Original Article

Molecular Detection of *Pneumocystis Jirovecii* in Patients with Respiratory Tract Infections

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Abstract

Background: *Pneumocystis jirovecii*, formerly known as *P. carinii,* is an opportunistic fungus causing *Pneumocystis carinii* pneumonia especially in immunocompromised patients. **Aim:** The aim of this study was to detect *P. jirovecii* in sputum samples from patients suspected of having respiratory tract infections. **Materials and Methods:** In this study, 230 acid fast bacilli negative sputum samples from 230 patients presenting with respiratory tract infections submitted to three teaching hospitals' medical microbiology laboratories in Osun and Oyo States, Nigeria for routine investigation were examined for *P. jirovecii* by microscopical and polymerase chain reaction methods. **Results:** *P. jirovecii* cysts were observed in 15 (6.5%) samples and polymerase chain reaction was positive for 29 (12.6%) samples out of 230 samples examined. It was observed that the detection of *P. jirovecii* was associated with age ($P < 0.05$) while there were no associations between diagnosis, sex, and prevalence of *P. jirovecii* ($P > 0.05$). Polymerase chain reaction was showed to be a better method for the detection of *P. jirovecii* based on the 51.7% sensitivity and 100% specificity of the microscopy. **Conclusion:** The study concluded that *P. jirovecii* is prevalent in patients with respiratory tract infections in hospitals from the southwestern part of Nigeria and should be included in diagnosis of these infections in this part of the world.

Keywords: Molecular identification, Nigeria, *Pneumocystis jirovecii (carinii)*, polymerase chain reaction

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Introduction

Pneumocystis jirovecii formerly called *Pneumocystis carinii* was once referred to as protozoan until recently classified as fungi due to overwhelming evidence from 16S ribosomal RNA sequencing, mitochondrion genome sequence, and amino acids sequence of some of the proteins.[1,2] The organism profile increased as pathogen at the beginning of human immunodeficiency virus (HIV) epidemics in the late 1980s as a result of immunocompromised nature of the people infected with HIV.[3] Prior to this, it has been described as an opportunistic pathogen in children with primary immune disorders and patients using immunosuppressive drugs where it caused pneumonia.[4] Depletion of CD4 T cells is a hallmark of HIV infection, and was recognized early in the acquired immune deficiency syndromes (AIDS) to correlate with the development of *Pneumocystis* pneumonia.[5] For example, HIV infection has been found to be associated with impaired phagocytosis, respiratory burst, and inflammatory activation of alveolar macrophages in response to *Pneumocystis*.[6] The infection is very serious in immunocompromised hosts resulting in multiplication of *Pneumocystis* in the alveoli lung as a result impedes meaningful gaseous exchange with dire consequence of death. Although this assumption has been challenged,[7] host immunity has been shown to be responsible to the damage to lungs.[8]

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Epidemiologically, in the United States, it was estimated that 75% of HIV-infected persons would develop *Pneumocystis* pneumonia during their lifetime.[9] The introduction of *Pneumocystis carinii* pneumonia (PCP) prophylaxis in 1989 with potent antiretroviral drugs in 1996 has reduced the number considerably. In Europe, a study undertaken about 10 years ago showed that

the incidence of *Pneumocystis* declined from 4.9 cases per 100 person-years before March 1995 to 0.3 cases per 100 person-years after March 1998.[10] Statistics on prevalence of *Pneumocystis* has been scanty for other regions or not available. The worrying trend is increasing reported cases in low middle income country; a study from Uganda reported that 38.6% of 83 HIV-infected patients who were admitted to the hospital with pneumonia and who had three expectorated sputum smears that were negative for acid fast bacilli had *Pneumocystis* pneumonia diagnosed on bronchoscopy with bronchoalveolar lavage (BAL).[11] No data on the prevalence of *P. jirovecii* are available in Nigeria in people living with HIV or patients with suspected lower respiratory tract infections (RTI). To compound the problem, the clinical significance of *Pneumocystis* infection is not cleared as this organism has been detected in non-HIV patients as colonization of the lower respiratory tract.[12] It was in view of these that this study was undertaken. This study was aimed at determining the prevalence of *Pneumocystis jirovecii* in sputum samples from patients suspected of having respiratory tract infections in southwestern of Nigeria.

