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Detection of *Pneumocystis* in the nasal swabs of immune-suppressed rats by use of PCR and microscopy

Authors' Contribution:
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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Detection of *Pneumocystis jiroveci* colonization in lungs or oral samples due to high sensitivity of PCR methods results in undue treatment of patients without any symptoms of *Pneumocystis* pneumonia. The aim of the present study is to demonstrate *Pneumocystis carinii* in rats, immune suppressed by oral and subcutaneous administration of dexamethasone.

Material/Methods: Blood, oral, nasal and eye swabs were collected prior to immune suppression and 2, 6, 12 weeks after administration of dexamethasone. Also, samples were collected from lung, heart, liver, kidney, diaphragm, brain, spleen, tongue, muscle, eye, intestine, and feces. Cysts and trophozoites were investigated in stained slides and MSG gene was detected by PCR.

Results: The results showed that weight loss is significantly higher in rats administered oral dexamethasone ($P<0.05$). Microscopy was positive only in lungs of rats orally administered dexamethasone. PCR was positive in lungs and oral swabs of rats prior to the administration of dexamethasone. After the administration of dexamethasone, the MSG gene was detected in oral swabs, lungs, spleen, kidney and (for the first time) in nasal swabs. PCR was positive in nasal swabs during the second and sixth weeks of oral and subcutaneous administration of dexamethasone, respectively.

Conclusions: Presence of *P. jiroveci* in nasopharyngeal aspirate, oropharyngeal wash, oral swab, induced sputum or BAL, and absence in nasal swab in a patient without symptoms of PCP may support clinician's decision regarding colonization. Overall, detection of *P. carinii* in nasal swabs of rats by PCR demonstrated that nasal sampling can be used for the diagnosis of *Pneumocystis* pneumonia.

key words: **nasal swab** ***Pneumocystis carinii*** **PCR** **diagnosis** **colonization**

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Background

Pneumocystis species are eukaryotic organisms detected worldwide and infect mammalian hosts including animals and humans. *Pneumocystis jiroveci* causes lethal Pneumocystis pneumonia (PCP) in immune-suppressed hosts such as patients with AIDS and those receiving immune suppressive treatment for malignancy treatment or transplantation [1,2]. Currently, PCP most commonly occurs as AIDS-associated illness in patients who are not aware that they are HIV-positive or those who are not receiving combined anti-retroviral therapy (CART) [1–3].

Although the most common infection site is the lungs, extrapulmonary infection also occurs [4–13]. Definitive diagnosis of pneumocystosis is usually achieved by the demonstration of *Pneumocystis* spp. using staining methods, PCR and immunofluorescence techniques [1,2].

The diagnosis of PCP has been achieved by non-invasive techniques such as induced sputum, oropharyngeal washes and nasopharyngeal aspirates [1,2,14,15]. Consequently, more invasive techniques such as bronchoalveolar lavage (BAL), transbronchoscopic or surgical lung biopsy used to increase the sensitivity [1,2].

Molecular diagnostic techniques are more sensitive than staining methods, but may have difficulty in distinction of infection versus colonization [16,17]. Demonstration of *P. jiroveci* in any specimen collected from the respiratory tract of an individual without signs and symptoms of PCP is defined as colonization [17]. Due to colonization, *P. jiroveci* is detected in nasopharyngeal aspirates of healthy immunocompetent infants by PCR [18]. Current research has focused on developing novel quantitative PCR techniques to distinguish between colonization and infection [19,20]. During oropharyngeal or nasopharyngeal sampling, colonization of *P. jiroveci* in the pharynx may be transported to the oral or nasal cavity. *Pneumocystis carinii* organisms have been detected in oral swabs of immune-competent rats using PCR [21,22]. Nasal sampling has also been used to determine the presence of other pathogens [23,24]. Nasal swab sampling has been used in comparison with samples obtained by oropharyngeal washing to determine colonization in elderly individuals, using PCR [25]. Therefore, non-invasive oral or nasal swab sampling emerges as a solution to determine the colonization of *P. jiroveci* in the uppermost parts of the respiratory tract.

