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RESEARCH ARTICLE

MOLECULAR METHODS FOR THE DIAGNOSIS OF PNEUMOCYSTIS JIROVECII IN IMMUNOCOMPROMISED IRAQI PATIENTS

AZHAR A. F. IBRAHIM[1], ISA S. TOUHALI*[1], HAIDER N. DAWOOD[2]

[1]Dept. of Microbiology, College of Medicine, Al-Nahrain University, Iraq, [2]Dept. of Respiratory, Medical AL- Imammian AL- Kadhmain City teaching Hospital / Baghdad, Ministry of health, Iraq.**Email:**isaswadi75@gmail.com

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ABSTRACT

Objective: The study were to: Determine the possible implication of Pneumocystis jirovecii in a sample of immunocompromised Iraqi patients and determine the prevalence of Dihydropteroate synthase gene related to treatment and prophylaxis failures. **Methods:** This study included a total of two hundred (n=200)clinical samples from 100 immunocompromised patients as 70 Bronchoalveolar lavage , 21sputum samples and 9 pleural fluids, with underlying different diseases. Control group included 100 samples(50 Bronchoalveolar lavage, 30 sputum samples and 20 pleural fluids) from immunocompetent individuals from immunocompetent individuals were collected from of in-and out patients who attended of Medical AL- Imammian AL- Kadhmain City teaching Hospital / Baghdad, Baghdad teaching Hospital / Baghdad, Al-Zahra teaching Hospital / Wasit province and other private Laboratories, during the period from May-2014 to March-2015. The detection pneumocystosis by molecular methods. **Result:**The organism was detected in bronchoalveolar lavage, and/or sputum of immunocompromised patients only. The highest detection rate was observed for the mtLSUrRNA gene in 19 (19%) samples, 15 (15%) of these samples of immunocompromised patients consist of DHPS genes**. Conclusion:** Pneumocystis jirovecii, is the fundamental opportunistic infection among immunocompromised patients. The primers target of mtLSUrRNA has the most specific and sensitive for the detection of P. jirovecii. In the present study showed the DHPS gene has served as a selective drug linked with sulfa resistance and PCR amplification target.

Keywords: Pneumocystis jirovecii(carinii), Dihydropteroate synthase (DHPS) gene, immunocompromised Iraqi patients.

INTRODUCTION

Pneumocystis jirovecii (previously known as *Pneumocystis carinii*) is an unusual opportunistic organism, *Pneumocystis jirovecii* most commonly causes *Pneumocystis* pneumonia (PCP) in patients with AIDS and patients receiving intensive or prolonged immune suppressive treatment for malignancy, transplantation and immune disorders [1-3]. which causes a severe and often fatal pneumonia in immunocompromised individuals [4]. The organism has a unique tropism for the lungs, where it exists primarily as an alveolar pathogen. Individuals with intact immunity control this primary infection, there are no apparent clinical manifestations of primary infection in immunocompetent individuals, and the organism likely remains latent in the lungs for long periods of time, clinically apparent pneumonia occurs when cellular or humoral immunity becomes severely deficient, the organisms proliferate, evoking a mononuclear cell response, alveoli become filled with proteinaceous material and intact and degenerating organisms [5-6]. *Pneumocystis jirovecii* inability to culture suggests that it has evolved to require a very specific environment that is not easy to reproduce outside its host [7]**.** The diagnosis of *P. jirovecii* disease requires the demonstration of cysts or trophozoites within tissue or body fluids via colorimetric or immunofluorescent stains since the human organism cannot be cultured in *vitro* and *vivo* [8]. More recently, molecular detection assays have been developed for detection *P. jirovecii* in bronchoalveolar (BAL) or induced sputum samples, polymerase chain reaction (PCR) have used a variety of gene target such as mitochondrial rRNA, PCR are still in development currently the diagnosis of PCP relies on [9]. *Pneumocystis*-PCR gains more and more importance in the diagnosis of a *Pneumocystis*-infection [10]. *Pneumocystis* is less studies in Iraq and because the mimicry of such disease with other pulmonary infection we tried to determine the incidence of such disease. The aims of this study were to: Determine the possible implication of *Pneumocystis jirovecii in* a sample of immunocompromised Iraqi patients and determine the prevalence of Dihydropteroate synthase gene (DHPS) related to treatment and prophylaxis failures.