Materials and Methods

This is a cross-sectional study of sputum samples from patients suspected with respiratory tract infections submitted to Medical Microbiology laboratories in teaching hospitals in Oyo and Osun states, Nigeria for routine investigation.

Sample collection

In this study, 230 acid fast bacilli negative sputum samples submitted in sterile universal containers to the Medical Microbiology laboratories of the University College Hospital (UCH), Ibadan Oyo State, Obafemi Awolowo teaching hospital (OAUTH), Ile- Ife, Osun state and Osun State Teaching hospital, Asubiaro, Osogbo, Osun State, from patients suspected of RTIs from January to June 2009 were examined for *P. jirovecii* infection by microscopical and molecular methods as described below. Two hundred and thirty samples were from 230 patients with 98 males and 132 females. The samples analyzed were from patients with less than a year old to 85 years old of age with mean age of 37.5 years, median and mode age of 35 years, with standard error of 1.7 years and 95% confidence level of 3.21. Samples positive for acid fast bacilli were excluded from the study in order to determine the true prevalence of *P. jirovecii* in patients with pneumonia and not tuberculosis and also from the health and safety point of view. Ethical approval was given by the Department of Biomedical Sciences, Ladoke Akintola University of Technology, Osogbo, Nigeria before embarking on this study.

Microscopy

Smears were made in duplicate from the acid fast bacilli negative sputum samples on a clean grease free slide, air dried, and fixed with methanol. A set of these were stained and examined by Toluidine blue-O staining according to a standard laboratory manual.[13] The stained smears were viewed using oil immersion objective of the microscope (×100). The other set, following fixation with methanol were stained with a Romanowsky stain Giemsa (1 in 20 dilution) for 30 minutes following standard laboratory manual.[13] Smears were rinsed with clean water and slides were air dried. *P. jirovecii* cysts were viewed using oil immersion objective of the microscope (×100).

DNA extraction

DNA extraction was carried out on the samples as described by Nakamura.[14] Sputum samples (~2 ml) were mucolysed by adding 10 µl of 1 M Dithiothreithol (DTT), 2 ml of distilled water and mixed, incubated at 37°C for 10 minutes. About 1.5 ml portions of the mucolysed samples were dispensed using micropipette into 1.5 ml Eppendorf tubes and centrifuged at 2,000 × g for 5 minutes at room temperature. The pellets formed were washed twice using 1 ml of distilled water. Fifty microliters of the digestion buffer was added and mixed by pipetting up and down using a micropipette with filter tips and placed in a 60°C water bath for 6 hours. The digests were boiled at 100°C for 10 minutes and snap cooled on ice. These were centrifuged at 12,000 × g for 1 minute.

Optimization of the PCR condition

Single round PCR was carried out using optimum concentration of the 5S ribosomal DNA oligonucleotide primers and amplification was performed in a thermal cycler with a three-step cycling program: 30-second denaturation at 94°C followed by a 2 minutes annealing at 51°C and a 30-second elongation at 72°C for a total of 40 cycles in a 30 µl volume reaction.

Optimization was carried out on varying amounts of primer concentration with a three-step cycling program: 30-second denaturation at 94°C followed by a 2 minutes annealing at 53°C and a 30-second elongation at 72°C for a total of 40 cycles involving diluted concentrations of the primers to allow 1\2, 1\4, 1\8 dilutions (i.e. 0.25 µM, 0.125 µM, and 0.063 µM) and the optimum concentration of the primer (0.50 µM) in a 30 µl volume reaction. Another round of amplification was performed by adding 1.5 mM to 3.0 mM magnesium chloride (MgCl2) in order to decrease the stringency of primer binding using a three-step cycling program: 30-second denaturation at 94°C followed by a 2 minutes annealing at 53°C and a 30-second elongation at 72°C for a total of 40 cycles per 30 µl volume reaction.