Dexamethasone is commonly used as an immune suppressive agent to induce protozoan parasitic diseases in animal models [26–29]. In the present study, oral and nasal swabs, blood samples and first-time eye swabs were collected from rats prior to administration of dexamethasone and at fixed time points after immune suppression. At the end of immune suppression, samples were collected from lung, heart, liver, kidney, diaphragm, brain, spleen, tongue, muscle, eye, intestine, and feces. Microscopic examination and PCR were performed on all samples to assess the presence of *P. carinii* infection and colonization.

Material and Methods

Animal, immune suppression and sampling

Two- to three-month-old male outbred *Rattus norvegicus* rats weighing 60–80 g were obtained from the Ege University Experimental Animal Production Facility and used during the experiments. Rats were housed under standard, suitable conditions and fed a 14% protein diet. The experimental plan was performed under the instructions and approval of the Institutional Animal Care and Use Committee of Ege University for animal ethical norms.

Immune suppression and sampling were performed as described [26,27]. During the study, a total of 12 rats were used. Two groups of rats (each containing 4 rats) were administrated oral (2 mg/l in drinking water) and subcutaneous injection (3 mg/week) of dexamethasone. Drinking water was supplemented with tetracycline (0.5–1 g/l) to suppress secondary bacterial infections. A third group of rats was used as control.

Prior to immune suppression and 2, 6, and 12 weeks after administration of dexamethasone, blood, nasal, oral and eye swab samples (collected by rubbing the cotton swab) were collected from anesthetized rats. During the collection of nasal swabs, 50 µl sterile 0.9% NaCl was pipetted into each nostril of the rats positioned face down and absorbed immediately by sterile dry cotton-tipped swabs. All oral swabs were collected by rubbing the dry cotton-tipped swab in the oral cavity and around the tongue. Each swab was broken in a sterile DNase/RNase-free 1.5 ml tube containing 200 µl serum physiologic. All swabs were vortexed vigorously, centrifuged at 14,000 × g for 5 minutes. After discarding the swabs, supernatants were used to prepare smears for microscopic examination and DNA extraction.

Rats were euthanized after 12 weeks of immune suppression. Lung, heart, liver, kidney, brain, spleen, eye, diaphragm, tongue, muscle, intestine samples and fecal material were collected from each rat. Tissues were homogenized using a sterile mortar and pestle in 200 µl sterile serum physiologic. Morbidity was confirmed by observation of dyspnea, loss of appetite, weight loss, and change in the color of the fur.

Giemsa and Gram Weigert staining

Smears of tissues, fecal material, blood, nasal, oral and eye swabs were stained by Giemsa and Gram Weigert as described [30–32] to examine the presence of *P. carinii* cysts and

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trophozoites, respectively. During Giemsa staining, slides were covered with methanol and air dried, then slides were covered with Giemsa solution [10% Giemsa (v/v) (Merck)] and incubated for 30 minutes at room temperature. Then, slides were washed with distilled water to remove excess dye and examined under light microscopy with immersion oil. In Gram Weigert staining, air dried slides were stained with 1% Eosin-Y (Merck, Germany) solution for 5 minutes. Then, slides were rinsed with distilled water for 2 minutes to remove excess dye and stained with crystal violet solution [5% crystal violet (w/v); 10% ethanol (v/v) (Applichem); 2% aniline oil (v/v) (Merck)] for 5 minutes. Excess crystal violet is rinsed off with Gram's iodine solution [3.61 mM potassium iodide; 1.18 mM iodine (Merck)]. Rinsed and blot dried slides were washed with aniline oil-xylene solution [50% aniline (v/v); 50% xylene (v/v) (Merck)] for decolorization. Further decolorization is stopped by xylene washing. Slides were examined by 2 qualified parasitologists, under light microscopy with immersion oil.