MATERIALS AND METHODS

Patient's selection

This study included a total of two hundred (n=200) clinical samples from 100 immunocompromised patients (43 men, 57 women; average age 16-90 years) as 70 Bronchoalveolar lavage (BAL), 21sputum samples and 9 pleural fluids, with underlying different diseases; As 22 (22%) leukemia, 17(17%) solid tumor, 15(15%) lymphomas, 12(12%) chronic obstructive pulmonary disease(COPD), 10(10%) asthma(steroid therapy), 10(10%) rheumatoid arthritis(cytotoxic therapy), 8(8%) solid-organ transplantation and 6(6%) Multiple myeloma with suspected of pneumocystosis. Control group included 100 samples(50 Bronchoalveolar lavage (BAL), 30 sputum samples and 20 pleural fluids) from immunocompetent individuals were collected from of in and out patients who attended of Medical AL-Imammian AL- Kadhmain City teaching Hospital / Baghdad, Baghdad teaching Hospital / Baghdad, Al-Zahra teaching Hospital / Wasit province and other private Laboratories, during the period from May-2014 to March-2015. The ethical aspects of this study have been approved by the ethical council in Medical college, Al-Nahrain University.

Samples collection

Bronchialveolar lavage(BAL)

Were performed by a bronchofibroscope (STORZ,Germany) wedged in segmental orifice of sedated spontaneous breathing patients or intubated patients, in most cases,20-50 ml warmed saline was infused into targeted segment followed by gentle suction by specialist physician. BAL fluids were directly collected by sterile syringe. About 10-15 ml were dispensed into sterile test tube and immediately placed on ice then transmitted into the laboratory for processing.

Induced sputum samples (IS)

Were obtained by induction in patients involved in the study. Sputum induction was done using an ultrasonic nebulizer (SN 2000, England) this was done in an open space using a 3ml saline as an inducing fluid, from each induced patient by nurse practitioner, this sputum sample(10-15ml) was directly collected by sterile screw cup bottles and immediately placed on ice then transmitted into the laboratory for processing. Induced sputum were divided into two portions and treated with either 0.1% Dithiothreitol or with 0.9% NaCl alone.

Pleural effusion samples (PE)

This method was done by aspirating pleural fluid with 25G needle after marking, cleaning the suspected area with antiseptic and

then local anesthetic (5-10 ml of 2% lidocaine) was injected locally, this method was done by specialist physician.Ten-15ml pleural fluid was aspirated into sterile test tube and immediately placed on ice then transmitted into the laboratory for further processing.

Samples processing

BAL, sputum and pleural fluid containing mucous martial were added to a 2-fold volume of 0.9% NaCl and were mixed vigorously vortexes for 5 minutes. Samples centrifuged at 3000 rpm for 5 minutes, supernatants were discarded and the precipitated pellets were placed into a 1.5 ml microcentrifuge tubes [11]. A portion of precipitate pellets (200µl) were stored at -20 for DNA extraction and amplification.

Molecular methods for diagnosis

DNA Extraction fluids

DNA was extracted from 200 µl body fluids using DNA isolation kit (QIAamp® DNA blood Mini Kit 50) x5 reaction (QIAGEN,Germany) according to [9] and manufacturer information.

Determination of genomic DNA concentration and purity

The concentration and purity of the purified DNA were quantified by nanodrop instrument, by following the instruction of the manufacturer(Act Gene NAS99) Briefly, 3µl was aspirated using special tips (Aeroject tips 10µl) and inserted in specified socket in the machine, DNA was quantified by the refractive index using the wave length 260nm, 280nm. DNA concentration was calculated with the OD_{260nm} . The purity was estimated with the OD_{260nm}/OD_{280nm} ratio, a ratio of ~1.8 was generally accepted as "pure" DNA, indicating a low degree of protein contamination.

