threshold increased, the specificity and PPV increased as well, as one would expect. However, the sensitivity and NPV remained at 100% despite increasing the threshold.

P. jirovecii DNA was detected in many of the IF negative samples, which is then reflected by the relatively low specificity and positive predictive values of the test – however this presumes that the samples positive on PCR yet negative on IF are all false positives (ie patients did not have infection with P. jirovecii). If the sensitivity of the IF method is less than 100% (which is likely), then calculation of the specificity of PCR is more problematic. For this reason, an additional group of negative control samples were investigated.

Results

Of the 175 samples positive by MSG real-time PCR using threshold of 1, a portion of the DHPS region in the fas gene was successfully amplified from 123 (70.3%) samples (figure 3.2). Despite at least 3 attempts to amplify the DHPS gene, amplification was unsuccessful in 52 samples and these were excluded from the study. Of the 52 excluded samples, 63.5%

(33/52) were MSG PCR positive at detection thresholds of 1.5 compared to 36.5% (19/52) positive at a threshold of 3. This is similar to the proportion of DHPS positive samples that were MSG PCR positive at a threshold of 3 (p=NS). The mtLSU gene was successfully amplified in 126 (72%) samples, while amplification failed in 49 samples despite at least 3 attempts at amplification. Of these 49 samples, 75.5% (37/49) were positive at MSG PCR detection thresholds of 1 and 1.5 while only 24.5% (12/49) samples were positive by all 3 (1,1.5 and 3) MSG PCR detection thresholds. Again, this was not significantly different from the proportion of mtLSU positive samples that were MSG PCR positive at a threshold of 3. Samples from the lower respiratory tract would be expected to contain greater quantities of target organism, and thus one would expect a greater yield on lower respiratory tract samples. However, the success of PCR for the both DHPS and mtLSU rRNA did not differ substantially based on specimen type, as shown in table 3.2.

The success rate for PCR amplification of the DHPS and mtLSU loci also depends on the primer hybridization sites on the target DNA, therefore mutations on the primer hybridization sites may have contributed to the unsuccessful amplification in some clinical samples. The nested PCR conditions used may also have influenced the amplification success.

Sequence alignment and analysis of the DHPS gene

The sequencing analysis was performed on the DHPS genes from the successfully amplified samples. The DHPS nucleotide sequence inserts obtained after direct sequencing were aligned with a wild type DHPS reference sequence (NCBI GenBank accession: AJ586567.1). The determination of genotypes for DHPS is dependent on the amino acid substitutions at codon positions 55 and 57 as described previously (Huang et al., 2004).

Figure 3.3 illustrates an example of electropherograms of a typical mixed infection (mutations at both codon 55 and 57).

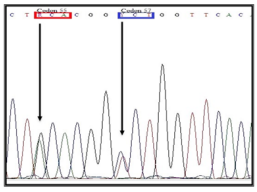

Figure 3.4: Electropherogram showing DHPS sequence data for a mixed genotype sample with double peaks, arrows pointing at indistinguishable nucleotide bases at both position 55 and 57 codon

In this study, the sequence variations were observed at codon positions 55 and 57. The DHPS gene mutation rate was 42% (52/123), and distribution of different genotypes are shown in table 3.3.

Table 3.3: *P. jirovecii* genotypes detected at the DHPS locus in 123 samples

DHPS Genotype	Nucleotide(amino-acid) position	No. of isolates	Percentage of Population (%)
1(Wt)	165 (55)/A (Thr); 171 (57)/C (Pro)	71	57.7
2	165 (55)/G (Ala); 171 (57)/C (Pro)	2	1.6
3	165 (55)/A (Thr); 171 (57)/T (Ser)	40	32.5
4	165 (55)/G (Ala); 171 (57)/T (Ser)	7	5.7
Mixed	-	3	2.4