DNA extraction and PCR analysis

Isolation of DNA from rat tissues, blood, nasal, oral and eye swabs was performed with the QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen). During the procedure, 200 µl blood, 100 µl nasal, oral and eye swab samples, 10 mg spleen and 25 mg from the remaining tissues were used. DNA extraction from fecal material was performed with ZR Fecal DNA kit according to the manufacturer's protocol (Zymo Research). During the procedure, 150 mg of fecal material was used. At the end of both procedures, eluted buffer yielded approximately 0.5 to 25 ng/µl purified DNA.

Conventional PCR targeting the major surface glycoprotein (MSG) gene of *P. carinii* (GenBank no. D82031.1) was performed as described [33]. Briefly, the primers to amplify 338 base pair (bp) gene fragment in PCR reaction were 5'-ATGGCACGGCCGGTTAAGAG-3' (20 nt, AUG forward primer) and 5'-ATACATTTTTCTTCATGTTTT-3' (21 nt, C2 reverse primer). The 25 µl amplification reactions included 3 µl template DNA, the primers (0.8 µM each), 1.25 U Platinum Taq DNA polymerase (Invitrogen), 200 µM dNTPs, and 1× Platinum Taq reaction buffer. The PCR amplification reaction was performed using the following calculated-control protocol: 5 minutes initial denaturation step at 95°C, followed by 30 cycles of 1 minute at 94°C, 2 minute at 50°C, and 1 minute at 72°C, and a final extension of 10 minute at 72°C. Each PCR included DNA from the lung of an infected rat as a positive control and negative control, prepared by the replacement of template DNA with distilled water. The PCR products were visualized by 2% agarose gel electrophoresis.

Sensitivity of the PCR was determined as described [21,22,26,34]. Briefly, the number of *P. carinii* organisms was counted in stained slides prepared from 20 µl lung homogenates of infected rats. DNA was isolated from 100 µl lung homogenate and PCR was performed as described above with 10-fold dilutions of DNA from 10^{-1}–10^{3} organisms.

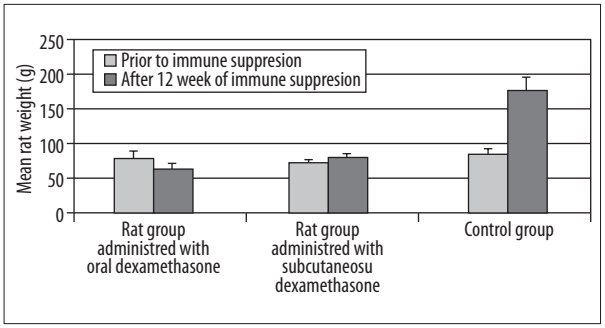

Figure 1. Comparison of immune suppressed and control group rats' weights prior and after 12 week of immune suppression.

Statistical analysis

Data obtained during the study were processed using Prism 3.03 (GraphPad, San Diego, CA). A two-tailed unpaired t test with 95% confidence interval was used to determine the significance between the results of assays. The weights of rats were expressed as mean±standard deviation (S.D.).

Results

Pneumocystosis in rats

Loss of appetite, dyspnea, and change of fur color from white to yellowish were observed at 3 and 5 weeks in orally and subcutaneously immune suppressed rats, respectively. After 12 week of immune suppression, the mean weight of the rat group administered oral dexamethasone significantly decreased from 79±10 g to 65±6 g ($P<0.05$); rats administered subcutaneous dexamethasone increased from 71±5 g to 77±2 g ($P=0.06$); and the control group increased from 86±7 g to 173±23 g ($P=0.0004$). The mean weights of oral ($P<0.0001$) and subcutaneous ($P=0.0002$) dexamethasone-administered rats were significantly lower than in control groups (Figure 1). The percent weight losses in rats administered oral and subcutaneous dexamethasone were 62.4% and 55.5%, respectively.