Gel electrophoresis

DNA samples were electrophoresed by horizontal agarose gel electrophoresis according to [12] as follows: Agarose at a concentrations of 2% was prepared, the agarose solution was left to cool at 55°C, then(0.5µl) of ethidium bromide solution was added, Agarose solution poured into the taped plate. A comb was placed near one edge of the gel. The gel was left to harden until it became opaque; each of the comb and tape were removed gently. TBE buffer (1X) prepared was poured into the gel tank and the slab was placed horizontally in electrophoresis tank. About 3 microliters of loading buffer prepared was applied to each 7 µl of DNA sample wells were filled with the mixture by a micropipette, PCR products were directly applied. Power supply was set at (5 V/cm (70) for 1 hr) for genomic DNA and PCR products electrophoresis. When the electrophoresis was finished the gel was exposed to UV light using UV transilluminator and then photographed using digital camera.

Polymerase chain reaction PCR

The specific of oligonucleotide primer sequences were used in conventional PCR to detect the presence of large subunit of mitochondrial ribosomal RNA (mtLSUrRNA) gene was taken from [13-15] Dihydropteroate Synthase (DHPS) gene was taken from [16-17] and human Beta-globin primers was taken from [18] used as an experimental control during protocols of PCR a positive control for confirming the acceptability of the extracted DNA to template, those genes synthesized in Alpha DNA® (Canada) as shown in table no.1-2. DNA template of *Pneumocystis jirovecii* was prepared . The primers (mtLSUrRNA, DHPS and β-globin genes) were diluted by adding nuclease free water according to the manufacturer instructions. The master mix contents were thawed at room temperature before use, and the PCR master mix was made on a separate biohazard safety cabinet with wearing hand gloves at all times to avoid contamination. For each reaction within each single pre-mixed PCR reaction tube, 2µl from each forward primer and reverse primer were added. Five microliter of DNA template was added for each reaction tube. Twelve and a half microliters of GoTaq® Green Master Mix was added for each reaction tube, the volume was completed to 25µl with Deionized Nuclease –Free as shown in table no.3, tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components. PCR mixture without DNA template(non-template negative control) were used as negative control. The tubes were placed on the PCR machine and the PCR program, with the right cycling conditions pre-installed, was started. Cleaver Scientific Thermal Cycler TC32/80 was used for all PCR amplification reactions. The PCR thermocycler program used with mtLSUrRNA and DHPS genes were designed on the basis of published paper as shown in table no. 4-5.

PCR Gel Electrophoresis

Electrophoresis was done as stated earlier**.** Five microliters of the 100bp DNA ladder were mixed with one microliter of blue/orange 6X loading dye and subjected to electrophoresis in a single lane. Served as marker during PCR products electrophoresis. The gel was exposed to UV using UV light transilluminator and then photographed using digital camera (Sony-Japan).

Statistical Analysis

The statistical analysis of data was done by using ANOVA then student's't' test. *P*<0.001 was considered as highly significant.

Table 1: Primers sequences with their relevant product size.

Gene target	Primer name		Primer sequence (5' ⟶ 3')	GenBank Accession No	Product Size(bp)
mtLSUrRNA	pAZ102-E	F	$^{5'}$GAT GGC TGT TTC CAA GCC CA$^{3'}$	Jf733748	346-350
	pAZ102-H	R	$^{5'}$GTG TAC GTT GCA AAG TAC TC$^{3'}$		
DHPS	A_{HUM}	F	$^{5'}$GCG CCT ACA CAT ATT ATG GCC ATT TTA AAT C$^{3'}$		
	B_{HUM}	R	$^{5'}$CAT AAA CAT CAT GAA CCC G$^{3'}$	U66279.1	702-720

Table 2: Primer sequences of Beta-globin gene(Internal control).

Gene target	Primer name		Primer sequence (5' ⟶ 3')	Reference	Product Size(bp)
β-globin	GH20	F	$^{5'}$ GAAGAGCCAAGGACAGGTAC $^{3'}$	[18]	408
	GH21	R	$^{5'}$GGAAAATAGACCAATAGGCAG $^{3'}$		

Table 3: Composition of PCR reaction mixture used for amplification of each mtLSUrRNA, DHPS and β-globin genes.

