Title of dissertation:

The molecular diagnosis of *Pneumocystis* pneumonia in children using nasopharyngeal aspirate samples

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Declaration by Dr Catherine Mary Samuel:

I hereby declare that I have read and am familiar with the current "MMed Part III: Guidelines for Candidates" and have complied with the instructions and stated conditions. The research manuscript, on which this dissertation is based, is my original work and neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. The manuscript is as yet unpublished but has recently been submitted to THORAX (British Medical Journal Group) and is awaiting editorial review. Signed by candidate

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PART A: PROTOCOL

Title of Research Project:

Molecular diagnosis of *Pneumocystis jirovecii* pneumonia in children

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Introduction

Pneumocystis pneumonia (PCP) is an important opportunistic infection caused by the fungus *Pneumocystis jirovecii*. The incidence of PCP in sub-Saharan Africa is on the increase. This is due to the progression of the HIV-pandemic and limited access to health care facilities, specific highly active anti-retroviral therapy and chemoprophylaxis. It is a major cause of hospitalization and mortality in HIV-infected children with in-hospital case-fatality rates ranging from 20 – 63%.[1-4]

The prevalence of PCP amongst HIV-infected South African children, hospitalized with pneumonia, has been reported to vary from 10 – 49%.[1-3] It is a common AIDS-defining illness in infants in South Africa.[1] Other risk factors for PCP in children, in addition to HIV infection, include protein-energy malnutrition and HIV exposure. A Zambian post mortem study found that 10% of children who died from PCP were HIV negative.[5] HIV-exposed children have been described to have a higher risk of PCP compared to unexposed children. Recent reports describe probable transmission of *P.jirovecii* from an HIV-infected mother to her HIV-exposed but uninfected infant.[6,7] Despite the national roll-out of the Prevention of Mother-To-Child-Transmission Programme in South Africa, many HIV-infected or HIV-exposed children continue to be hospitalized for presumptive PCP infection. Many of these children require admission into an intensive care facility.

When managing patients with suspected PCP, it is important to make an accurate laboratory diagnosis to confirm that *Pneumocystis jirovecii* is the cause of their lower respiratory tract infection. In HIV-infected patients, the differential diagnosis of diffuse pulmonary infiltrates, in addition to PCP, includes bacterial pneumonia and pulmonary tuberculosis. The case-fatality rate in children with untreated PCP approximates 100%,[1-4] making a rapid laboratory diagnosis essential in the management of these patients. A missed or an incorrect diagnosis may result in inappropriate treatment, which in addition to poor clinical outcomes, may have both cost implications and result in unnecessary adverse drug reactions. A rapid diagnostic test for PCP using non-invasive approaches for specimen collection will aid the laboratory diagnosis in resource-limited settings and allow early initiation of appropriate treatment, thereby improving patient outcome.

The standard method for diagnosing PCP has been microscopic examination using Gomori Grocott's methenamine silver nitrate stain which stain *Pneumocystis* cystic or sporocyte forms, or the more sensitive commercialized immunofluorescence (IF) tests to demonstrate the organism in respiratory samples. These tests have been used on bronchoalveolar lavage (BAL) or lung biopsy specimens in developed countries to diagnose PCP; however these methods of obtaining specimens are invasive, carry the risk of significant morbidity and mortality and are not routinely available in most developing countries. Sputum induction (IS) may be useful[1-3] and nasopharyngeal swabs or aspirates (NPA) may yield *P.jirovecii* in severe infection[3,4] . The combination of IS with NPA may provide a higher yield. Ruffini et al report a higher sensitivity of 70-80% when the results of both induced sputum and NPA were combined.[3]

The development of DNA amplification by polymerase chain reaction (PCR) techniques, has provided a more sensitive and specific diagnostic method of detecting *Pneumocystis* in a variety of respiratory specimens, with the potential to improve diagnostic accuracy.[8,9] PCR may also prove useful for detection of *P.jirovecii* on specimens obtained using non-invasive approaches from the upper airways including nasopharyngeal or oropharyngeal aspirates. In oral washes from HIV –positive patients, *P.jirovecii* DNA-amplification yielded a sensitivity of 70-80% using a standard PCR protocol compared with PCR on IS or BAL.[8] This non-invasive procedure may reach 90% sensitivity when touch-down PCR is used. [8,9] This would be an advantage in children as the procedure risk is minimal and it is quick and simple to perform compared with sputum induction and BAL, which requires specialized equipment and expertise. In addition, serial non-invasive sampling can be performed in order to monitor response to therapy. In adults, PCR analysis of oropharyngeal aspirates may be useful for diagnosis but reported sensitivities have ranged from 40-80%.[10,11] The efficacy of PCR for diagnosis of PCP in children, however, has not been well studied.

In a study by Durand-Joly et al, patients with negative microscopy results but positive touch-down PCR on BAL but negative in oral washes, the absence of PCP was confirmed clinically and colonization was concluded.[8] Real-time PCR allows for quantification of the

organism concerned. A positive PCR result with a low organism load may represent colonisation with *P. jirovecii*. Cut-off values representing the difference between colonization and infection has yet to be determined but will improve the specificity of the qualitative assay.

Aims of the study

- To establish a quantitative real-time PCR assay for the detection of *P.jirovecii* in respiratory samples based on a previously described method.[12]

- To evaluate the performance of the real-time PCR assay for the detection of *P.jirovecii* in different clinical specimens in children with presumed PCP in comparison with the current standard method of immunofluorescence. *We hypothesize that PCR is a more sensitive diagnostic method than immunofluorescence for the diagnosis of PCP in children.*

- To compare the performance of the PCR assay when performed on non-invasive nasopharyngeal aspirate samples with lower respiratory tract samples (bronchoalveolar lavage, induced sputum) from children with presumed PCP. *We hypothesize that PCR on NPA samples will be more sensitive than immunofluorescence in the detection of P.jirovecii; and sensitivity and specificity of the assay on NPA samples may approach that of PCR performed on lower respiratory tract samples.*

Study population

The study population comprises children (<14 years), admitted to the Red Cross Children's War Memorial Hospital, with suspected PCP. Consecutive children, with a primary diagnosis of presumed PCP, will be prospectively enrolled. Induced sputum and NPA samples will be obtained, and non-directed BAL will be done in intubated children. The study will aim to enrol a minimum of 1 child per week over a 2-year period.

A clinical case definition of presumptive PCP will include:

- Acute onset (<2 weeks) of respiratory illness requiring hospitalization
- Presence of tachypnoea (RR> 60 if less than 2 months, > 50 if less than a year, >40 if older than a year)
- Hypoxia (room air saturation < 90%)
- Diffuse bilateral lung disease, not associated with wheezing
- One of the following risk factors for PCP:
    - HIV-infected or clinically suspected or
    - HIV-exposed or
    - Malnourished or
    - On immunosuppressive therapy or
    - Presence of immunodeficiency disease other than HIV

Exclusion criteria will be treatment for PCP in the preceding 2 weeks, on PCP treatment for more than 48 hours or consent not obtainable.

Control group

The negative controls will consist of respiratory samples obtained for routine diagnostic purposes, from patients admitted to Red Cross Children's War Memorial Hospital, that adhere to the following conditions:

- Acute onset (<2 weeks) of respiratory illness, severe enough to warrant medical attention at a tertiary institute
- Presence of respiratory compromise with or without hypoxia (room air saturation < 90%)
- Presence or absence of HIV status or other co-morbidities
- Has not been on treatment for PCP in the preceding 2 weeks or on PCP treatment for more than 48 hours prior to obtaining the specimen
- Clinical improvement without being on treatment for PCP
- Positive multiplex respiratory viral panel PCR performed on appropriate respiratory sample (nasopharyngeal aspirate or BAL)

The clinical records of patients, who have had routine respiratory specimens submitted, will be reviewed in order to collect the above information. The samples will be made anonymous prior to analysis so that the results cannot be linked back to individual patients**.**

Investigations

Nasopharyngeal aspirates representing upper respiratory tract specimens and induced sputum or bronchoalveolar lavage fluid from the lower respiratory tract will be submitted for detection of *P. jirovecii* by:

- **Immunofluorescence**

Direct immunofluorescence (Detect IF, Axis-Shield Diagnostics, UK) using a monoclonal antibody will be performed on sputum, NPA and BAL specimens to detect *P. jirovecii*. This is the currently employed standard diagnostic test to detect PCP in patients admitted to Groote Schuur Hospital and Red Cross Hospital.

- **Silver stain**

Grocott's methenamine silver nitrate stain will be performed by the Cytology Department at Red Cross Hospital on certain of the trial BAL specimens, as per clinicians request. The results we be used as part of our analysis.

- **PCR**

The study will be conducted at the National Health Laboratory Service (NHLS) laboratories at Groote Schuur Hospital. All work will be performed in an ISO-accredited molecular laboratory, which employs strict precautions to prevent contamination. The investigators performing PCR methods will be blinded to the immunofluorescence results.