PCR condition

Based on the optimization result, PCR master mix was made to allow for a 30 µl reaction per tube. For a typical 10 reaction, PCR master mix contained 30 µl of 10 µM solution of the forward primer {AGTTACGGCCATACCTCAGA} and 30 µl of 10 µM solution of the backward primer {GTGTACGTTGCAAAGTACTC}, 60 µl taq master mix polymerase and 160 µl volume of PCR water. Twenty-eight microliters of the master mix was aliquoted into PCR tubes and 2 µl of the DNA template was added and mixed. Amplification was performed in a thermal cycler with a three-step cycling program: 30-second denaturation at 94°C followed by a 2 minutes annealing at 53°C and a 30-second elongation at 72°C for a total of 40 cycles. Precautions were taken to avoid PCR contamination by ensuring strict physical barrier methods and use of multiple negative controls. Electrophoresis was done using the 1% agarose gel slab in 0.5 × Tris borate EDTA buffer (44.5 mM Tris-borate and 1 mM EDTA, pH 8.3). Ten microliters of the PCR product was mixed with loading buffer (containing 0.25% bromphenol blue, 0.25% xylene cyanole and 40% sucrose) and loaded into the wells on the agarose gel. DNA ladder was also loaded to one end of the gel using a micropipette. Electrophoresis was done at 70 V for 90 minutes. Bands were visualized with short wave ultraviolet transilluminator and photographs were taken using a syngene gel documentation system (Syngene, UK).

Detection of PCR inhibition

To detect inhibition, amplification was performed using PCR products as DNA template including both positive and negative controls, another round of amplification was repeated with a three-step cycling program: 30-second denaturation at 94°C followed by a 2 minutes annealing at 53°C and a 30-second elongation at 72°C for a total of 40 cycles in a 30 µl volume reaction by adding 2 µl of the PCR product in 28 µl of the PCR master mix.

Statistical analysis

Collation of data were carried out using Epi-info from Centre for Disease control and prevention, USA. Data were analyzed using statistical package within the Epi-info software. A chi-square test was used to determine the effect of sex, age, hospital location, and diagnosis on the data obtained. The *P* value less than 0.05 was considered to be significant.

Results

In this study, two hundred and thirty (230) acid fast bacilli negative sputum samples were examined from patients suspected of respiratory tract infection. These patients were between the ages of 4 months and 75 years, 98 were males, and 132 were females. One hundred and forty eight (148) samples were obtained from the University College Hospital (UCH), Ibadan, 42 sputum samples were obtained from Osun State Teaching Hospital (OSTH), Osogbo, and 40 samples from Obafemi Awolowo University Teaching Hospital Complex (OAUTHC) Ile-Ife. Of these, 167 were samples suspected of having lower respiratory tract infection; 40 were from immunosuppressed patients; 8 samples from chronic obstructive pulmonary disease (COPD); and 15 samples from other diagnoses.

Using the Giemsa staining technique, no cyst was seen. Rather, slides were congested with numerous and nonspecific structures. Following Toluidine blue-O staining, characteristic cysts were observed with oil immersion objective (×100). Fifteen (6.5%) out of the 230 samples were positive for microscopy while the remaining 215 (93.5%) sputum samples showed no cysts. Of those positive for microscopy, 9 out of 132 (6.8%) of the samples were from females and 6 (6.1%) out of 98 from males. Four (57.14%) were from the samples obtained from UCH, Ibadan, 2 (28.5%) from OAUTHC, Ile-Ife and 1 (14.29%) from OSTH, Osogbo. It was observed that most of the patients from whom cysts were seen had chronic infections such as chronic obstructive pulmonary disease (COPD), immunosuppressed and some were suspected of pulmonary tuberculosis or lower respiratory tract infection.

