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Molecular diagnosis of *Pneumocystis jirovecii* pneumonia by using External PCR in immunocompromised patients .

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ABSTRACT

Pneumocystis jirovecii is an opportunistic eukaryotic pathogen causing life threating pneumonia (PJP) in immunosuppressed patients.Detection of *Pneumocystis jirovecii* Pneumonia in immune suppressed patients like hematological cancer with or without chemotherapy, patients on corticosteroid therapy, other malignancy like bronchogenic cancer by using External Polymerase Chain Reaction (External PCR) and to compare the results with other control group (patients who are immunocompetent suffer from pneumonia).A total of 220 specimens were obtained from patients admitted to Al- Ramadi Teaching Hospital, Clinic excitability of respiratory disease in Al- Ramadi and Oncology Teaching Hospital in Baghdad during the period from February to July 2013. This number composed of 71(32%) specimens were from female while the male specimens were 149(68%). A total of 184 (84%) were immunocompromised patients, They were considered immunocompromised patients because they have (hematological cancer with or without chemotherapy, patients on corticosteroid therapy, other malignancies like bronchogenic cancer). The other 36 (16%) patients with pneumonia were included as immunocompetent patients (No hematological cancer, No chemotherapy, No immunosuppressive drug like corticosteroid therapy and No malignancy like bronchogenic cancer). A total of 220 specimens which were collected, (consisting of 115 blood, 65 sputum, 40 BAL Specimens) the total 220 specimens (115 blood, 65 sputum and 40 BAL Specimens) were examined PCR technique (External and Nested PCR) to identify *Pneumocystis Jirovecii* DNA based on the amplification of specific primers. The result of agarose gel electrophoresis revealed that small DNA diagnostic bands were detected at 346 bp for External PCR in all positive specimens. Out of 220 specimens 26 (11.8%) were detected by the mtLSU rRNA External PCR. Regarding the use of External PCR technique for sputum specimens 8 (12.3%) were positive, While BAL 8 (20%) and Blood 10 (8.7%). PCR is more accurate and better indicative for the presence of *Pneumocystis jirovecii* Pneumonia. In addition this technique can use specimens which were not stored properly and became non suitable for microscopy.

Introduction:

Pneumocystis jirovecii is an opportunistic eukaryotic pathogen causing life threating pneumonia *Pneumocystis jirovecii* pneumonia (PJP) in immunosuppressed patients. Since its discovery in the early 1900s, it was thought to be a protozoon; then in the 1980s, Deoxyribonucleic Acid (DNA) analysis showed that this organism is in fact, a fungal species.

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Pneumocystis jirovecii pneumonia (PJP) is a major cause of morbidity and mortality in the immunosuppressed patients and an increasing number of cases of pulmonary pneumocystosis has been reported in immunosuppressed patients[1]. (PCP) was described in the first half of this century in premature infants, immunodeficient subjects with hematologic cancers, graft recipients, and others [2,3].The disease known as *Pneumocystis jirovecii* pneumonia (PJP) is one of the leading causes of illness and death in persons with impaired immunity. The disease has been described in immunocompromised patients having the immune response attenuated by administration of