Specimens will be stored at -70ºC. The PCR assay will be carried out as described below with standards for quantification, a positive control (DNA extracts of a known *P. jirovecii* positive sample) and a negative extraction control in each PCR run.

PCR Methodology:

Deoxyribonucleic acid extraction

DNA will be extracted from using the NucliSENS Magnetic Extraction kit (bioMerieux) in accordance with the manufacturer's recommendation. These will be performed on the NucliSENS easyMAG platform.

Quantitative real-time PCR

P. jirovecii DNA will be detected using a quantitative, touch-down, real-time PCR assay targeting the major surface glycoprotein (MSG) gene based on the publication by Larsen et al. This assay has been shown to be as sensitive as the nested PCR assay, but more specific.[9]

Tenfold serial dilutions of a cloned target will be used as standards for quantification. The *P.jirovecii* MSG gene has been cloned into the pCR 2.1 vector and this has been donated to us by J. Kovacs M.D. (NIH). The plasmid will be propagated in *E.coli* competent cells using a standard transformation protocol and will be purified using the QIAGEN Plasmid Midi Kit (QIAGEN). Deoxyribonucleic acid concentration will be evaluated and tenfold serial dilutions will be prepared as standards for quantification

MSG primers which have been commercially synthesized, JKK14/15 (5_-GAA TGC AAA TCY TTA CAG ACA ACA G-3_) and JKK17 (5_-AAA TCA TGA ACG AAA TAA CCA TTG C-3_),[12] which amplify a 250-bp segment of the multicopy MSG gene family, will be obtained for use in the assay. Fluorescence resonance energy transfer (FRET) probes designed as per publication by Larsen et al. will be used for detection. [12] The assay will be performed on the. Lightcycler platform (Roche) and each PCR reaction will have a final volume of 20ul. The assay conditions utilised will be based on those used by Larsen et al.[12] Each run will contain 4 standards of cloned target template (10^6, 10^5, 10^4, 10^3) which will generate an

external standard curve required for quantification of any positive patient samples that might appear in the run. Fluorescence measurements will be analysed using the Lightcycler software

External controls: A sample with a positive immunofluorescence result will be included as a positive control in addition to the cloned target being used as standards for quantification. A negative extraction control will also be included in each PCR run.

Specificity studies: Respiratory samples from 25 patients with respiratory symptoms similar to PCP, who have improved clinically without treatment targeted against *P.jirovecii,* will be analyzed as a control group.

- Other investigations

Routine laboratory investigations will be done on respiratory specimens including immunofluorescence for *P. jirovecii*, bacterial culture, microscopy and culture for *Mycobacterium tuberculosis* and viral detection, as indicated clinically. Other investigations will be at the discretion of the attending physician.

Treatment

All patients with suspected PCP will be treated empirically according to the hospital's standard guidelines. This will include intravenous Cotrimoxazole (TMP-SMX), corticosteroids and a broad-spectrum antimicrobial agent. The decision to admit to an intensive care unit facility will be at the discretion of the attending doctor.

Analysis

The performance of the PCR assay will be assessed by determining the sensitivity and specificity of the test. The result of each PCR reaction on a specific patient sample will be compared to the corresponding immunofluorescence result (and silver stain result, if available). The PCR results of the nasopharyngeal aspirates will be compared to the results obtained from the lower respiratory tract sample types, namely induced sputa and bronchoalveolar lavages. A comparison of yield from different clinical specimens will

be made using a chi square test. If the child is discharged from medical care or dies or for another reason, only one sample is obtained, the sample will still be included in the analysis.

Primary Outcomes

- To optimise and validate the use of a quantitative PCR assay for detection of *P. jiroveci* in respiratory samples.
- To evaluate the usefulness of the direct immunofluorescence test compared to that of PCR on respiratory samples from patients with presumed PCP.
- To describe the value of real-time PCR on non-invasive nasopharyngeal aspirate samples compared to specimens of the lower respiratory tract (induced sputa or bronchoalveolar lavage).

Impact

With the rising HIV-pandemic in South Africa, the ability to offer a rapid, reliable, diagnostic test for *Pneumocystis* could prove clinically invaluable. PCR could gain acceptance as the standard technique available for the diagnosis of PCP.

If the quantitative, touch-down, real-time PCR assay proves to be a sensitive and specific diagnostic test, as anticipated, it would be possible to offer the test to institutes other than Groote Schuur Hospital and Red Cross Hospital. It would also be offered for testing in adult patients. This would further enhance and expand the repertoire of molecular diagnostics offered at our laboratory.

This is also an important opportunity to increase collaboration between clinicians and the NHLS laboratory.

Caveats and potential limitations

1. Contamination may result in false positives. The molecular laboratory layout ensures strict separation of the extraction and amplification steps. Aerosol-barrier pipette tips will be used in steps involving reagent transfer in the PCR process. The design of this PCR method should, however, carry a lower risk of contamination as the assay is performed in a closed system with limited manipulation of PCR products. Quality control measures will include relevant positive and negative controls in each PCR run.

2. If the patient sample obtained is inadequate for all microbiological investigations requested by the attending clinician, then only the current standard diagnostic method (IF) will be performed. Some patients may only be able to provide one respiratory sample, however this will still be included in the analysis.

3. In asymptomatic patients, particularly those that are immunocompromised, the relevance of a positive PCR result is not known. A positive result may represent colonisation or sub-clinical infection and the decision on whether or not to treat the patient, can be difficult. This real-time PCR method allows for quantification and therefore the determination of organism load or concentration per microlitre of sample. Patients with infection will have a higher organism load than patients that are colonised. Determining the cutoff values representing the difference between colonization versus infection may, however, prove to be challenging but will improve the specificity of the qualitative assay.

4. Currently there is no known ideal gold standard for the laboratory diagnosis of PCP. Most clinicians treat empirically if clinical symptoms and signs and radiological evidence suggests PCP. Culture is not possible and the poor performance of microscopy means that a positive result is confirmatory however a negative result cannot exclude the presence of PCP. It is, therefore, not possible to accurately determine the sensitivity of our PCR assay as we currently have no gold standard by which to compare our results. However, quantification may help

to distinguish between clinical true and false positivity and thereby improve the sensitivity of the assay.

Ethics

The patient samples that shall be obtained during the study will most often form part of the routine diagnostic work-up on the patient to exclude bacterial, fungal and viral infections. If additional study-specific samples need to be taken in order to obtain patient sample pairs, it most commonly will be a nasopharyngeal aspirate. The investigation is considered to be a low-risk procedure and shall not result in the deterioration in the patient's respiratory condition.

Informed consent will be obtained from a parent or legal guardian prior to inclusion into the study. The study conduct will adhere to the Declaration of Helsinki 2008. [13]

The study will commence after approval from the Department of Clinical Laboratory Science and the Ethics Committee at the University of Cape Town.

Funding has been approved and will be provided by the NHLS Research Trust.

This study forms part of a larger collaborative study involving clinicians at Red Cross Children's Hospital and the NHLS Laboratory. Further objectives to be included in the larger clinical study will be to:

- To determine the prevalence of mutations in the P jirovecii DHPS gene in South African children and to investigate whether the presence of mutations is associated with disease severity, prior TMP-SMX exposure or treatment failure
- To investigate the epidemiology of resistant P jirovecii isolates over time
- To investigate the serological responses to pneumocystis antigens in children with PCP.

Both institutional approval (Institute of Child Health) and UCT Research Ethics Committee approval (REC REF 363 / 2006) for the larger clinical study, has already been obtained.

Collaborators

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2. Dr Peter Walzer

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PART B: LITERATURE REVIEW

Objectives of the literature review

The objective of this literature review was to describe and evaluate current tests used for the detection of *Pneumocystis jirovecii* in different clinical specimens in children with presumed pneumocystis pneumonia (PCP) and to identify areas where further research may be needed, particularly in the context of diagnosis in resource-limited settings. In order to achieve this it was also necessary to review (1) the incidence of PCP in HIV-infected South African children; (2) the prevalence of the condition amongst HIV-infected South African children hospitalized with pneumonia; (3) the mortality in children hospitalized with PCP and (4) risk factors, other than HIV, associated with the development of PCP infection.

Literature search strategy

The literature search was performed using PubMed which comprises biomedical literature from MEDLINE, life science journals, and online books, all up to November 2008. The following search string was used to identify studies: (*Pneumocystis* [medical subject headings {MeSH}] OR *Pneumonia, Pneumocystis* [MeSH] OR *Pneumocystis Infections* [MeSH] OR *Pneumocystis carinii* [MeSH] OR *Pneumocystis jirovecii* [MeSH] OR *pcp*) AND (*diagnosis* [Subheading] OR *immunofluorescence* [MeSH]) OR *PCR* [MeSH] OR *real-time* OR *molecular diagnosis.* General search terms were then replaced with more specific ones (eg. incidence, nasopharyngeal aspirate, bronchoalveolar lavage, induced sputum) and additional terms were added to the search (e.g. HIV). Citations were limited by age group (all children: 0-18 years), language (to sources in the English language) and the search was confined to human studies. Due to the paucity of studies, especially in Africa, looking at the incidence of PCP and new diagnostics, no limit was applied to publication date or geographical specificity and therefore both local and international data were included in the review. References of identified studies were further inspected to identify any additional evidence.