Microscopy

In Gram Weigert and Giemsa stained slides, *P. carinii* was observed only in lungs of immune-suppressed rats. Numerous cysts and trophozoites were detected in lung smears of rats administered oral dexamethasone, compared to few cysts observed in rats administered subcutaneous dexamethasone.

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Table 1. Results of PCR obtained from immune suppressed and control group rats' tissues, blood, nasal, oral and eye swabs.

	Rats administered oral dexamethasone *				Rats administered subcutaneous dexamethasone *				Control group rats *			
	Day 0	2nd week	6th week	12th week	Day 0	2nd week	6th week	12th week	Day 0	2nd week	6th week	12th week
Oral swab	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Nasal swab	– – – –	++++	++++	++++	– – – –	– – – –	++++	++++	– – – –	– – – –	– – – –	– – – –
Eye swab	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –
Blood	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –	– – – –
Lung				++++				++++				++++
Liver				– – – –				– – – –				– – – –
Heart				– – – –				– – – –				– – – –
Brain				– – – –				– – – –				– – – –
Eye				– – – –				– – – –				– – – –
Kidney				++++				++++				++++
Spleen				++++				++++				++++
Intestine				– – – –				– – – –				– – – –
Tongue				– – – –				– – – –				– – – –
Muscle				– – – –				– – – –				– – – –
Diaphragm				– – – –				– – – –				– – – –
Feces				– – – –				– – – –				– – – –

* Each group contains 4 rats.

Trophozoites or cysts of *P. carinii* were not observed in the stained smears of remaining rat samples.

Detection of *P. carinii* MSG gene with PCR

The *P. carinii* MSG gene was detected in immune suppressed and control group rats' oral swab samples prior to dexamethasone administration, 2, 6 and 12 week after immune suppression. In addition, PCR was positive in lungs of all rats, including the control group, after 12 week of immune suppression (Table 1).

PCR was positive in nasal swabs of rats administered oral dexamethasone at 2, 6 and 12 weeks of immune suppression. The *P. carinii* MSG gene was detected in nasal swabs of rats administered subcutaneous dexamethasone after 6 and 12 week of immune suppression. At 12 weeks of immune suppression, PCR detected MSG gene in kidney and spleen tissues of immune-suppressed rats, in addition to lung tissues (Table 1). Each PCR reaction amplifying 338 bp fragment of MSG gene has a detection limit of 10 organisms (data not shown).

Discussion

Colonization is defined as detection of *P. jiroveci* in respiratory tract specimens without signs and symptoms of PCP [17]. Positive PCR results for *P. jiroveci* in patients without any symptom of PCP may cause undue treatment [1,2,17]. To assist clinicians in the management of patients, current research has focused on developing novel quantitative PCR or sampling techniques to distinguish colonization from infection [1,2,14,15,18–20]. Colonization of *P. jiroveci* is demonstrated in nasopharyngeal aspirates and oropharyngeal washes of immune-competent individuals as well as immune-suppressed patients [17,18]. Due to its presence in the pharynx and lungs, detection of *P. jiroveci* by PCR in induced sputum, oropharyngeal or nasopharyngeal sampling does not definitively indicate that the oral or nasal cavity contains *P. jiroveci*, and therefore does not determine if the patient has active infection or colonization. To clarify the patient's clinical status, nasal and eye swabs, in addition to oral swabs, were first-time collected from a rat model in the present study.