Gene	Components	Volume /µl	Final concentration
	Green Master Mix 2x	12.5 µl	1x
mtLSUrRNA gene	Forward primer, 10µM	2 µl	0.2 µM
DHPS gene	Reverse primer, 10µM	2 µl	0.2 µM
β-globin gene			
	DNA template	5 µl	
	(DNAse free) water	3.5 µl	
	Total volume	25 µl	

Table 4: The PCR thermo cycler program for mtLSUrRNA gene according to [38].

Steps	Temperature	Time	Cycles
Initial denaturation	94°C	5 min	
Denaturation	94°c	20 sec	
Annealing	56°c	20 sec	40
Extension	72°c	20 sec	
Final extension	72°c	5 min	
Hold	4°c		

Table 5: PCR thermo cycler program for DHPS gene according to [17].

Steps		Temperature	Time	Cycles
Initial denaturation		**94°C**	**3 min**	
	Denaturation	94°c	30 sec	
1	Annealing	52°c	1 min	10
	Extension	72°c	1 min	
	Denaturation	94°c	30 sec	
2	Annealing	42°c	1 min	25
	Extension	72°c	1 min	
Final extension		72°c	5 min	
Hold		4°c		

RESULTS AND DISCUSSION

Descriptive data on study subjects

Among those 100 immunocompromised patients 43 (43%) were males and 57 (57%) were females as shown in table no.6, their mean ages was 54.56 ±16.46 years which ranged from 16 to 90 years, compared with 100 samples from immunocompetent individuals used as a control group as shown in table no.7.

Table 6: Gender distribution among patients and control group.

Gender type	Patients	Control
Female	57(57.0%)	50 (50.0%)
Male	43(43.0%)	50(50.0%)
Total	100(100.0%)	100(100.0%)
p value	0.198*[NS]	

*[NS]: **No-significant difference was demonstrated.**

Table 7: Descriptive data according to the age of patients and control group.

Age	Median	Mean	S.D	Maximum	Minimum
Patients	55.00	54.56	16.46	90.00	16.00
Control	34.00	37.33	13.24	66.00	16.00
*p value	<0.001				

***Significant difference was demonstrated.**

Laboratory Diagnosis of *Pneumocystis jirovecii*

Pneumocystis jirovecii is the causative agent of *Pneumocystis* pneumonia, one of the most frequent and severe opportunistic infections in immunocompromised patients. As *P. jirovecii* cannot be grown in culture from clinical specimens [19-20]. The current laboratory diagnosis of *Pneumocystis* pneumonia has relied mainly upon microscopic techniques. Polymerase chain reaction (PCR) is the more sensitive and specific than the conventional methods for the detection of *P. jirovecii* [21- 22].

Molecular methods for diagnosis of *Pneumocystis jirovecii*

DNA extraction

DNA was extracted from respiratory samples(BAL and or sputum) using DNA isolation kit (QIAamp® DNA blood Mini Kit 50, QIAGEN, Germany), according to [9] and manufacturer information with little modification (when the concentration of the DNA is small, the volumes of Buffer AE or distilled water should be decreased to 50-100 µl.). The final DNA concentration was increased if the elution volume was less than 200 µl. All samples enrolled in this study (n=200) were tested for the presence of genomic DNA by gel electrophoresis as shown in figure no.1. Purity and concentrations of all DNA samples were tested by nanodrop. The purity was ranged from (1.7-2) while the concentrations was ranged from (20-200) ng/ul. No apparent signs of contamination in any of the tested samples have been seen.