immunosuppressive drugs, irradiation, malnutrition, or by certain disease processes (e.g., cancer). And also in patients with acquired immune deficiency syndrome (AIDS) those who are on immunosuppressive drugs [(4)]. Pneumocystosis is a form of pneumonia, caused by the yeast-like fungus (which had previously been erroneously classified as a protozoan) *Pneumocystis jirovecii*. This pathogen is specific to humans; it has not been shown to infect other animals. Other species of *Pneumocystis* that parasitize other animals have not been shown to infect humans. *Pneumocystis* is found in the lungs of healthy people, but, being a source of opportunistic infection, it can cause a lung infection in people with a weak immune system. *Pneumocystis jirovecii* pneumonia is especially seen in people with cancer, AIDS and the use of medications that affect the immune system [(5)]. The diagnosis of *Pneumocystis jirovecii* is usually achieved by examining stained smears of bronchoalveolar lavage (BAL) for the presence of the organism[(6)]. Diagnosis of PJP in the laboratory was until a few years ago, dependent on visualization of *Pneumocystis* organism in stained preparation of appropriate respiratory specimen using the Giemsa and Florescent techniques. The Sensitivity of the staining techniques, is considered acceptable (70-92%) for bronchoalveolar lavage (BAL) specimens[(7)]. In the last ten years , Polymerase Chain Reaction (PCR) has considerably increased sensitivity of detection of *Pneumocystis* , which is now 86-100% in BAL, aspirates and induced sputum specimens[(8)]. The laboratory diagnosis of *Pneumocystis jirovecii* pneumonia (PJP), caused by the opportunistic fungal pathogen *Pneumocystis jiroveci*, still relies on tinctorial and/or Immunoflourescent staining of bronchoalveolar lavage (BAL) fluid samples, Now Nucleic acid amplification tests can overcome the difficulties of microscopic examination[(9)]. *Pneumocystis jirovecii* remains a potentially life-threatening cause of pneumonia among immunocompromised persons because of prolonged survival of patients with hematologic malignancies, solid organ and blood stem cell transplant recipients, and patients undergoing chemotherapy and prolonged corticosteroid therapy. Furthermore, *Pneumocystis jirovecii* pneumonia (PJP) can be the initial manifestation of (AIDS) in patients who do not have access to highly active antiretroviral therapy (HAART) or patients in whom HAART fails [(10)].

Patients and methods :

- Immunocompetent Group: Thirty six (36) immunocompetent patients with pneumonia were (No hematological cancer, No chemotherapy, No immunosuppressive drug like corticosteroid therapy and No malignancy like bronchogenic cancer). These patients were diagnosed as pneumonia by physician on clinical radiological back ground of respiratory disease. The samples of patients (Blood, Sputum, BAL) were collected from Al-Ramadi teaching hospital, Clinic excitability of respiratory disease in Al-Ramadi and Oncology Teaching Hospital in Baghdad.
- Immunocompromised Group: One hundred eighty four (184) immunocompromised patients were considered immunocompromised patients because of they have (hematological cancer with or without chemotherapy, patients on corticosteroid therapy, other malignancy like bronchogenic cancer), who were attended to Al-Ramadi teaching hospital, Clinic excitability of respiratory disease in Al-Ramadi and Oncology Teaching Hospital in Baghdad, during the period from February to July 2013. They were of different sex, 71 specimens were female while 149 specimens were male, the ages from 17 to 80 years. Samples (blood, morning sputum and BAL taken during bronchoscopy) were taken from them. These samples were studied by the following staining procedure and molecular study (PCR) to detect *Pneumocystis jirovecii*.

Results:

A total of 220 specimens, (consisting of 115 blood, 65 sputum, 40 *BAL* Specimens) . The total 220 specimens (115 blood, 65 sputum and 40 BAL Specimens) were examined by PCR technique (External and Nested PCR) to identify *Pneumocystis Jirovecii* DNA based on the amplification of specific primers. The result of agarose gel electrophoresis revealed that small DNA diagnostic bands were detected at 346 bp for External PCR in all positive specimens.

1. The detection of *Pneumocystis jirovecii* pneumonia according to Gender group by External PCR: The types of clinical Specimens were studied and observed that, out of 220 specimens, 26 (11.8%) were detected by the mtLSU rRNA External PCR. Out of 115 Blood Specimens, 10 (8.7%) were

found to be positive *Pneumocystis jirovecii* DNA, while 65 sputum Specimens 8 (12.3%) were positive, and out of 40 BAL Specimens, 8 (20%) were positive (Table 1).