On optimization, PCR carried out using the 5S ribosomal DNA oligonucleotide primers and amplification performed in a thermal cycler with a three-step cycling program: 30-second denaturation at 94°C followed by a 2 minutes annealing at 51°C and a 30-second elongation at 72°C for a total of 40 cycles gave non-specific bands and no amplification of the 120 bp product was visible at this condition. Amplification performed with a three-step cycling program: 30-second denaturation at 94°C followed by a 2 minutes annealing at 53°C and 30-second elongation at 72°C for a total of 40 cycles gave a stable and specific band at the expected 120 bp thus subsequent amplifications were performed using this parameters. On amplification, using oligonucleotide primers specific for 5S rDNA; all negative controls (water and master mix) and positive controls (sputum samples) showed appropriate amplification (i.e. positive control showed amplification and the negative control showed none). Sequencing of the 120 bp products indeed confirmed the amplification of part of the 5S rDNA of *P. jirovecii*. The limit of detection for DNA was found to be 0.1 ng of DNA which was equivalent to 10^3 *Pneumocystis*. Twenty nine (13.0%) out of the 230 samples after accounting for PCR inhibition gave a specific band corresponding to 120 bp with an annealing temperature of 53°C using the primer targeting the 5S ribosomal DNA

(rDNA) [Figure 1]. PCR inhibition was evidenced in this study but this was surmounted with the appropriate dilution of samples. Ten of the 29 positive samples were initially inhibited in PCR. The primers did not amplify 5S ribosomal DNA (rDNA) from other pathogens involved in pulmonary disease such as *Candida spp* and other forms of yeast. All samples were checked for evidence of internal PCR inhibition by making a tenfold dilution of the DNA extract. A total of 29 PCR positive samples were detected out of the 230 sputum samples examined, thus 12.6% prevalence. The number of positive samples detected included the 15 samples equally detected by TBO method with additional 14 samples. The specificity and sensitivity of TBO (microscopy) were 100% and 51.7%, respectively using PCR as the "gold" standard. PCR was found to be more superior to microscopy; hence the rest of the results were described using PCR. Fourteen (14.3%) of the 98 males were positive for PCR for *P. jirovecii* while 16 (12.1%) of the 132 females were positive for PCR for *P. jirovecii*. There was no association between sex and pneumocystis infection in this study ($P > 0.05$; $\chi^2 = 0.08$). The distribution of *P. jirovecii* according to diagnosis showed that three main clinical diagnoses highly featured in this study as shown in Figure 2. *P. jirovecii* was highly prevalent in COPD - 2 (25%) out of 8; followed by immunosuppressed patients – 8 (19%) out of 41, and lower respiratory tract infections which included diagnoses such as pneumonia constituted 11.5% (19 were positive for *P. jirovecii* out of 167). *Pneumocystis* was not detected in samples from patients with persistent cough, asthma, upper respiratory tract infections, and suppurative lung disease. Statistically, there was no association between diagnosis and pneumocystis infection ($P > 0.05$; $P = 0.14$; $\chi^2 = 5.46$) in this study. OAUTHC accounted for the highest (15.0%) prevalence (6 out of 40 samples processed) with OSUTH responsible for the lowest (9.5%) prevalence (4 out of 42 samples processed) of *P. jirovecii* [Figure 3]. There was no association between location of the hospitals and pneumocystis infection ($P > 0.05$; $P = 0.73$; $\chi^2 = 0.62$). The study also examined the effect of age on the prevalence of *P. jirovecii*. Figure 4 shows that the age group between 0 and 10 years old accounted for the highest (36.4%) relative frequency (i.e., 8 positives out of 22 samples examined) of *P. jirovecii* while the lowest frequencies were recorded for 11-20, 41-50, and 71-80 age groups. Pneumocystis infection was not equally distributed in this study ($P < 0.05$; $P = 0.0036$; $\chi^2 = 21.11$). No linear trend was observed with respect to different age groups ($P > 0.05$; χ^2 for linear trend = 2.63).