Polymerase Chain Reaction (PCR)

Conventional PCR screening for mtLSUrRNA gene

Purified DNA was used as a template to amplify a part of mitochondrial gene encoding for large subunit of rRNA (mtLSUrRNA) by conventional PCR by using specific primer sequences. PCR assays targeting the mitochondrial rRNA region (which is present in high copy numbers) were found to have a higher sensitivity than methods that targeted other gene, the (mtLSUrRNA) most specific and sensitive for the detection of *P. jirovecii* [23]. In this study, it has been found that the mtLSUrRNA gene was present in respiratory samples of immunocompromised patients only, as nineteen samples with a PCR product with a size of approximately 350 bp., while eighty one samples of the immunocompromised patients showed negative results for the presence of this gene as indicated by the absence of the PCR products in their relevant lanes as shown in figure no. 2. In 1996, Wakefield [24] has developed all primers necessary for DNA amplification of *Pneumocyctis jirovecii*. When these primers used in human-oriented samples are ability to detect only *P. jirovecii* DNA but they cannot detect microorganisms in the lung tissues of other mammals.

Conventional PCR screening for DHPS gene

The presence of DHPS gene was also searched for confirming the identity of sulfamethoxazole resistant *Pneumocystis jirovecii* and the results of the present study indicated that , this gene was

successfully amplified in fifteen out of nineteen samples of PCR amplification products of the mtLSUrRNA gene, with a product size of 720 bp, while only four sample lacked this gene and gave negative amplification results as shown in figure no.3. DHPS gene analysis has a major importance for monitoring (SMX-TMP) resistance development, sulfamethoxazole(SMX) inhibits dihydropteroate synthase (DHPS), which is not present in mammals, trimethoprim (TMP) is an inhibitor of dihydrofolate reductase, which leads to the inhibition of the thymidine synthesis pathway and therefore DNA synthesis, both enzymes were involved in the biosynthesis of folic acid. The DHPS gene has served as a selective drug and PCR amplification target [25]. The combination of trimethoprim (TMP) and sulfamethoxazole (SMZ) is the first-line agent for the treatment of mild to severe and prophylaxis of *P. jirovecii* pneumonia, although intravenous pentamidine has been recommended as the main alternative to (TMP-SMX) for moderate to sever pcp a recent study has found a greater risk of death when pentamidine was used as first and second line therapy for pcp as compared with (SMX-TMP) and clindamycin-primaquine these finding could be due to toxicities related to pentamidine and the absence of antibacterial effect in contrast to (SMX-TMP) or clindamycin-primaquine which might act against concomitant bacterial co-infection [26-27]. Experiments in animal models by Walzer *et al.*[28] and kunz *et al*.[29] had suggested that the widespread use of drugs of SMX-TMP in the prevention and treatment of PCP in recent years has been found to be associated with an increase in the prevalence of specific mutations in the gene coding for DHPS. In so far as no routine in *vitro* drug assay is available for *Pneumocystis*, the estimation of *P. jirovecii* potential drug resistance by using molecular methods [30-31].

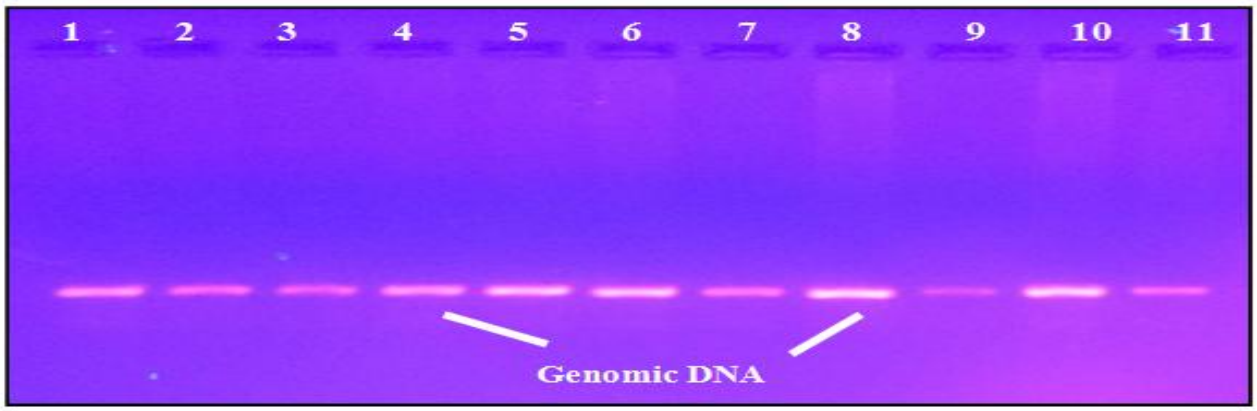

Figure 1: Gel electrophoresis (2% agarose, 7v/cm^2, 1.5hrs) of Genomic DNA; Lane (1-11).