2. The detection of *Pneumocystis jirovecii* pneumonia according to Age group by External PCR: Higher percentages was found in the External PCR technique in all age groups, but without significant difference (P value > 0.05) as shown in table 2.
3. The detection of *Pneumocystis jirovecii* pneumonia according to Residence by External PCR:Table 3. showed The detection of *Pneumocystis jirovecii* DNA according to residence. In Sputum and BAL specimens, Urban residence revealed higher rate of *Pneumocystis jirovecii* pneumonia [5 (13.9%) & 4 (26.7%) , respectively] than rural residence [3 (10.3%) & 4 (16.0%), respectively]. All results were statistically not significant (P value > 0.05).
4. The detection of *Pneumocystis jirovecii* pneumonia according to Immunity groups by External PCR:Table 4. studied immunocompetent and immunosuppressed patients diagnosed external PCR technique. In sputum and BAL specimens higher percentage of Pneumocystis jirovecii pneumonia was observed, when examined sputum specimens in immunosuppressed patients 7 (12.5%) in compare with that of immunocompetent patients 1 (11.1%). There was no significant difference between two groups (P value > 0.05).
5. The detection of *Pneumocystis jirovecii* pneumonia according to history of treatment by External PCR: The history of treatment according to *Pneumocystis jirovecii* pneumonia diagnosed external PCR was studied in table 5. Patients with history of treatment were higher percentages of *Pneumocystis jirovecii* pneumonia in sputum and BAL [6 (9.2%) & 7 (17.5%), respectively] than those without treatment [2 (3.1%) & 1 (2.5%), respectively]. There was high significant difference between two groups (P value < 0.05).

● Identification of *Pneumocystis jirovecii* DNA bands by External PCR: A total 220 specimens (115 blood, 65 sputum and 40 BAL Specimens) were examined by External PCR to identify *Pneumocystis jirovecii* DNA Based on the amplification of the specific primers. The result of 2% agarose gel electrophoresis revealed that small DNA diagnostic bands were detected at 346 bp (Figure 1.).

Discussion:

1. Suspected person study: Despite widespread use of Highly Active Antiretroviral Therapy (HAART) and chemoprophylaxis in industrialized countries, *Pneumocystis jirovecii* remains a potentially life-threatening cause of pneumonia among immunocompromised persons because of prolonged survival of patients with hematologic malignancies, solid organ and blood stem cell transplant recipients, and patients undergoing chemotherapy and prolonged corticosteroid therapy. Furthermore. *Pneumocystis jirovecii* pneumonia (PJP) should be strongly suspected when any immunocompromised patient has respiratory tract signs and systems and extensive, patchy, upper-lobe-predominant ground glass opacity on High- Resolution Computed Tomography images (HRCT) [(10)].
2. *Pneumocystis jirovecii* infection according to diagnostic methods: In the present study, the total positive rate of *Pneumocystis jirovecii* for sputum , BAL specimens diagnosed by External PCR for sputum and BAL were 8 (12.3%) ; 8 (20%), respectively, PCR depend on the amplification of the DNA extracted from the fungus using specific primer. This study was in agreement with [(11)], who he was evaluated the usefulness of single and nested PCRs as diagnostic tools for the diagnosis of PCP and to compare them to conventional microscopy, It could be attributed to that the molecular methods such as PCR, which can amplify low copy numbers of the organisms DNA using single or nested PCR increases the sensitivity of detection, and also increases the risk of detecting subclinical colonization of *Pneumocystis jirovecii* [(12)].
3. The detection of *Pneumocystis jirovecii* DNA by External PCR: Sputum, BAL and blood were analysed and observed that, out of 220 specimens, 26 (11.8%) were detected by the mtLSU rRNA External PCR this result was in agreement with [(13)], who also found the positive samples in his study were sequenced based on the mt LSU rRNA gene and these isolates were definitely *Pneumocystis jirovecii*, and most Iranian isolates were similar to Indian isolates (99% identity). The external PCR higher rate positivity may be attributed to the fact

that PCR, which can amplify low copy numbers of the organisms DNA .