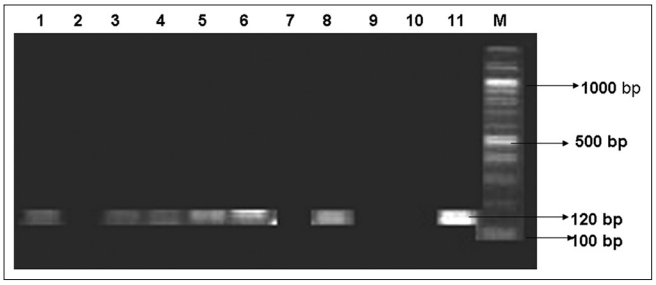

Figure 1: PCR for detection of P. Jirovecii from clinical samples. Representative agarose gel electrophoresis of PCR products after PCR for detection of P. Jirovecii. Lanes 1-9: samples, lane 10- Negative control; Lane 11: positive control; lane M: 100 bp DNA ladder size marker; and arrow indicating the expected 120 bp PCR product

Discussion

Choosing the right diagnostic method for diagnosis can go a long way in improving patients' prognosis. This study was conceptualized in order to determine the prevalence of *P. jirovecii* from acid fast bacilli negative sputum samples in this environment using the most sensitive technique available. In this study, two methods of diagnosing PCP (microscopy and molecular method such as PCR) were used to determine the presence of *P. jirovecii* in sputum samples submitted to three teaching hospitals in southwestern part of Nigeria. Carry-over contaminations were reduced by strict physical separation of all various steps and employing unidirectional work flow in PCR protocols.

Microscopic examination using a Romanowsky stain, Giemsa (1 in 20 dilution) as described by a standard laboratory manual[13] showed undefined structures and no cysts were identifiable from the congested slides. This is in agreement with Fishman's finding who reported difficulty in identification of cysts in Giemsa stained slides.[15] Using Toluidine blue O (TBO) staining method, specific and defined but scanty cysts were observed in 15 samples from 230 sputum samples examined

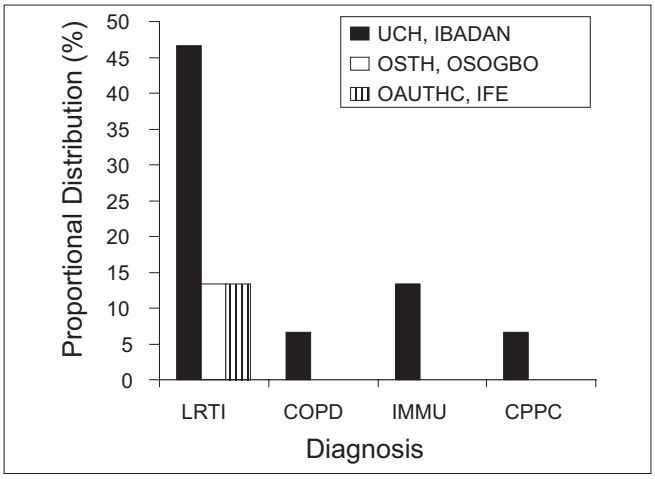

Figure 2: The distribution of *P. jirovecii* in sputum samples from patients with respiratory tract infection according to location and clinical diagnosis, LRTI: Lower respiratory tract infection, COPD: chronic obstruction pulmonary disease, IMMU: Immunosuppressive patients, CPPC: Chest pain and persistent cough

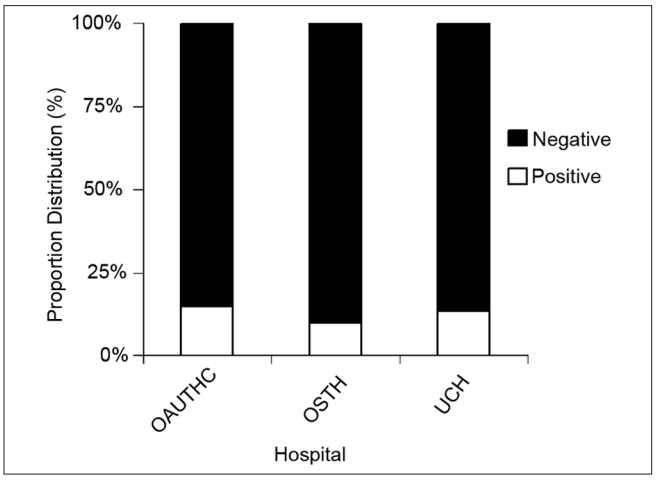

Figure 3: The hospital distribution of *P. jirovecii* in sputum samples from patients with respiratory tract infection