This review included studies that prospectively and retrospectively investigated the incidence of PCP in HIV-infected children as well as studies that assessed patient mortality and that described risk factors for the condition. Studies comparing a diagnostic test with a reference standard or studies that compared the performance of the same diagnostic test on a variety of clinical specimens were included. Patient populations in these diagnostic studies included those with known risk factors for acquiring the infection, such as those with cancer, on immunosuppressive therapy or prolonged corticosteroid treatment, those receiving solid organ or bone marrow allografts and children with protein-energy malnutrition.

Summary of the literature

Introduction

Pneumocystis pneumonia (PCP) is an important opportunistic infection caused by the fungus *Pneumocystis jirovecii*. The organism was discovered in 1909 by Chagas, but the cystic forms were mistakenly considered to be part of the trypanosome life cycle. It was named *Pneumocystis carinii* (Greek: *pneumon* 'lung', *kystis* 'cyst') after Antonio Carini in 1912 (Dail et al., 2008). Prior to 1988, the organism was considered to be a protozoan. The controversy regarding its taxonomy and classification continued for decades and recently, based on ribosomal RNA sequences homologous to that found in fungi, it was re-classified as a fungus. Advances in molecular technology led to 5 species being described with *P. carinii* and *P. wakefieldiae* described as infecting strains in rats (Gigliotti, 2005).

Pneumocystis organisms infecting humans were re-named *P. jirovecii* in 1999, in honor of Otto Jirovec, who described *Pneumocystis* pneumonia in humans in 1952 (Dail et al., 2008). *P. jirovecii* infection is well known to cause interstitial plasma cell pneumonia, primarily in immune-compromised patients. The incidence seems to be on the increase in sub-Saharan Africa due to the progression of the HIV-pandemic and limited access to health care facilities, specific highly active anti-retroviral therapy and chemoprophylaxis. It has been considered an under-diagnosed condition due to the difficulties in diagnosing the infection particularly in resource-poor settings.

Epidemiology

In a Zimbabwean post-mortem study of children who died at home, PCP was found in 16% of children found to be HIV-positive (Ikeogu et al., 1997). A study in Côte d'Ivoire reported PCP in 23% of HIV-positive children during necropsy studies performed on children who died in the community or at hospital prior to a clinical diagnosis being made (Lucas et al., 1996). However, the occurrence of PCP in HIV-infected infants and children in Africa was largely unavailable until Zar et al. (2000) conducted the first large, prospective study to investigate the incidence of PCP in HIV-infected children with pneumonia in Cape Town, South Africa. In this study, PCP accounted for 10% of acute pneumonia episodes in hospitalized HIV-infected children and was the AIDS-defining infection in 20% of those newly diagnosed as HIV-positive. This figure was probably an underestimate however, as the diagnosis was made by microscopy of induced sputum in most subjects and not on bronchoalveolar lavage samples, as the study was performed in a resource-poor setting. In another study, Zar et al. (2001) showed that in HIV-infected children admitted to intensive care in a developing country, 38% had evidence of PCP on induced sputa and non-directed BAL samples from intubated patients.

PCP is considered a major cause of mortality in HIV-infected children and in-hospital case-fatality rates range from 20 – 63% (Madhi et al., 2002; Ruffini et al., 2002; Graham et al., 2000). In Zar's study in 2000, 7 of 15 (47%) children with PCP died while hospitalized compared with 24 of 136 (18%) without PCP [RR 1.21 (0.99 - 1.47)] (Zar et al., 2000). In the 2001 study of patients admitted to intensive care, 31 of 151 (20%) HIV-positive and 8 of 99 (8%) HIV-negative children died [RR 1.16 (95% CI 1.05-1.28), p=0.008] and using multiple logistic regression, PCP was the only risk factor for mortality (p = 0.03) (Zar et al., 2001).

In addition to HIV infection, other risk factors identified for PCP in children include protein-energy malnutrition and HIV exposure (Ikeogu et al., 1997; Su et al., 2008). In a Zambian post mortem study, Chintu (2002) found that 7% of children who died from PCP were HIV negative. All these children were identified as being less than six months of age but apart from HIV status, other risk factors were not sought for. HIV-exposed children

have been described to have a higher risk of PCP compared to unexposed children, which may be due to transmission of *P. jirovecii* from an HIV-infected mother to her HIV-exposed but uninfected infant (Heresi et al., 1997; McNally et al., 2005).

Despite the national roll-out of the Prevention of Mother-To-Child-Transmission (PMTCT) Programme in South Africa, many HIV-infected or HIV-exposed children continue to be hospitalized for presumptive PCP infection (Morrow et al., 2010). Management of these patients with suspected PCP ideally includes making an accurate laboratory diagnosis to confirm *P. jirovecii* as the cause of their lower respiratory tract infection. This is essential in HIV-infected patients, as the differential diagnosis of diffuse pulmonary infiltrates is broad and in addition to PCP, includes viral and bacterial pneumonia, and pulmonary tuberculosis, the management and prognosis of which varies depending on the infection.

Diagnosis

The current gold standard test most commonly used for diagnosing PCP is microscopy using either the Gomori methenamine silver nitrate stain which stains *Pneumocystis* cystic and sporocyte forms, or the commercial (direct fluorescent-antibody) immunofluorescence (IF) assay (Caliendo et al., 1998). Other conventional tests available that morphologically demonstrate the organisms in respiratory specimens include Giemsa or Giemsa-like rapid stains (e.g. Diff-Quik) and the toluidine blue O stain. Of all these microscopy methods, immunofluorescence has the advantage of requiring less time, fewer reagents and may be the most sensitive (Kovacs et al., 1988). A major limitation to the use of immunofluorescence is the need for a fluorescent microscope, as well as, experienced microscopists. Valerie (1990) examined a variety of respiratory specimens from known or suspected HIV-infected patients with modified Giemsa and IF stains. The organism was detected in 92% of the specimens using the modified Giemsa and in 97% of the specimens by use of the fluorescent-antibody stain.

Microscopy is usually performed on bronchoalveolar lavage (BAL) fluid obtained via fiberoptic bronchoscopy or on lung biopsy specimens (transbronchial biopsy or open-lung biopsy) in developed countries (Nyamande et al., 2005). However, obtaining these specimens, particularly in HIV-infected infants is difficult. Performing bronchoalveolar

lavages in sick infants may cause deterioration in the respiratory status of the child (Morrow et al., 2004). In addition these techniques require expertise and specialized equipment which may not be available in resource-limited settings. This form of diagnosis is thus not routinely available in most developing countries and many patients may be treated empirically without a laboratory-confirmed diagnosis.

Less invasive approaches to obtaining respiratory specimens from children include sputum induction (IS) and nasopharyngeal swabs or aspirates (NPA). Sputum induction has been reported as useful by Zar (2000), Madhi (2002) and Ruffini (2002) when compared to the above-mentioned reference methods of BAL or lung biopsy. Silver stain had a similar sensitivity whether used on IS or BAL (Pinlaor et al., 2004).

Examination of NPA specimens may yield *P. jirovecii* in severe pulmonary infection as shown by Ruffini (2002). The sensitivity and specificity of microscopy on a combination of induced sputum and NPA were 75% and 80% respectively, as compared to post-mortem lung histology. The ability to detect *P. jirovecii* in NPA samples may be due to the high organism load in the respiratory tract in severe disease or the fact that the organisms may reach the upper airways during the process of coughing. However, not all studies have found examination of NPA samples useful. Zar et al. (2000) using IF found no positive results on NPA samples in a group of 15 children, 9 of which were positive by examination of IS and 6 positive by examination if BAL fluid.

Molecular diagnostics

The advent of molecular technology has revolutionized laboratory diagnostics. Nucleic acid amplification techniques, such as polymerase chain reaction (PCR), have provided more sensitive and specific diagnostic methods to detect *P. jirovecii* (Nyamande et al., 2005; Brancart et al., 2005; Fischer et al., 2001; Flori et al., 2004; Larsen et al., 2002; Palladino et al., 2001). Commonly used target genes in conventional nested PCR include the mitochondrial large subunit ribosomal RNA (mtLSU rRNA) and the internal transcribed spacer (ITS) region and the dihydropteroate synthase (DHPS) gene (Helweg-Larsen et al., 1997; 1998; Ribes et al., 1997; Olssen et al., 2001 Flori et al., 2004; Gupta et al., 2008; Su et al., 2008). The DHPS gene has also been a target in real-time PCR (Alvarez-Martinez et

al., 2006) and more recently the multicopy major surface glycoprotein (MSG) gene of *P. jirovecii* has improved the performance of real-time assays as discussed below (Larsen et al., 2002; Larsen et al., 2004).