Conclusions

The results of this study revealed that real time PCR is more sensitive in detecting P. jirovecii than the current gold standard IF method. The SYBR green PCR improved the detection of P. jirovecii; especially in URT samples, compared to IF and this is consistent with previous studies. Our results suggest that rtPCR may be able to replace IF for diagnostic purposes. The

ability of rtPCR to detect P. jirovecii in URT samples with high sensitivity, offers a good diagnostic technique as this will reduce the risk of complications associated with the use of bronchoscopy and biopsy, which requires specialiazed personel and equipment for sample collection, especially in children and critically ill adults. The high analytical sensitivity of the PCR has been a concern, as PCR may detect P. jirovecii DNA in asymptomatic individuals without PCP. Therefore, the rtPCR methods must be further evaluated in order to set an accurate quantitative cut-off value which will be able to discriminate PCP from asymptomatic colonization. However, in our study we tested 50 samples from patients with suspected respiratory illness other than PCP, as our negative control group, of which no samples tested positive for P. jirovecii DNA. After further validation and evaluation rtPCR has a potential to replace microscopic methods, and to become the gold standard method for diagnosis of PCP, especially in high burden HIV and resource limited settings. For our molecular epidemiology study, the targeted genes were successfully amplified in 123 (70.3%) samples for DHPS; and 126 (72%) mtLSU rRNA samples. The DHPS mutation prevalence increased to 42% from the 27% recorded from a previous study in this setting in 2013. The increased DHPS mutation rate in this study may be a result of on-going sulfa-drug use, for prophylaxis or treatment of PCP or other infections. The need to continue efforts to estimate the true burden of PCP in South Africa must be emphasised. In 126 samples analyzed for the mtLSU gene, a mutation prevalence of 58% was observed. The high frequency of mtLSUrRNA polymorphisms among PCP patients in our study population may be important for phylogenetic or population genetic models to elucidate P. jirovecii interspecies strain relatedness.

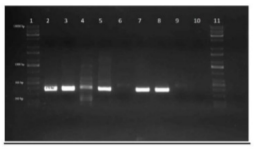

Figure 3.2: Agarose gel showing PCR-amplified 370 bp product of the *P. jirovecii* DHPS gene. Lanes 1and 11: 1000 bp KAPA universal ladder, lanes 2-5, 7-8 positive samples, lanes 6 and 9 negative samples and lane 10 PCR negative control

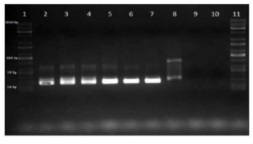

Figure 3.3: Agarose gel showing PCR -amplified 267 bp product of *P. jirovecii* mtLSU gene. Lanes 1 and 11: 1000 bp KAPA universal DNA ladder, Lane 2 positive control, Lanes 3-8 positive samples, Lane 9: Negative sample, Lane 10: Negative control

Ethical approval

The patient samples obtained during the course of the study form part of the routine diagnostic work-up. Samples were anonymous and minimal clinical information was collected for analysis of the data. The PCR results were not reported to the clinicians or on the NHLS Laboratory Information System, but only used to validate and assess the assay.

This study was approved by the Health Research Ethics Committee of the University of Stellenbosch (#: S15/07/141)