Figure 2: Gel electrophoresis(2% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane2: Internal control(402bp); lane(3,6,8-10): Postive sample for *P. jirovecii* (mtLSUrRNA gene 350bp); lane4-5: Negative sample; lane 7: Negative control.

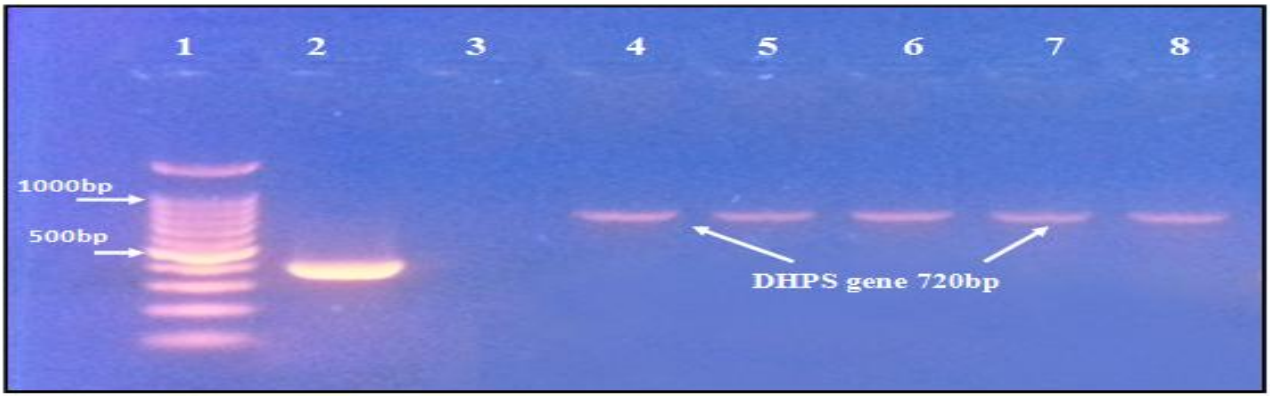

Figure 3: Gel electrophoresis (2% agarose,7v/cm^2,1.5hrs) of the PCR products, lane1(MW): One hundred base pairs DNA ladder; lane2: Internal control(402bp); lane(4-8): Postive sample for *P. jirovecii* (DHPS gene 720bp); lane 3: Negative control.

Overall results of molecular methods

The highest detection rate was observed for the mtLSUrRNA gene in 19 (19%) samples which included the presence of DHPS gene in 15 (15%) samples of immunocompromised patients by PCR reaction as shown in figure no.4, in those patients with leukemia (six patients), solid tumor (four patients), lymphoma (three patients), chronic pulmonary obstructive disease (two patients), and one only sample for each of asthma (steroid therapy), rheumatoid arthritis (cytotoxic therapy), solid-organ transplantation and multiple myeloma disease all of which were positive for mtLSUrRNA gene. The above results reflect the inability to successfully amplify the DHPS gene from all positive mtLSUrRNA P. jirovecii specimens which has been reported by other researchers [32-33]. Robberts *et al*.[23] and Beard *et al*.[34] both concluded that a possible explanation as to why the DHPS methods failed to amplify all mtLSUrRNA positive samples was due to: (a) mitochondrial DNA serves as a specific and reproducible template and (b) multiple mitochondria are present within individual organisms. The DHPS gene is a single copy, nuclear encoded gene, which will inevitably result in a reduction in PCR performance compared to a multicopy gene. Costa *et al*.[25] and Medrano *et al*. [35] reported that the amplification of the DHPS gene has also been hampered by the used of relatively non-invasive pulmonary specimens, which characteristically is associated with a lower fungus load and PCR inhibitors. Other studies by Helweg-Larsen *et al.* [36] and Santos et al. [37] who mention that the *P.jirovecii* dihydropteroate synthase (DHPS) gene showed mutations associated with impaired prognosis, which apparently resulted from exposure to sulfa drugs, as a nonsynonymous DHPS point mutations at nucleotide positions. In the present study probably showed *Pneumocystis* DHPS mutations, this will may lead to a mismatch with the specific primers of the gene and thus not getting amplification of this gene. It is concluded from this study that the *Pneumocystis jirovecii*, is the fundamental opportunistic infection among immunocompromised patients. The primers target of mtLSUrRNA has the most specific and sensitive for the detection of P. jirovecii. In the present study showed the PCR detection of P. *jirovecii* is a very sensitive test and will offer a powerful technique in clinical laboratories for the routine diagnosis of Pneumocystis pneumonia in BAL and sputum specimens and Dihydropteroate synthase (DHPS) gene has served as a selective drug linked with sulfa resistance and PCR amplification target.