As with microscopy, PCR assays have been used on both lower and upper respiratory tract samples. The motivation for these studies was that if PCR had good sensitivity and specificity in oropharyngeal washings, it could be a useful substitute in situations where sputum induction or fiberoptic bronchoscopy is either unavailable or contraindicated. The utility of DNA amplification to detect *P. jirovecii* in oropharyngeal washes in adult patients was described by Wakefield (1993) who reported a sensitivity of 56% in comparison to microscopy with methenamine silver staining of bronchoscopic lavage samples. A similar study in Kwa-Zulu Natal (Nyamande et al., 2005) assessed the diagnostic utility of PCR as a rapid screen for *P. jirovecii* DNA in non-invasive oropharyngeal samples from adult patients and compared their results to the gold standards of histology and microscopy on BAL fluid, unlike Wakefield et al. (1993) who only compared their results to microscopy on BAL. The sensitivity of DNA amplification using oropharyngeal washes in this study was found to be 44% and 40% (Nyamande et al., 2005). Prior to this, no study had compared the sensitivity and specificity of PCR on oropharyngeal washes to histology of BAL fluid.

Nyamande's study showed that the use of primers targeting the gene encoding the large subunit mitochondrial ribosomal RNA of *P. jirovecii* had a low sensitivity and specificity when compared with microscopy on lung tissue or microscopy on BAL fluid. The questions raised by these results are whether the target was appropriate, or whether oropharyngeal washes are actually poor specimen types for detection of *P. jirovecii*. There are other genetic loci for which PCR primers are available which may have given better results. The conclusion drawn from this study was that PCR is still useful and that refinements to the methods of sample collection, storage, and laboratory techniques may result in higher sensitivities.

Another study using a standard nested PCR protocol targeting the mitochondrial 23S rDNA region of *P.* in oral washes from HIV-positive adult patients, yielded a sensitivity of 78% (Tsolaki, et al., 1999). This is certainly lower than that of PCR coupled with Southern-

or dot-blot hybridization applied to BAL samples, which is usually higher than 95% (Tamburrini, et al. 1998), indicating that both the amplification method as well as the method of detecting amplification products, plays a large part in determining the performance of the PCR assay.

In 2004, Larsen et al. used a quantitative touch-down PCR assay targeting the multicopy major surface glycoprotein (MSG) gene of *P. jirovecii* on oral-wash samples from HIV-infected adults to demonstrate a sensitivity of 88% and a specificity of 85%. He compared this to direct microscopy of induced sputum or BAL by use of Diff-Quik (Dade Behring) and concluded that PCR of oral-wash samples can be an accurate and noninvasive method for diagnosis of PCP. Touch-down PCR is a method by which primers avoid amplifying non-specific sequences by the use of higher annealing temperatures, above the projected melting temperature. The annealing temperature is decreased by 1°C every cycle until a "touchdown" annealing temperature is reached. This specific touchdown temperature is subsequently used for the remaining cycles (Larsen et al., 2002).

Alvarez-Martinez (2006) reported a sensitivity as high as 90% using touch-down PCR. In this study, both nested and quantitative real-time PCR methods for the amplification of the *P. jirovecii* dihydropteroate synthase (DHPS) gene were evaluated using a variety of stored clinical lower respiratory samples from children and adults in Spain, South Africa, and Brazil. The sensitivities of both assays was high, ranging from 62.5 - 100% depending on the type of specimen that was analyzed. In a subset of 71 microscopically confirmed PCP cases and 70 negative cases, there was no significant differences in sensitivity between real-time and nested PCR (both were 94%), however, the specificity of real-time PCR was significantly higher than that of nested PCR (96% vs. 81%; $p = 0.015$). The carryover problem in nested PCR could perhaps explain the high number of false-positive results and this risk of contamination and consequently lower specificity is one of the most important problems of nested PCR. Real-time PCR methods offer other advantages in addition to decreased contamination rates, such as rapidity and dynamic detection. In this study, the results were reported as being obtained in less than 3 hours compared with more than 12 hours required by nested PCR.

The issue of *Pneumocystis* colonization has recently been a topic of interest. Colonization may be defined as isolation of a microbe that does not result in sufficient damage to cause clinical disease but that may alter host homeostasis (Calderon et al., 2010). For *Pneumocystis,* in particular, colonization may be the explanation when there is detection of the organism or its DNA in respiratory samples from individuals who do not have signs or symptoms of pneumonia. Among adults, *Pneumocystis* colonization has been demonstrated in both HIV-infected and non–HIV-infected individuals. Certain groups appear to have a higher risk of colonization. These include, older individuals who do not have predisposing illnesses, and those who are not immunosuppressed but have chronic lung disease. These colonized individuals may be a major species-specific reservoir of infection (Maskell et al., 2003; Calderon et al., 2010)

In the study by Durand-Joly (2005), patients with negative microscopy results, positive touch-down PCR on BAL and negative PCR in oral washes, the absence of PCP was confirmed clinically and colonization was concluded. Since real-time PCR allows for quantification of the organism, it may be possible to differentiate colonization from infection based on organism load. Larsen et al. (2002) developed a rapid (time to completion <4 hours, including DNA extraction), quantitative touch-down real-time assay to study this. The assay utilized an associated internal control and fluorescence resonance energy transfer (FRET) probes for detection. The touch-down procedure significantly increased the sensitivity of the assay compared to a non-touch-down procedure. *P. jirovecii* DNA was detected in respiratory specimens from patients with PCP and from patients without clinical evidence of PCP. These latter patients were thought to be either colonized or to have subclinical infection. They hypothesized that quantification might prove helpful in distinguishing between infected and colonized patients: the latter group would have lower copy numbers than patients with PCP. A blinded, retrospective study of 98 respiratory samples (49 lower respiratory tract specimens and 49 oral washes), from 51 patients with 24 episodes of PCP and 34 episodes of other respiratory disease, was conducted. PCR-positive samples from colonized patients contained lower concentrations of DNA than samples from PCP patients. The data obtained from that study suggested that this quantitative PCR could be used to determine if *P. jirovecii* is present in respiratory samples and to help distinguish between colonization and

infection. Cut-off values representing the difference between colonization and infection would therefore improve the specificity of the assay. Unfortunately, no reliable cut-off values have been determined to date.

Problems identified and the need for further research

The analysis had a number of limitations. Many of the studies were not clinically up to date but were included because of the paucity of literature on the subject of incidence and diagnostics. The true incidence of PCP in South Africa may have increased with the progression of the HIV-pandemic and limited access to health care facilities or decreased since the advent of PMTCT and the wider use of chemo-prophylaxis.

One problem in many of the earlier trials was the use of different microscopic methods, performed in different laboratories, as the "gold standard", making comparisons between studies difficult. The different staining methods themselves could have varying sensitivities and specificities. In addition, the reference standards previously used did not have high sensitivities.

Methodological issues also arose. Some studies may not have been adequately powered to determine significant differences between tests. Logistical problems such as storage, freezing, and thawing of samples or poor calibration of equipment may have affected the accuracy of some of the diagnostic tests. Several factors also threatened the internal and external validity of the studies. Some of these factors had to do with the design of the studies, others with the selection of patients, the execution of the tests, or the analysis of the data. When analyzing the results of these diagnostic studies, the possibility for bias had to be considered, specifically, publication bias where there seemed to be the preferential publication of studies that show improved detection of PCP infection versus those that show no effect.

In conclusion, PCP is a common AIDS-defining infection in infants in South Africa, with a high case fatality rate in children with untreated PCP. A rapid laboratory diagnosis is thus essential in the management of these patients and a rapid diagnostic test using non-

invasively collected specimens would facilitate the laboratory diagnosis in resource-limited settings and allow early initiation of appropriate treatment, thereby improving patient outcome. Although sputum induction has been successfully used to diagnose PCP in HIV-infected adults using microscopy, this technique has not commonly been used in children and not diagnostically evaluated in infants. Use of less invasive samples, such as nasopharyngeal aspirates would be an advantage in children as the procedure risk is minimal and it is quick and simple to perform compared with sputum induction and BAL, which requires specialized equipment and expertise. In adults, PCR analysis of oropharyngeal aspirates has been described as useful for diagnosis although reported sensitivities range from 40-80%. The efficacy of PCR for diagnosis of PCP in children, however, has not been well studied. Data from developed countries indicate that the peak incidence of paediatric PCP occurs before 1 year of age. Further trials are thus needed in a representative paediatric population using methodology and techniques that can be implemented in high-burden, developing countries, like South Africa.