Funding

Beit Trust and National Health Laboratory Service (NHLS) funded this project

REFERENCES

1. Aderaye G, Bruchfeld J, Olsson M, Lindquist L. Occurrence of Pneumocystis carinii in HIV- positive patients with suspected pulmonary tuberculosis in Ethiopia. Journal Acquired Immune Deficiency Syndrome 2003; 17:435–40.
2. Alanio A, Bretagne S. Diagnosis of Pneumocystis jirovecii Pneumonia: Role of 𝛃-D-Glucan Detection and PCR. Current Fungal Infection Reports 2014; 8(4): 322-330
3. Alanio A, Desoubeaux G, Sarfati C, Hamane S, Bergeron A, Azoulay E et al. Real-time PCR assay-based strategy for differentiation between active Pneumocystis jirovecii pneumonia and colonization in immunocompromised patients. Journal of Clinical Microbiology and Infection 2011; 17: 1531–7.
4. Alanio A, Gits-Muselli M, Mercier-Delarue S,Dromer F and Bretagne F. Diversity of Pneumocystis jirovecii during Infection Revealed by Ultra-Deep Pyrosequencing. Frontiers in Microbiology 2016: 7(733)
5. Aliouat-Denis , Chabe´ M , Demanche C, Aliouat EL M, Viscogliosi E, Guillot J, Delhae L, Dei-Cas E. Pneumocystis species, co-evolution and pathogenic power. Journal of Infection, Genetics and Evolution 2008; 8: 708–726
6. Alli OTA, Ogbolu DO, Ademola O, and Oyenike MA et al. Molecular Detection of Pneumocystis Jirovecii in Patients with Respiratory Tract Infections. North America Journal of Medical Sciences 2012; 4(10): 479–485.
7. Alvarez-Martínez MJ, Miró JM, Valls ME, et al. Prevalence of dihydropteroate synthase genotypes before and after the introduction of combined antiretroviral therapy and their influence on the outcome of Pneumocystis pneumonia in HIV-1–infected patients. Journal of Diagniostic Microbiology and Infectious Diseases 2010; 68(1):60-65.
8. Alvarez-Martinez MJ, Miro JM, Valls ME, Moreno A, Rivas PV, Solé M, Benito N, Domingo P, Muñoz C, Rivera E, Zar HJ, Wissmann G, Diehl A R, Prolla JC, De Anta MT, Gatell JM, Wilson PE, Meshnick SR & Spanish P.C.P. Working Group et al. Sensitivity and specificity of
9. nested and real-time PCR for detection of Pneumocystis jiroveci in clinical specimens. Journal of Diagnostic Microbiology and Infectious Disease 2006; 56(2): 153-160.
10. Alvarez-Martínez MJ, Moreno A, Miró JM et al. Pneumocystis jirovecii pneumonia in Spanish HIV-infected patients in the combined antiretroviral therapy era: prevalence of dihydropteroate synthase mutations and prognostic factors of mortality. Journal of Diagnostic Microbiology and Infectious Diseases 2008; 62:34-43
11. Ansari NA, Kombe AH, Kenyon TA, Mazhani L, Binkin N, Tappero JW, Gebrekristos T, Nyirenda S and Lucas SB. Pathology and causes of death in a series of human immunodeficiency virus-positive and -negative pediatric referral hospital admissions in Botswana. Journal of Pediatric Infectious Diseases 2003; 22(1): 43-47
12. Arcenas RC, Uhl JR, Buckwalter SP, Limper AH, Crino D, Roberts GD. et al. A real-time polymerase chain reaction assay for detection of Pneumocystis from bronchoalveolar lavage fluid. Journal of Diagnostic Microbiology and Infectious Diseases 2006; 54: 169-175.
13. Bandt D, Monecke S. Development and evaluation of a real-time PCR assay for detection of
14. Pneumocystis jiroveci. Journal of Transplantation and Infectious Diseases 2007; 9:196–202
15. Beard CB, Carter JL, Keely SP, Huang L, Pieniazek NJ, Moura IN, Roberts JM, Hightower AW, Bens MS, Freeman AR, Lee S, Stringer JR, Duchin JS, del Rio C, Rimland D, Baughman RP, Levy DA, Dietz VJ, Simon P, Navin TR et al. Genetic variation in Pneumocystis carinii isolates from different geographic regions: implications for transmission. Journal of Emerging Infectious Diseases 2000; 6: 265–272
16. Beard CB, Roux P, Nevez G, et al. Strain typing methods and molecular epidemiology of
17. Pneumocystis pneumonia. Journal of Emerging Infectious Diseases 2004; 10:1729– 35.
18. Beck JM, Cushion MT. Pneumocystis workshop: 10th anniversary summary. Eukaryotic Cell 2009; 8:446–60.

19. Beser J, Dini L, Botero-Kleiven S. Krabbe M, Lindh J, Hagblom P. et al. Absence of dihydropteroate synthase gene mutations in Pneumocystis jirovecii isolated from Swedish patients. Journal of Medical Mycology 2012; 50:320–323

20. Botterel F, Cabaret O, Foulet F, Cordonnier C, Costa JM, Bretagne S. Clinical significance of quantifying Pneumocystis jirovecii DNA by using real-time PCR in bronchoalveolar lavage fluid from immunocompromised patients. Journal of Clinical Microbiology 2012; 50: 227–31.