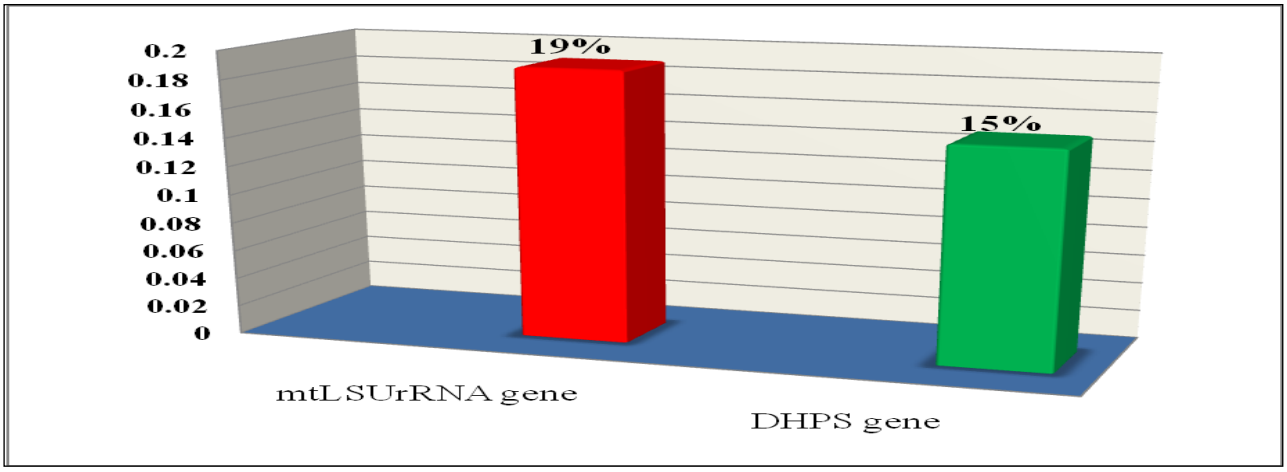

Figure 4: *Pneumocystis jirovecii* detection used PCR screening for mtLSUrRNA and DHPS genes.

CONCLUSION

It is concluded from this study that the *Pneumocystis jirovecii*, is the fundamental opportunistic infection among immunocompromised patients. The primers target of mtLSUrRNA has the most specific and sensitive for the detection of *P. jirovecii*. In the present study showed the PCR detection of P. *jirovecii* is a very sensitive test and will offer a powerful technique in clinical laboratories for the routine diagnosis of Pneumocystis pneumonia in BAL and sputum specimens and Dihydropteroate synthase (DHPS) gene has served as a selective drug linked with sulfa resistance and PCR amplification target.

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Author contributions

Isa PhD student conducted the sampling, isolation, molecular work and write the manuscript. Prof. Dr Azhar supervised the research work and co-advisor Consultant Dr. Haider guided, finished writing and editing the study.

Declaration of interest

The authors declare no conflict of interest.

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