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PART C: MANUSCRIPT

TITLE PAGE

Title:

The molecular diagnosis of *Pneumocystis* pneumonia in children using nasopharyngeal aspirate samples

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Keywords: *Pneumocystis* pneumonia (PCP); *Pneumocystis jirovecii*; children; polymerase chain reaction; nasopharyngeal aspirate

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ABSTRACT

Background: *Pneumocystis* pneumonia (PCP) is a major cause of hospitalization and mortality in HIV-infected children in Africa. Diagnosis relies predominantly on silver or immunofluorescent staining of a lower respiratory tract specimen, which may be difficult to obtain.

Objectives: (1) To compare the diagnostic yield of PCR with immunofluorescence (IF) and (2) to investigate the usefulness of upper compared to lower respiratory tract samples for diagnosing PCP in children.

Methods: Children hospitalised at an academic hospital with suspected PCP were prospectively enrolled between November 2006 and August 2008. An upper respiratory sample (nasopharyngeal aspirate, NPA) and a lower respiratory sample (induced sputum, IS or bronchoalveolar lavage, BAL) were submitted for real-time PCR and direct IF for the detection of *Pneumocystis jirovecii*.

Results: 202 children (median age 3.3 [interquartile range, IQR 2.2 - 4.6] months) were enrolled. The overall detection rate by PCR was higher than by IF [180/349 (52%) vs. 26/349 (7%) respectively; p<0.0001]. PCR detected more infections compared to IF in lower respiratory tract samples [93/166 (56%) vs. 22/166 (13%); p<0.0001] and in NPAs [87/183 (48%) vs. 4/183 (2%); p<0.0001]. Detection rates by PCR on upper (87/183; 48%) compared with lower respiratory tract samples (93/166; 56%) were similar (OR, 0.71; 95% CI, 0.46 - 1.11).

Conclusion: Real-time PCR is more sensitive than IF for the detection of *P. jirovecii* in children with PCP. NPA samples may be used for diagnostic purposes when PCR is utilised. Wider implementation of PCR on NPA samples is warranted for diagnosing PCP in children.

Abstract Word Count: (249 words)

TEXT

INTRODUCTION

Pneumocystis pneumonia (PCP), caused by *Pneumocystis jirovecii,* is an important opportunistic infection in HIV-infected children. [1, 2] The incidence of PCP in developed countries has declined since the introduction of highly active anti-retroviral therapy and use of chemoprophylaxis. However, PCP remains a major cause of hospitalization and mortality in HIV-infected children in low or middle income countries, [1, 3-5] with reported incidence rates of 10 – 49%, [1, 3, 6] and in-hospital case-fatality rates of 20 – 63%. [1, 3-4, 6] Apart from HIV infection, there are other factors that predispose children to developing PCP including malnutrition, other immune deficiencies or HIV exposure. Untreated, the case fatality rate in children with PCP approximates 100%. [1, 3-4, 6] However, the diagnosis can be difficult as clinical and radiological findings are non-specific. Therefore, a rapid, accurate laboratory diagnosis is important for timely use of appropriate medication.

Detection of *P. jirovecii* is hampered by the lack of a sustainable *in-vitro* culture method. [7] Standard laboratory diagnostic methods are microscopic examination of a lower respiratory tract sample with Gomori Grocott's methenamine silver nitrate stain or the more sensitive immunofluorescence assay (IF) [8] on bronchoalveolar lavage (BAL) or induced sputum (IS) specimens. Using microscopy, the yield from IS has been reported to be similar compared to that from BAL. [9] However, sputum induction in children is not widely performed, requires staff trained to do the procedure and may result in clinical

deterioration or nosocomial transmission of respiratory pathogens. Diagnosis using a non-invasive sample such as a nasopharyngeal aspirate (NPA) is, therefore, desirable.

The clinical sensitivity from a NPA has been reported to be low and variable when microscopy is utilised. [4-5, 10] The development of polymerase chain reaction (PCR) techniques has provided a more reliable diagnostic method. In adult studies, PCR is as specific and more sensitive than microscopy for diagnosis, when performed on respiratory specimens, including oral washes. [7, 9, 11-20] In a study of oropharyngeal washes from HIV-infected adult patients, *P.jirovecii* DNA-amplification had a sensitivity of 44% using a nested PCR protocol compared to trans-bronchial biopsy, [15] increasing to 90% when touch-down real-time PCR was utilised. [7, 9] Real-time PCR also allows for quantification of the organism load and with application of cutoff values, could improve the specificity by distinguishing between colonization and infection. [12]

The aims of this study were (1) to compare a real-time quantitative PCR assay with IF for the diagnosis of PCP in children and (2) to evaluate the reliability of PCR for the diagnosis of PCP in upper compared to lower respiratory tract secretions.

METHODS

Participants

Consecutive children (<14 years old) with suspected PCP, hospitalized at Red Cross War Memorial Children's Hospital, Cape Town, South Africa, were enrolled from November 2006 to August 2008. Clinical criteria for suspected PCP were an acute onset of a respiratory illness, presence of age-specific tachypnoea and hypoxia, bilateral lung disease (not associated with wheezing) and a risk factor for PCP (HIV-infected, HIV-exposed, malnourished, receiving immunosuppressive therapy or immunodeficiency disease other than HIV). Patients were excluded if they had received treatment for PCP in the preceding 2 weeks or were on PCP treatment for more than 48 hours. A child was defined as HIV-infected if they had a positive HIV PCR (Amplicor HIV-1 DNA test version 1.5, Roche Diagnostics GmbH, Mannheim, Germany) if younger than 18 months or a positive HIV ELISA (Architect HIV Ag/Ab Combo ELISA, Abbott Laboratories, Abbott Park, IL) in older children. HIV exposure in infants less than 18 months was defined as being HIV seropositive with a negative HIV PCR. All children were treated according to a standard protocol for severe pneumonia that included intravenous cotrimoxazole and oral corticosteroids, as well as broad-spectrum antibiotics. Other antimicrobial therapy was added at the discretion of the attending clinician. Written informed consent was obtained from a parent or legal guardian. The study was approved by the Research Ethics Committee of the Faculty of Health Sciences at the University of Cape Town.

Sample collection

An upper respiratory tract sample (NPA) and a lower respiratory tract sample (IS in non-intubated patients or BAL in intubated patients) were obtained using standardised methods. [10, 21] After routine laboratory investigations had been performed, an aliquot of sample was frozen at -70°C for analysis by PCR.

Control group

Respiratory samples (NPA or BAL) from 30 children hospitalized with a confirmed viral lower respiratory tract infection, and who improved clinically without specific treatment for PCP, were investigated with PCR for PCP, as a control group.

Laboratory investigations

Direct immunofluorescence (IF) (Detect IF PC, Axis-Shield Diagnostics, Cambridgeshire, United Kingdom) to detect *P. jirovecii* using a monoclonal antibody immunofluorescent stain was performed according to the manufacturer's instructions. In addition, Grocott's methenamine silver nitrate stain was performed on BAL specimens, as per clinician's request, if sufficient sample volume was available.

DNA was isolated using the Nuclisens EasyMAG platform (bioMérieux, Boxtel, Netherlands). *P. jirovecii* DNA was detected using a quantitative, touch-down, real-time PCR assay, targeting the major surface glycoprotein (MSG) gene as described by Larsen *et al.* [12] Commercially synthesized primers, amplifying a 250-bp segment of the multicopy MSG gene family, were utilized in a PCR reaction containing fluorescence resonance energy transfer (FRET) probes for detection of the amplified product [12].

The *P. jirovecii* MSG gene was cloned into the pCR 2.1 vector. Four external standards of the cloned target template, corresponding to 10^6, 10^5, 10^4 and 10^3 copies per microlitre, were used in each PCR run to generate an external standard curve, required for quantification. The reactions were performed on the LightCycler platform (Roche Diagnostics GmbH, Mannheim, Germany) and results were calculated using the LightCycler software and expressed in copies/mL. A *P. jirovecii* positive and a negative control were included in each PCR run. Laboratory testing was conducted in an ISO-accredited molecular laboratory of the National Health Laboratory Service (NHLS) at Groote Schuur Hospital, Cape Town. The investigators who performed the PCR testing were blinded to the IF or silver stain results.

Data Analysis

Statistical analysis was performed using statistical software Stata (version 10.0, StataCorp, Houston, USA). Continuous data was tested for normality using the Shapiro-Wilk test. Detection rates of PCR were compared by Pearson's chi-squared test and quantification results were compared by Wilcoxon rank-sum (Mann-Whitney) test. Paired analysis of continuous and binary variables was performed using t test and chi-squared test, respectively.