4. The detection of *Pneumocystis jirovecii* pneumonia according to Gender group by External PCR: The positive rates of *Pneumocystis jirovecii* pneumonia by External PCR technique for sputum, BAL specimens were high rates. These results were agreed with[14], who found that PCR is more sensitive in detecting low levels of *P. carinii* that may be found in sputum. This may be due to the differences of proportion between trophozoites and cysts (the presence of DNA-containing forms trophozoites less easily detectable by cytological stains could explain the better results obtained when using PCR) in upper and lower airways and found the intensity of infection for males was higher than for female. The higher positive results of BAL specimens over sputum specimens is probably due to the fact that BAL fluid are less likely to contain inhibitors compared to sputum specimens [15].
5. The detection of *Pneumocystis jirovecii* pneumonia according to Age group by External PCR: The age group 20-50 years showed higher percent of *Pneumocystis jirovecii* pneumonia detected by external PCR for sputum, While higher percent of positive infection in BAL specimens was found in age group greater than 50 years. The higher positivity rate of BAL specimens in the age greater than 50 years is probable due to method of obtaining the specimens which is bronchoscopy which give more accessible way to the site of infection. This study was in agreement with [16] , who was showed the majority of pneumonia cases were in the age group 20-39 years and he found 24 positive sputum specimens from 30 specimens in his study.
6. The detection of *Pneumocystis jirovecii* pneumonia according to Residence by External PCR: Our study showed that higher positive results of *Pneumocystis jirovecii* pneumonia in urban residence regarding sputum and BAL specimens [5 (13.9%) ; 4 (26.7%) , respectively] in comparison with rural residence who showed less positive results [3 (10.3%) ; 4 (16.0%), respectively]. It could be attributed to most people in urban area were expose to pollutions, smoking, higher incidence of malignancy and different life style.
7. The detection of *Pneumocystis jirovecii* pneumonia according to Immunity groups by External PCR: Higher percentages of *Pneumocystis jirovecii* pneumonia were detected by using external PCR for sputum, BAL specimens in immunosuppressed patients in comparison with immunocompetent. These results were agreed with[11], who showed that higher infection in Immunosuppressed than Immunocompetent patients groups. This may be explain by the fact that *Pneumocystis jirovecii* in opportunistic infection that favorite immunosuppressed individuals. The higher incidence of *Pneumocystis jirovecii* pneumonia in immunosuppressed patients is probable due to modification of immune system, such the lack of a T-cell blastogenic response to P. jirovecii organisms, low CD4 cell counts, reduced gamma interferon production by activated T cells, and reduced interleukin-1 secretion by macrophages, may contribute to PCP [17,18,19].
8. The detection of *Pneumocystis jirovecii* pneumonia according to history of treatment by External PCR: Patients with history of treatment with immunosuppressed drug (cytotoxic and steroid) has higher percentage of *Pneumocystis jirovecii* pneumonia detected by external PCR in sputum and BAL [6 (9.2%) ; 7 (17.5%), respectively] than those without treatment [2 (3.1%); 1 (2.5%), respectively]. This result agreed with [20], in Mosul who studied identification and treatment on a patient with lymphoma under cytotoxic therapy for 5 years.

In conclusion:

PCR can use for specimens which are not stored properly and become unusable for microscopy. PCR is more accurate diagnostic tool for indicative of the presence of *Pneumocystis jirovecii* Pneumonia in samples of individuals. Higher percent of *Pneumocystis jirovecii* Pneumonia was in males than in females. The highly percent of *Pneumocystis jirovecii* Pneumonia in sputum Specimens are found in age group of 20-50 years, While BAL Specimens in age group greater than 50 years. BAL specimen is the best specimen for detecting *Pneumocystis jirovecii* by PCR technique. It is better to use the highly specific primers in PCR to achieve amplification with minimum amount of *Pneumocystis jirovecii* template

DNA in clinical specimens (BAL, Sputum and blood).Urban residence showed higher Pneumocystis jirovecii Pneumonia than rural residence. High percentage of *Pneumocystis jirovecii* Pneumonia is observe in immunosuppressed in comparison with that of immunocompetent patients. Patients with history of treatment have higher percentage of *Pneumocystis jirovecii* Pneumonia than without history of treatment. The significance of positive PCR assay should be interpreted in conjunction with the patient's clinical findings.