21. Brancart F, Hector Rodriguez-Villalobos, Pierre-Alain Fonteyne, Daliana Peres-Bota, Corinne Liesnar. Quantitative TaqMan PCR for detection of Pneumocystis jirovecii. Journal of Microbiological Methods 2005;61: 381–387

22. Calderon E, De la Horra C, Medrano F, López-Suárez A, Montes-Cano M, Respaldiza N, et al. Pneumocystis jirovecii isolates with dihydropteroate synthase mutations in patients with chronic bronchitis. European Journal of Clinical Microbiology Infectious Diseases 2004; 23(7): 545-9.

23. Calderon EJ, Regordan C, Medrano FJ, Ollero M, Varela JM et al. Pneumocystis carinii infection in patients with chronic bronchial disease. Lancet 1996; 347(9006): 977. 21. Caliendo AM, Hewitt PL, Allega JM, Keen A, Ruoff KL, Ferraro MJ. Performance of a PCR assay for detection of Pneumocystis carinii from respiratory specimens. Journal Clinical Microbiology 1998; 36:979–82.

24. Carmona EM and Limper AH. Update on the diagnosis and treatment of Pneumocystis

25. pneumonia. Journal of Therapeutic Advances in Respiratory Disease 2011; 5(1): 41-59

26. Cartwright CP, Nelson NA, Gill VJ. Development and evaluation of a rapid and simple procedure for detection of Pneumocystis carinii by PCR. Journal of Clinical Microbiology 1994; 32:1634–1638

27. Chabe M, Dei-Cas E, Creusy C, Fleurisse L, Respaldiza N, et al. Immunocompetent hosts as a reservoir of pneumocystis organisms: histological and rt-PCR data demonstrate active replication. European Journal of Clinical Microbiology and Infectious Disease 2004; 23(2): 89-97.

28. Chagas C. Nova trypanozomiaze humana. Mem. Inst. Oswaldo Cruz, vol. 1, 1909, pp. 159- 218 (cited in Kovacs et al, 2009).

29. Chaisson RE, Keruly J, Richman DD, Moore RD. Pneumocystis prophylaxis and survival in patients with advanced human immunodeficiency virus infection treated with zidovudine. The Zidovudine Epidemiology Group. Arch Intern Med 1992; 152(10):2009-2013.

30. Charles FT and Limper AH. New England Journal of Medicine 2004; 350(6):2487

31. Chintu C, Mudenda V, Lucas S, Nunn A, Lishimpi K, Maswahu D, Kasolo F, Mwaba P, Bhat G, Terunuma H and Zumla A. Lung diseases at necropsy in African children dying from respiratory illnesses: a descriptive necropsy study, Lancet 2002;360;985-990.

32. Costa MC, Helweg-Larsen J, Lundgren B, Antunes F & Matos O. Mutations in the dihydropteroate synthase gene of Pneumocystis jirovecii isolates from Portuguese patients with Pneumocystis pneumonia. International Journal of Antimicrobial Agents 2003; 22 (5): 516-520.

33. Cregan P, Yamamoto A, Lum A, VanDerHeide T, MacDonald M, Pulliam L. Comparison of four methods for rapid detection of Pneumocystis carinii in respiratory specimens. Journal of Clinical Microbiology 1990; 28: 2432–6.

34. Udani P, Parekh U, Dastur D. Neurological and related syndromes in CNS tuberculosis Clinical features and pathogenesis. J Neurol Sci. 1971; 14(13):341-57.

35. Burstein L, Breningstall GN. Movement disorders in bacterial meningitis. J Pediatr. 1986; 109(2):260-64.

36. Janavs JL, Aminoff MJ. Dystonia and chorea in acquired systemic disorders. J Neurol Neurosurger Psychiatry.1998; 65:436-45.

37. Namer I, Tan E, Akalin E,et al. A case of hemiballismus during cryptococcal meningitis. Rev Neurol (Paris). 1990;146(2):153-54.

38. Weeks R, Clough C. Hemichorea due to cryptococcal meningitis. Mov Disord. 1995; 10(4):522.

39. Coppen EM, Roos RA. Current pharmacological approaches to reduce chorea in Huntington's disease. Drugs. 2017;77(1):29-46.

40. Burgunder J-M. Recent advances in the management of choreas. Ther Adv Neurol Disord. 2013;6(2):117-27.

41. Rezai AR, Machado AG, Deogaonkar M et al. Surgery for movement disorders. Neurosurgery. 2008;62:809-39.