RESULTS

Patient characteristics

212 children were enrolled (Table 1); 10 patients were excluded as there was insufficient respiratory sample left for PCR, thus 202 children were included in this analysis. Of these, 92 (46%) were male; the median (IQR) age was 3.3 (2.2 - 4.6) months. HIV status was determined in 200 (99%) patients, of whom 129 (65%) were HIV-infected. Of the 71 (35%) HIV-uninfected children, 32 (45%) were HIV-exposed. Twenty-seven (21%) of the 129 HIV-infected children were on cotrimoxazole prophylaxis at presentation. The median (IQR) CD4 percentage of HIV-infected children was 16.9 (10.1 - 27.1)

Table 1. Baseline characteristics of children admitted to hospital with suspected *Pneumocystis jirovecii* pneumonia

Characteristic	All Patients (n = 202)	Patients with positive PCR for PCP (n = 110)
Male	92 (46%)	49 (45%)
Median age, IQR, months	3.3 (2.2-4.6)	3.4 (2.7-3.9)
HIV positive	129/200 (65%)	92/109 (84%)*
Median CD4 percentage**, IQR, %	16.9 (10.1-27.1)	13.6 (9.0-18.0)
Cotrimoxazole prophylaxis**	27 (21%)	10 (11%)

* Odds-ratio OR, 7.9; 95% CI 3.9-16.3 (PCR positive vs. HIV positive); p < 0.0001

** In HIV-infected children

PCR and IF on patient samples

349 respiratory samples were obtained. One hundred and forty-seven (73%) children had paired samples consisting of a NPA with either a BAL or IS. Fifty-five children produced a single respiratory sample (Table 2).

Table 2. Sources of the 349 samples from 202 patients included in the study

	Unpaired Samples	Paired Samples		Total Samples
		NPA+IS	NPA+BAL	
NPA	36	92	55	183
IS	8	92	-	100
BAL	11	-	55	66
Total Samples	55	184	110	349

URT, upper respiratory tract; LRT, lower respiratory tract; NPA, nasopharyngeal aspirate; IS, induced sputum; BAL, bronchoalveolar lavage

Real-time PCR performed on 349 respiratory samples detected *P. jirovecii* in 180 specimens (52%) (Table 3). Of the 202 patients, 110 (54.5%) had one or more specimens positive by PCR. Overall, *P. jirovecii* DNA was detected in 37 of 66 (56%) BAL specimens, 56 of 100 (56%) IS and 87 of 183 (48%) NPA. There was no significant difference between the detection by PCR on lower respiratory tract samples compared to NPA (odds-ratio OR, 0.71; 95% CI, 0.46 – 1.11; Table 3).

Table 3. Quantitative PCR and Direct IF performed on 349 respiratory samples collected from 202 patients

	TOTAL (n = 349)	URT (n = 183)	LRT (n = 166)	OR (95% CI)
Positive PCR	180 (52%)	87 (48%)	93 (56%)	0.71 (0.46 - 1.11)
Positive IF	26 (7%)	4 (2%)	22 (13%)	0.15 (0.04 - 0.45)

URT, upper respiratory tract; LRT, lower respiratory tract; OR, Odds-ratio; CI, confidence interval; PCR, polymerase chain reaction; IF, immunofluorescence

In contrast, *P. jirovecii* was detected by IF in 26 (7%) of samples, consisting of 13 (50%) BAL, 9 (35%) IS and 4 (15%) NPA samples. All 26 IF-positive samples were positive on PCR and thus no additional cases were detected using IF. The overall yield on upper tract samples was 48% (87/183) by PCR, as compared to the yield of 2% (4/183) by IF ($p < 0.0001$). For lower tract samples, the yield from PCR was 56% (93/166) compared to 13% (22/166) by IF ($p < 0.0001$).

P. jirovecii was also demonstrated on silver stain in 8 of the 25 (32%) BAL samples sent for analysis. All 8 were also PCR positive, and 3 were IF positive. Of the 17 patients with a negative BAL silver stain, 5 (29%) were PCR-positive and 3 were also IF positive.

Comparison of yield by PCR on paired upper and lower respiratory tract samples

Of the 147 paired upper and lower respiratory tract samples, 70 pairs were concordant PCR-positive, 63 pairs were concordant PCR-negative and 14 pairs had discordant PCR

results. Of the discordant pairs, most were PCR positive on the lower tract sample but negative on the upper tract sample (Table 4). Overall, the detection from upper and lower specimens by PCR was similar with 81 of 147 (55%) lower respiratory tract samples positive by PCR vs. 73 of 147 (50%) NPA samples (OR, 1.4; 95% CI, 0.91-2.19). Only 8 (5%) additional cases were detected by PCR when performed on a lower respiratory tract sample compared to a NPA specimen (p = 0.11). Using a positive PCR result on a lower respiratory tract sample as a gold standard, the sensitivity, specificity, positive predictive value and negative predictive value for PCR on a NPA sample was 86%, 95%, 96% and 85% respectively.

Table 4. Real-time PCR results on paired upper and lower respiratory tract samples (n=147)

	LRT Sample (IS/BAL) Positive	**LRT Sample (IS/BAL) Negative**	Total
URT Sample (NPA) Positive	70 (48%)	3 (2%)	73 (50%)
URT Sample (NPA) Negative	11 (7%)	63 (43%)	74 (50%)
Total	81	66	147

URT, upper respiratory tract; LRT, lower respiratory tract; NPA, nasopharyngeal aspirate; IS, induced sputum; BAL, bronchoalveolar lavage

Concentrations of *P. jirovecii* DNA ranged from 3.2 to 9.4 log copies/mL. The median

(IQR) organism load detected in NPA samples was significantly lower than that detected in lower respiratory tract samples [5.9 (5.4 - 6.7) log copies/ml compared to 6.6 (5.8 - 7.5) log copies/mL; p = 0.0002).

Control group

Of the 30 children in the control group, 18 (60%) were male; the median (IQR) age was 6.0 (4.0 – 12.0) months. HIV status was determined in 21 (70%) patients, of whom 2 (10%) were HIV-infected, 6 (28%) were HIV-exposed and 13 (62%) were HIV-unexposed. Twenty-eight (93%) of 30 control samples were PCR-negative. The 2 PCR positive samples (both NPA samples), were from HIV-unexposed infants, younger than 6 months old, with DNA concentrations of 6.1 and 6.7 log copies/mL.

DISCUSSION

This study found that real-time PCR has a much higher sensitivity than the current standard diagnostic test of IF, with more than double the number of children diagnosed with PCP using PCR. The different staining methods, namely IF and silver stain, against which PCR was compared, have variable reliability especially when used on a NPA. However, these staining methods are the current standards for diagnosis. When IF on lower tract samples was used as the reference standard, the sensitivity of the PCR assay was 100%. The large proportion of IF-negative, PCR-positive samples suggests that the sensitivity of IF is poor; and the PCR results are likely to reflect true positive cases of PCP as children had severe respiratory illness, in the context of a known risk factor for PCP. It is therefore difficult to comment on specificity of PCR, as there is no 'gold standard' for comparison. The increase in diagnostic yield using PCR occurred in both upper and lower respiratory specimens, but was especially marked in samples from the upper respiratory tract. Therefore, using current methods of detection, PCP may be substantially under-diagnosed, accounting for the relatively low laboratory confirmation rate in clinical studies of approximately 20%. [1, 3]

A second advantage of PCR is that an upper respiratory tract specimen was as reliable as a lower respiratory tract specimen for diagnosis. This study provides good evidence that in children, a diagnosis of PCP can be reliably made using PCR on a specimen obtained non-invasively from the upper respiratory tract. Obtaining a NPA is much easier than performing sputum induction, and carries a lower risk of nosocomial transmission of infection, thus making the diagnosis of PCP feasible in many health-care settings

including in primary care. The detection of organism in an upper respiratory tract sample may be explained by infection extending to these airways in severe disease or by organisms that are propelled to the upper airway during coughing. [15]

A further advantage of PCR is the rapidity of the test, with results obtainable within hours. Children with PCP are often seriously ill with a rapidly progressive course, thus timely diagnosis allows early initiation of treatment, with potential reduction in mortality. The usual management for patients with clinically suspected PCP is empiric treatment with co-trimoxazole and corticosteroids. Corticosteroids have been reported to reduce mortality in HIV-infected adults with PCP when initiated early; thus rapid confirmation of the diagnosis is also useful for initiating early corticosteroid therapy, especially as there may be concern about the use of corticosteroids in other infections such as CMV pneumonitis. [22, 23]

A potential difficulty with PCR is distinguishing colonization from disease due to *P. jirovecii.* The organism has been increasingly reported to colonize the airways of adults with underlying chronic obstructive airway disease, with such individuals forming the reservoir for infection of susceptible hosts. [24] Moreover, positive PCR results have been reported in BAL samples from adults without clinical PCP, HIV, or other infections. [25] However, in the context of a severely ill, immunocompromised child with diffuse pneumonia and hypoxia, as occurred in the study patients, it is likely that a positive PCR represents disease. In addition, *P. jirovecii* infection in young children with immune compromise usually results in primary infection, with resultant severe illness rather than colonization (as may occur in adults who may then develop reactivation disease later).Of

the 30 control children, only 2 tested positive by PCR. These 2 positive samples may represent true infection or disease, as primary infection is very common in children with most infected within the first few years of life. A further explanation may be laboratory contamination. However, the molecular laboratory layout in our institution is designed to allow unidirectional work-flow with strict separation of all steps involved in the PCR process. The real-time PCR was performed in a closed system, with detection occurring on the same platform thus minimizing the risk of contamination.