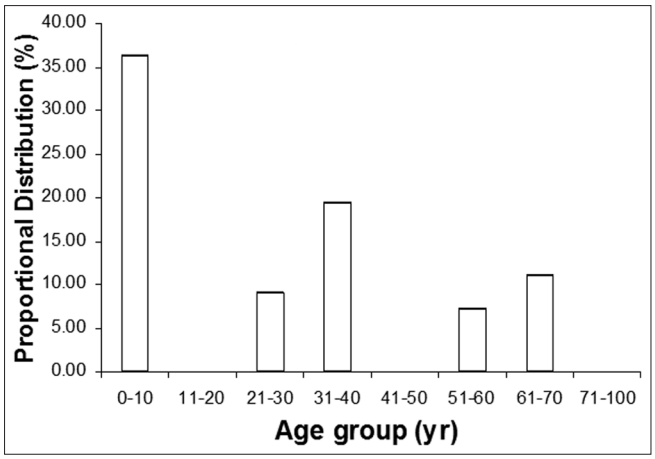

Figure 4: The age distribution of *P. jirovecii* in sputum samples from patients with respiratory tract infection

giving 6.5% prevalence with 51.7% sensitivity and 100% specificity using PCR result as the gold standard.

The PCR methodology adopted was adapted from Kitada *et al*.[16] and Nakamura *et al*.[14] In this study, the optimum condition for the detection of *P. jirovecii* was a three-step cycling program: 30-second denaturation at 94°C followed by a 2 minutes annealing at 53°C and a 30-second elongation at 72°C for a total of 40 cycles as used by Nakamura[14] although with slight modification by lowering the annealing temperature from 55°C to 53°C. At 55°C annealing temperature, no specific band was seen and the expected 120 bp product was not amplified. This slight modification may be due to variations in the climatic conditions and PCR machine used.

PCR detected *P. jirovecii* DNA in 29 (12.6%) out of the 230 sputum samples from patients suspected of respiratory tract infections. Correlating the PCR result with microscopy, all microscopy positive samples were also PCR positives. PCR detected additional 14 samples that were microscopy negative. These data suggest the high sensitivity and specificity of PCR over the microscopy methods used in this study and confirmed other previous studies.[16,17] All PCR negative but microscopy positive samples were re-amplified by making a 10-fold dilution of the DNA template to check for evidence of internal inhibition of the *Taq* polymerase. It was not surprising to find out that some of the samples were internally inhibited. This may be due to the presence of blood, mucus and other inhibitory agents in the samples, as the use of PCR in clinical samples has been shown to be prone to PCR inhibition.[18] It is recommended that an internal standard should be included in the PCR protocol to reduce the risk of false negative result or a 10-fold dilution of template DNA be made before a negative PCR result can be confirmed as true negative.

The use of nested PCR is recommended as this has been shown to increase sensitivity in comparison with the single-round PCR or an optimized single round PCR protocol (touch-down PCR) can be used routinely.[17,19] PCR use in the diagnosis of *P. jirovecii* is highly sensitive and specific than other diagnostic methods even though the pathogen cannot be routinely cultured. PCR detection of *P. jirovecii* in sputum samples offers a good diagnostic technique as this reduces the risk of complications associated with the use of bronchoalveolar lavage. Several studies have evaluated the use of PCR in the detection of *P. jirovecii*.[17,20] Most studies have found that PCR is more sensitive than the conventional staining methods with ability to detect cysts in oral washing, sputum and tracheal aspirates, in which convectional staining has inadequate sensitivity.