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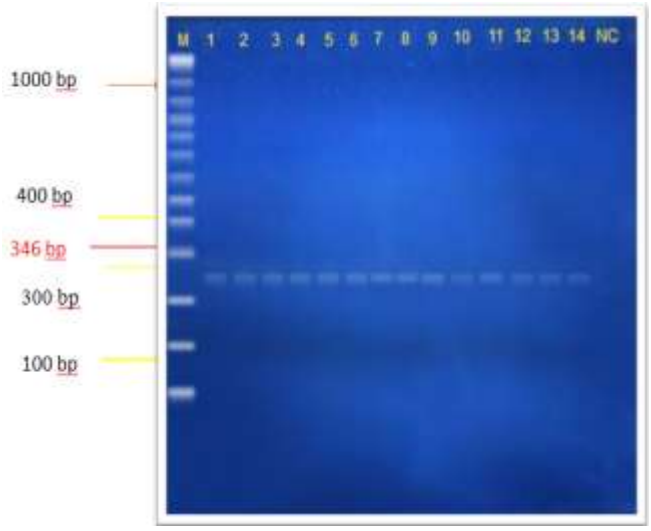

Figure 1. External Polymerase Chain Reaction (External PCR) amplification of *Pneumocystis jirovecii* DNA extracted from suspected patients for *Pneumocystis jirovecii* pneumonia using specific primers for the diagnosis of the DNA fragment detected on 2% agarose gel electrophoresis. **Lane-M,** molecular weight marker (100-bp ladder).**Lanes 1-14** positive Specimens (*Pneumocystis jirovecii* DNA) at 346-bp diagnostic band., **Lane- NC** Negative Control (using distilled water) no DNA band seen.

Table-1: The detection of Pneumocystis jirovecii pneumonia by External PCR according to gender group

Gender Group	Sample Type	External PCR No.Inf (%)
Male	Sputum N= 47	5 (10.6%)
	BAL N= 34	7 (20.6%)
Female	Sputum N= 18	3 (16.7%)
	BAL N= 6	1 (16.7%)
``Total	Sputum N= 65	8 (12.3%)
	BAL N=40	8 (20%)

Table-2: The detection of Pneumocystis jirovecii pneumonia by External PCR according to Age groups.

Age Groups	Sample Type	External PCR No.Inf.(%)
< 20 Years	Sputum N= 4	1 (25%)
	BAL N= 0	0 (0%)
20-50 Years	Sputum N= 32	4 (12.5%)
	BAL N= 17	3 (17.6%)
>50 Years	Sputum N= 29	3 (10.3%)
	BAL N=23	5 (21.7%)

Table-3: The detection of Pneumocystis jirovecii pneumonia by External PCR according to Residence.

Residence	Sample Type	External PCRNo.Inf.(%)
Rural	Sputum N= 29	3 (10.3%)
	BAL N= 25	4 (16.0%)
Urban	Sputum N= 36	5 (13.9%)
	BAL N= 15	4 (26.7%)
Total	Sputum N= 65	8 (12.3%)
	BAL N=40	8 (20%)

Table-4: The detection of Pneumocystis jirovecii pneumonia by External PCR according to Immunity groups

Immunity groups	Sample Type	External PCR No.Inf.(%)
Immunocompetent	Sputum N= 9	1 (11.1%)
	BAL N= 2	0 (0%)
Immunosuppressed	Sputum N= 56	7 (12.5%)
	BAL N= 38	8 (21.1%)
Total	Sputum N= 65	8 (12.3%)
	BAL N=40	8 (20%)

Table-5: The detection of Pneumocystis jirovecii pneumonia by External PCR according to history of treatment

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History of Treatment	Sample Type	External PCRNo.Inf.(%)
With Treatment	Sputum N= 44	6 (9.2%)
	BAL N= 37	7 (17.5%)
Without Treatment	Sputum N= 21	2 (3.1%)