The quantitative results obtained in our study could not be accurately interpreted as data was not normalized by use of a reference gene as an internal control. Normalization would, in theory, control for variations in the extraction process, patient variability and inter-sample variability by accounting for the dilution factor in the BAL fluid. Unfortunately, although there is an awareness of the importance of this component in a quantitative PCR assay, there is still some debate about the choice of the most appropriate reference gene. [26] If normalization could be reliably performed, it may allow for more accurate estimation of colonization rates and perhaps the determination of cut-off values thus improving the specificity of the assay.

Potential difficulties in achieving widespread implementation of PCR for PCP diagnosis include cost and the need for a specialized molecular laboratory, equipment, and personnel. However, much progress has been made in developing closed molecular amplification platforms for the diagnosis of TB at point-of-care facilities. Given the scale of the HIV pandemic and the substantial mortality associated with PCP particularly in children, a similar approach for the diagnosis of PCP, should be undertaken.

In summary, this real-time PCR-based assay has a much higher sensitivity than current diagnostic tests and allows for the use of a NPA sample for reliable detection of *P. jirovecii* in children. PCR on a NPA should replace the traditional diagnostic method of IF or silver stain performed on lower respiratory tract samples. Widespread implementation of this technology is needed especially in high burden HIV and resource limited settings.

ACKNOWLEDGEMENTS

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PART D: APPENDICES

- Patient Information and Consent Form
- "Instructions for Authors" from THORAX
- Ethics Approval Letter

PATIENT INFORMATION AND CONSENT FORM

***Pneumocystis jirovecii* pneumonia in children – diagnosis & emergence of resistance**

You and your child are requested to participate in a medical research study that is being done at Red Cross Children's hospital. This study is being done by a team of researchers in the Department of Paediatrics and Child Health of the University of Cape Town. The following information will describe the study and your child's role as a participant. Please read this carefully and feel free to ask any questions. The study will be conducted according to the Declaration of Helsinki.

Background

Your child has been hospitalised for a chest infection. This may be caused by a germ called *Pneumocystis jirovecii* which can cause a severe chest infection or pneumonia (PCP) in children. The aim of this study is to do a number of tests for PCP to investigate whether your child does have PCP. Your child will be treated for PCP with a medicine called **cotrimoxazole (Bactrim).** Sometimes, other germs especially viruses can occur at the same time as PCP, causing a severe chest infection. Cytomegalovirus (CMV) is a germ that often causes infection with PCP. We will also therefore check whether your child has any other germ in their chest or blood, as some of these germs must be treated with additional medicines.

What is the reason for the study and how will it be done?

The aim of this study is to find out the cause of your child's chest problem, particularly to test for PCP. In order to check for PCP, we will do a number of tests, which will be sent to the laboratory for testing for this germ. We will also do additional tests for other germs especially to test for a virus called cytomegalovirus (CMV). The tests that will be done as part of this study are:

1) A sample of mucus from the chest or from the lungs (bronchoalveolar lavage, if your child is so ill that he/ she needs a tube to breathe) will be taken. To get a sample of mucus from the chest, a breathing treatment to open your child's lungs will be given, after which your child will be nebulised with very salty water. After this, mucus will be suctioned out. To do bronchoalveolar lavage, a few millilitres

of salt water will be put down the breathing tube and then suctioned back. These tests are usually done in children who may have PCP and are part of the usual way in which we look for PCP in children with chest infections. The mucus will also be sent to check for CMV. This is the usual way in which we check for germs causing a bad chest infection

2) A sample of mucus from the nose will be taken by doing a nose swab. To do the nose swab we will put a few drops of salt water in the nostrils and then gently suction it out. The mucus samples are sent to the laboratory to check for germs in the mucus. This will include checking for PCP and for CMV. A sample of the mucus specimens may also be stored, if there is mucus left over after testing for the germs. The stored mucus will be used should a better test for PCP become available in the future
3) A blood test will be done to check if your child is making blood cells (antibodies) to protect him/ her against PCP. Less than half a teaspoonful of blood will be taken for this test. This test may be repeated during the time your child is in hospital
4) A blood test will be taken to test for CMV. Less than half a teaspoonful of blood will be taken for this test.

What does the study mean for your child?

We will look at your child's hospital folder for her/his medical information and results of tests that have been done. Your child will receive the usual investigations and treatment in hospital; in addition, study tests as listed above will be done.

What are the possible benefits to your child?

From this study, we may be able to be more certain about whether your child has PCP or not, thus ensuring that he/ she gets the necessary treatment. We will also be more certain whether your child has CMV. Your child will be treated with a medicine for CMV if the tests show that CMV is causing his/ her illness.

The study may also benefit other children with chest infections, as we hope it will help us to find the best test for PCP, which can then be used for other children who may have PCP.

What are the possible risks to your child?

This study poses very little risk to your child. Besides the usual blood tests, a small amount of additional blood (less than half a teaspoon) will be drawn to check for antibodies for PCP and for

CMV. Your child may have more coughing or develop a small amount of nose bleeding or get a tight chest when the nurse is obtaining a sample of mucus from his / her lungs. If your child develops a tight chest then we will give him / her a breathing treatment to open the chest. If your child develops nose bleeding, then we will push on the nose and apply ice to stop this.

Confidentiality

Your child's study records will be kept confidential. Neither you nor your child's name will appear in any publication that may arise from this study.

Voluntary participation

You may choose for your child to be in this study. If you choose not to be in the study then your child will get regular treatment for his/ her chest infection including treatment for PCP. Being in the study will not affect any other treatment that your child will receive.

I have read and understood this form. My questions have been answered. I voluntary consent to have my child participate.

I, _____, the parent/ legal guardian of _____ agree to allow her/him to participate in this study.

Signed: _____ Witness: _____

Date: _____ Date: _____

Patient sticker:

Instructions for Authors

Editorial policy

Thorax seeks to publish significant advances in scientific understanding which are likely to impact on clinical practice. Articles concerning clinical topics, critical care, and those on basic mechanisms with application to clinical material, will be welcomed. We aim to cover all areas of respiratory medicine (epidemiology, paediatrics, immunology, pharmacology, pathology, surgery and critical care) through publishing original papers, editorials, reviews, case reports and images. The priorities are originality and excellence.

All submissions are subject to peer review. All papers that are potentially acceptable undergo statistical analysis.

Submissions to Thorax, once peer reviewed, are discussed at the weekly editorial committee prior to decision. We aim to ensure a fair and independent peer review system and to publish articles which follow the highest ethical standards concerning research conduct.

Open access/Unlocked articles

Authors are able to make their articles freely available online, immediately on publication, for a fee, using the Unlocked service. This service is available to any author publishing original research in a BMJ Journal for a fee of £1,700(+VAT)/€2,515(+VAT)/$3,145.

Article types and word counts

- Original Research
- Case Reports
- Pulmonary Puzzles
- Images in Thorax
- Reviews
- Letter to the editor (original research)
- Correspondence
- Editorials
- Lung alerts
- Supplements

The word count excludes the title page, abstract, tables, acknowledgements and contributions and the references.

Original Research

Full papers should follow the basic structure of abstract, introduction, methods, results, discussion, references, and tables and figures as appropriate. They should not normally exceed 250 words for the abstract, 3000 words for the content and include no more than 35 references. There is an online data repository for extra information, tables, figures and appendices.

Manuscript format

All manuscripts must be submitted via Bench>Press.

All material submitted is assumed to be submitted exclusively to the journal unless the contrary is stated. Submissions may be returned to the author for amendment if presented in the incorrect format.

If you are submitting a randomised controlled trial, please send with your manuscript the following:
The registration number of the trial and the name of the trial registry - in the last line of the paper's structured abstract. Trials that begin enrolment of patients after 1 July 2005 must register in a public trials registry at or before the onset of enrolment to be considered for publication. Trials that began patient enrolment on or before 1 July 2005 must register before 13 September 2005 to be considered for publication. Please see the Statement from the International Committee of Medical Journal Editors.

Cover letter

Your cover letter should inform the Editor of any special considerations regarding your submission, including but not limited to:

1. Details of related papers published or submitted for publication.

    - Copies of related papers should be submitted as supplementary data to help the Editor decide how to handle the matter.

2. Details of previous reviews of the submitted article.

    - The previous Editor's and reviewers' comments should be submitted as supplementary data along with your responses to those comments. Editors encourage authors to submit these previous communications and doing so may expedite the review process.

Whether any of the material could be published as data supplements rather than in the print version of the article.

Title page

The title page **must** contain the following information:

1. The title.
2. The name, postal address, e-mail, telephone and fax numbers of the corresponding author.
3. The full names, institutions, city and country of all co-authors.
4. Up to five keywords or phrases suitable for use in an index (it is recommended to use MeSH terms).
5. Word count - excluding title page, abstract, references, figures and tables.