The results from this study [Figure 4] pointed to association between age and pneumocystis infection as depicted by higher relative distribution of *P. jirovecii* in 0- to 10-year-old age group ($P < 0.05$). This finding is in line with previous studies that showed that *P. jirovecii* was more prevalent in young children including the one from Africa continent[12,21] and contradicts recent study by Dini *et al*.[22] where they found 93% of *P. jirovecii* infection in age more than 15 years old. The high prevalence in children from previous study has been shown not to have anything to do with CD4+ count from children because the CD4+ counts have been shown to be greater than 1,000 per µl [CDC, 1991 #29]. These individuals may represent a possible reservoir for the circulation of pneumocystis in the community. Age and presence of concurrent disease were associated with the finding of a positive *P. jirovecii* by PCR. Furthermore, this study has found no association between clinical diagnosis and prevalence of *P. jirovecii* infection ($P > 0.05$). Highest relative distribution (25%) of *P. jirovecii* was found in samples from patients diagnosed for COPD, followed by

immunosuppressed (19.1%), and LRTI (11.5%). *P. jirovecii* remains an opportunistic pathogen causing severe pneumonia in immunosuppressed patients, such as those with AIDS[23-25] or undergoing cancer therapy or organ transplantation. It has been estimated that 60% of AIDS patient will suffer from PCP at some time in the course of the diseases.[23] Lower respiratory tract infections cover wide range of infections caused by variety of organisms ranging from *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Chlamydophila pneumoniae*, *Legionella pneumoniae*, to mention a few. Medical microbiology diagnostic laboratory plays important roles in ruling in or out LRTI caused by any of these infectious agents aforementioned. It is possible for clinicians to use differential diagnosis including chest x-ray to diagnose some of these conditions. The confirmation of the diagnosis cannot be overemphasized because of the wide spectrum of organisms responsible for LRTI. Data from this study has supported the need for screening of *P. jirovecii* from this group of patients in order to improve patients' prognosis. The high relative distribution (25%) of *P. jirovecii* in sputum samples from COPD patients in this study has confirmed the role of this agent in this condition as suspected by other researchers.[26-28] Epidemiologically, COPD has been shown to be the fourth leading cause of death in the United States[29] and is expected to rank third in the world by 2020.[30] The significance of this finding to pathology of COPD cannot be underestimated.

Data from this study suggest that *P. jirovecii* is prevalent (13.0%) in the southwestern part of Nigeria, this result is in agreement with a study carried out in Ethiopia by Aderaye *et al*.[24] who reported in 2003 that of 119 outpatients with respiratory symptoms and negative acid fast bacilli cultures, only 11% had PCP, although another study reported by Malin *et al*.,[31] involving a studied group of 64 hospitalized HIV-infected patients in Zimbabwe in 1995, twenty-one (33%) of these patients had PCP. More so, *P. jirovecci* has also been reported in Gambia[12] – a country in the same West Africa as Nigeria. Reasons for a higher rate of PCP among these patients included use of definitive diagnosis and probable selection bias by including only patients with severe pneumonia when other diagnoses, such as tuberculosis had been excluded. These disparities in the prevalence rate may be due to the low sensitivity and specificity of the diagnostic method used, i.e. Immunofluorescent and TBO staining respectively, which are not as sensitive and specific as PCR. Further studies on the colonization and the possible pathogenic role of *P. jirovecii* in the etiology of disease in patients with or without HIV may be needed. It is worthwhile to say something on the applicability of this study to Africa countries or developing countries. PCR technology can easily be adapted to this environment because the enzyme can withstand the rigor of climatic change as well as epileptic power supply experience by many of these countries. The cost of doing it might be slightly expensive but this can easily be accommodated if the political will is there or the policy makers take note of the advantage this technique has over microscopy method. Of course this may also requires manpower development. There are various workshops on PCR Technology organized by universities in Africa in addressing the issue of human capacity development.

The study concluded that *P. jirovecii* is prevalent (13.0%) in patients with respiratory tract infection in hospitals from the southwestern part of Nigeria; and based on the sensitivity and specificity of the polymerase chain reaction (PCR) over microscopy methods used in this study, PCR will offer a good diagnostic method for the detection of *P. jirovecii* and it is recommended for diagnosis of *P. jirovecii* in patients with respiratory tract infection irrespective of the patient's immune status.

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