Total	BAL N= 3	1 (2.5%)
	Sputum N= 65	8 (12.3%)
	BAL N=40	8 (20%)

التشخيص الجزيئي لداء ذات الرئة بالمتكيسات الرئوية لفطر *Pneumocystis jirovecii* باستخدام PCR الخارجي في مرضى العوز المناعي

اركان عبدالله القيسي علاء عباس الخفاجي حميد ابراهيم الزكروط

الخلاصة:

درس مرض ذات الرئة بالمتكيسات الرئوية لفطر Pneumocystis jirovecii الذي هو مرض انتهازي يصيب كثير من المرضى ضعيفي المناعة.للكشف عن وجود مرض ذات الرئة بالمتكيسات الرئوية لفطر Pneumocystis jirovecii في مرضى العوز المناعي (سرطان الدم مع أو بدون العلاج الكيميائي ، والمرضى على العلاج كورتيكوستيرويد ، والأورام الخبيثة الأخرى مثل سرطان قصبية المنشأ) . باستخدام تقنية ال PCR الخارجية ومقارنة النتائج مع نتائج الاصابة بمرض ذات الرئة بالمتكيسات الرئوية في مرضى المناعة الكاملة.جمعت 220 عينه من المرضى الراقدين في مستشفى الرمادي التعليمي والعيادة الاستشارية للإمراض الصدريه في الرمادي و مستشفى الأورام التعليمي في بغداد خلال الفترة من فبراير حتي يوليو 2013. وهذا العدد يتكون من 71 (32 ٪) من العينات من الاناث بينما كانت عينات الذكور 149 (68 ٪) . اخذت 184 (84 ٪) من مرضى العوز المناعي (سرطان الدم مع أو بدون العلاج الكيميائي ، والمرضى على العلاج كورتيكوستيرويد ، والأورام الخبيثة الأخرى مثل سرطان قصبية المنشأ) . اما مرضى المناعة الكاملة كانت نسبتهم 36 (16 ٪) من المرضى المصابين بالالتهابات الرئوية ويتميزون بأنه ليس لديهم سرطان الدم ، العلاج الكيميائي ، الادوية المثبطة للمناعة مثل العلاج كورتيكوستيرويد ، اورام مثل سرطان قصبي المنشأ . قسمت العينات الكلية (220 عينة) الى 115 عينة دم ، 65 عينة قشع ، 40 عينة غسيل قصبي حويصلي).فحصت جميع العينات (220 عينة) المتمثلة بالإعداد التالية 115 عينة دم ، 65 عينة قشع ، 40 عينة غسيل قصبي حويصلي بواسطة تقنية PCR (الخارجية و المتداخلة) لتحديد الحامض النووي للمتكيسات الرئوية الفطرية اعتمادا على تضخيم اجزاء معينه من الحامض النووي لفطر Pneumocystis jirovecii. كشفت نتائج الترحيل الكهربائي عن حزم صغيره من الحامض النووي في 346 زوج قاعدة باستخدام PCR الخارجية في جميع العينات الإيجابية. فيما يتعلق بصبغة. العينات الكلية (220 عينة) 26 (11.8 ٪) كشف عنها بواسطة جين mtLSU rRNA باستخدام PCR الخارجية. لم تكن هناك فروق معنوية (قيمة P> 0.05) في التوزيع بين الجنسين. فيما يتعلق باستخدام تقنية PCR الخارجية لعينات القشع 8 (12.3 ٪) كانت ايجابية ، في حين عينات الغسيل القصبي الحويصلي 8 (20 ٪) وكانت عينات الدم 10 (8.7 ٪) عينات إيجابية. خلاصة النتائج المذكورة أعلاه أن PCR هو أكثر دقة من طرق الصبغ المباشر وأفضل مؤشرا لوجود مرضى ذات الرئة بالمتكيسات الرئوية. بالإضافة هذه التقنية يمكن استخدام العينات التي لم يتم تخزينها بشكل صحيح و أصبحت غير مناسبة للفحص المجهري.