Manuscript format

The manuscript format must be presented in the following order:

1. Title page
2. Abstract (or summary for case reports)
3. Main text (tables should be in the same format as your article and embedded into the document where the table should be cited; images must be uploaded as separate files)
4. Acknowledgments, Competing interests, Funding
5. Copyright licence statement
6. References
7. Appendices

Do not use the automatic formatting features of your word processor such as endnotes, footnotes, headers, footers, boxes etc.

Provide appropriate headings and subheadings as in the journal. We use the following hierarchy: **BOLD CAPS, bold lower case**, Plain Text, *Italics*.

Cite illustrations in numerical order (fig 1, fig 2 etc) as they are first mentioned in the text.

Tables should be in the same format as your article and embedded into the document where the table should be cited.

Images **must not** be embedded in the text file but submitted as individual files (view further details in File Formats.)

Filenaming convention

Where possible, please name your manuscript and image files as shown below. (Please note: the manuscript ID # appears at the top of each submission page as soon as you start your submission; author refers to the corresponding author's last name.)

1. Your manuscript file should be named as: **yr_manuscript id number_author**
(for example: 2005_001234_clark)

2. Your image file should be named as: **yr_manuscript id number_F#**
(for example: 2005_001234_F1)

Statistics

Statistical analyses must explain the methods used.
Guidelines on presenting statistics.
Guidelines on RCTs: CONSORT, QUORUM, MOOSE, STARD, and Economic submissions.

Style

Abbreviations and symbols must be standard and SI units used throughout except for blood pressure values which are reported in mm Hg.

Whenever possible, drugs should be given their approved generic name. Where a proprietary (brand) name is used, it should begin with a capital letter.
Acronyms should be used sparingly and fully explained when first used.

View more detailed style guidelines.

Figures/illustrations

Black and white images should be saved and supplied as **GIF, TIFF, EPS or JPEG** files, at a **minimum resolution of 300 dpi** and an image size of 9 cm across for single column format and 18.5 cm for double column format.

Colour images should be saved and supplied as **GIF, TIFF, EPS or JPEG** files, to a **minimum resolution of 600 dpi** at an image size of 9 cm across for single column format and 18.5 cm for double column format.

Images should be mentioned in the text and figure legends should be listed at the end of the manuscript.

During submission, when you upload the figure files please label them as Figure 1, Figure 2, etc. The file label will not appear in the pdf but the order in which the figures uploaded should be sufficient to link them to the correct figure legend for identification.

We can accept multi-page Powerpoint files. Alternatively, Powerpoint files can be saved as JPEG files and submitted as a standard image file.

Histograms should be presented in a simple, two-dimensional format, with no background grid.

Please note: Do not submit colour figures unless you are willing to pay the cost of publishing your figures in colour. If you do not wish to pay the colour charges please submit your figures in black and white.
The journal charges authors for the cost of reproducing colour images on all unsolicited articles. This charge is heavily subsidised by the journal and covers origination costs only. If an image is supplied as a **composite figure** that contains numerous parts (for example, fig 1A-D), the image will be considered as a single image, provided that all the parts are supplied within a single file that prints out at an overall size no larger that A4 (210 mm x 297 mm). The charge for **colour** processing will be **£100 + VAT** for the figure. Multi-part colour images supplied as **separate files will be charged at £100 + VAT for each file**. The charge only applies to images accepted for print publication and not online only or data supplement files.
Care should be taken in planning composites because combining different images with widely varying colours can lead to contamination or loss of colour and poor quality results. When submitting your manuscript, please ensure to include a name and address where the invoice should be sent for the colour reproduction costs. If an address is not included, the invoice will be sent to the corresponding author.

Unacceptable file formats
Any file using OLE (Object Linking and Embedding) technology to display information or embed files, Bitmap (.bmp), PICT (.pict), Photoshop (.psd), Canvas (.cnv), CorelDRAW (.cdr); Excel (.xls); and locked or encrypted PDFs are not acceptable.

Tables

Tables should be submitted in the same format as your article and embedded into the document where the table should be cited. Please note: Bench>Press **cannot** accept Excel files. If your table(s) are in Excel, copy and paste them into the manuscript file. In extreme circumstances, Excel files can be uploaded as supplementary files; however, we advise against this as they will not be acceptable if your article is accepted for publication.

Tables should be self-explanatory and the data they contain must not be duplicated in the text or figures.

References

Authors are responsible for the accuracy of references cited: these should be checked against the original documents before the paper is submitted. It is vital that the references are styled correctly so that they may be hyperlinked.

In the text

References must be numbered sequentially as they appear in the text. References cited in figures or tables (or in their legends and footnotes) should be numbered according to the place in the text where that table or figure is first cited. Reference numbers in the text must be given in square brackets immediately after punctuation (with no word spacing) - for example, .[6] not [6].

Where more than one reference is cited, separate by a comma - for example, [1, 4, 39]. For sequences of consecutive numbers, give the first and last number of the sequence separated by a hyphen - for example, [22-25]. References provided in this format are translated during the production process to superscript type, which act as hyperlinks from the text to the quoted references in electronic forms of the article.

In the reference list

References must be double spaced (numbered consecutively in the order in which they are mentioned in the text) in the [slightly modified] Vancouver style. Only papers published or in press should be included in the reference list. (Personal communications or unpublished data must be cited in parentheses in the text with the name(s) of the source(s) and the year. Authors should get permission from the source to cite unpublished data.)

Punctuation of references must follow the [slightly modified] Vancouver style:

12 Surname AB, Surname CD. Article title. Journal abbreviation. Year;Vol:Start page-End page.

Use one space only between words up to the year and then no spaces. The journal title should be in italic and abbreviated according to the style of Medline. If the journal is not listed in Medline then it should be written out in full.

Check journal abbreviations using PubMed.

List the names and initials of all authors if there are 3 or fewer; otherwise list the first 3 and add et al.

Example references:

Journal
13 Koziol-Mclain J, Brand D, Morgan D, et al. Measuring injury risk factors: question reliability in a statewide sample. Inj Prev 2000;6:148-50.

Chapter in book
14 Nagin D. General deterrence: a review of the empirical evidence. In: Blumstein A, Cohen J, Nagin D, eds. Deterrence and incapacitation: estimating the effects of criminal sanctions on crime rates. Washington, DC: National Academy of Sciences 1978:95-139.

Book
(personal author or authors) (all book references should have specific page numbers)
15 Howland J. Social norms and drunk driving countermeasures. In Graham JD, ed. Preventing automobile injury: new findings from evaluative research. Dover, MA: Auburn House Publishing Company 1988:163-96.

Abstract/supplement
16 Roxburgh J, Cooke RA, Deverall P, et al. Haemodynamic function of the carbomedics bileaflet prosthesis [abstract]. Br Heart J 1995;73 (suppl 2):P37.

Electronic citations
Basically, websites are referenced with their URL and access date, and as much other information is given as is available. Access date is important as websites can be updated and URLs change. The "date accessed" can be later than the acceptance date of the paper, and it can be just the month accessed. See the 9th edition of the AMA Manual of Style for further examples.

electronic journal articles:
Morse SS. Factors in the emergency of infectious diseases. Emerg Infect Dis 1995 Jan-Mar;1(1). www.cdc.gov/nciod/EID/vol1no1/morse.htm (accessed 5 Jun 1998).

Use as much information as the author gives. The volume/number information in the URL will take the user to the start of the individual document; ask the author to supply or confirm. Also ask authors to supply the date they accessed the file.

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AFTER the article has appeared in an issue
Use the citation format:
Sabin MA, Ford AL, Holly JMP, Hunt LP, Crowne EC, Shield JPH. Characterisation of morbidity in a UK, hospital based, obesity clinic. Arch Dis Child 2006; 91:126-130 doi:10.1136/adc.2005.083485 [published Online First: 24 October 2005].

Electronic Letters
Author. Title of letter. Journal name Online [eLetter] Date of publication. url

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1. Vole P, Smith H, Brown N, et al. Treatments for malaria: randomised controlled trial. Ann Rheum Dis 2003;327:765-8 doi:10.1136/ard.2003.001234 [published Online First: 5 February 2004].

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UNIVERSITY OF CAPE TOWN

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22 September 2009

REC REF: 387/2009

Dr CM Samuel
Medical Microbiology

Dear Dr Samuel

PROJECT TITLE: MOLECULAR DIAGNOSIS OF PNEUMOCYSTIS JIROVECII PNEUMONIA IN CHILDREN.

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted for one year till the 30th September 2010.

1. Amendment to 363/2006 is approved
2. In future please set up a research registry for stored samples

Please submit an annual progress report if the research continues beyond the expiry date. Please submit a brief summary of findings if you complete the study within the approval period so that we can close our file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

Wesley Manley

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

PP

Federal Wide Assurance Number: FWA00001637.

S Thomas

Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

S Thomas