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The immune-suppressed rat model has been frequently used in *P. carinii* studies, and administration of corticosteroids initiates deadly PCP in rats [35]. In the present study, 2 groups of rats were administrated with oral and subcutaneous dexamethasone to initiate pneumocystosis. After 12 week of immune suppression, the mean weights of rat groups significantly decreased in oral ($P<0.0001$) and subcutaneous ($P=0.0002$) dexamethasone-administered rats compared to control rats (Figure 1). The percent weight losses in rats administered oral and subcutaneous dexamethasone were 62.4% and 55.5%, respectively. The percent weight loss of various type of rats (Sprague-Dawley, Wistar, Long-Evans, Brown Norway) was between 60–70% after 12 week of subcutaneous methylprednisolone acetate administration (22). The percent weight loss of Sprague-Dawley rats administered oral dexamethasone for 12 weeks was 47.4%, comparable with results of the present study [26]. Comparison of the weight losses of immune-suppressed rat groups in the present study showed that weight loss was significantly higher in rats administered oral dexamethasone ($P<0.05$).

To determine the presence of *P. carinii*, stained slides containing smears of tissues, fecal material, blood, nasal, oral and eye swabs were examined with microscopy; *P. carinii* was only detected in lung smears of immune suppressed rats. Examination of the slides showed that numerous cysts and trophozoites were detected in lung smears of rats administered oral dexamethasone compared to few cysts observed in rats administered subcutaneous dexamethasone. However, *P. carinii* was detected by PCR in oral swabs of all rats prior to immune suppression and 2, 6 and 12 week after immune suppression. In addition, PCR was positive in lungs of all rats, including the control group, after 12 week of immune suppression.

P. carinii DNA was investigated in rat fetuses, pups and their placentas just after birth. PCR was positive in all oral swabs of pups by 48 hours and was negative in fetuses and their placentas, indicating that *P. carinii* is acquired just after birth, but is not transplacental [21].

In the present study, *P. carinii* DNA was detected by PCR in nasal swabs of all immune-suppressed rats, but not in control rats. Similarly, Oz et al investigated nasal and pharyngeal aspirates and lung samples of rats using PCR. In immune-competent rats, *P. carinii* was not detected in any respiratory sample. In immune-suppressed rats, *P. carinii* DNA was detected in nasal and pharyngeal aspirates, as well as in lung samples [36]. In a study conducted in elderly individuals without any symptoms of PCP, *P. jiroveci* was detected in 12.8% of oropharyngeal washes and 10.6% in nasal swabs collected with a modified technique to avoid the nasopharynx [25]. However, in another study, conducted in humans without PCP, *P. jiroveci* DNA was scarce or absent in the oropharyngeal secretions [37]. These results demonstrate that nasal sampling technique is adequate in rat models, but it has to be improved in humans.

In the present study, in addition to lungs, *P. carinii* was also detected by PCR in kidney and spleen tissues of all immune-suppressed rats (Table 1). *P. carinii* dissemination was investigated in rats (n: 12) administered oral dexamethasone for 12 weeks and a control group (n: 2) using PCR and microscopic examination. *P. carinii* DNA was detected in immune-suppressed rats' lungs (100%), spleen (58.3%), liver (50%), and kidney (50%) and in control group rats' lungs (100%), spleen (50%), and kidney (100%). *P. carinii* was detected by microscopic examination in immune-suppressed rats' lungs (83.3%), spleen (16.6%), liver (8.3%), and kidney (8.3%) [26]. In another study, rats were fed an 8% protein diet and were administered methylprednisolone acetate for 8 weeks to exacerbate PCP. *P. carinii* was detected by PCR in rats' adrenal gland (60%), bone marrow (60%), blood (40%), heart (70%), kidney (80%), liver (90%), lymph node (100%), lungs (100%), spleen (60%), and thyroid tissue (20%) [34]. The intense involvement of tissues detected in this study was associated with low-protein (8%) diet, compared to the present study using a 14% protein diet. In a similar study, rats (n: 6) were administered intra-muscular injection of hydrocortisone for 6 weeks; PCR was only positive in lungs and was negative in blood, liver, spleen, and kidney [38]. In the present study, weight losses was significantly higher in rats administered oral dexamethasone ($P<0.05$), and nasal swab of rats administered oral and subcutaneous dexamethasone were PCR positive after 2 and 6 week of immune suppression, respectively.

Conclusions

The results of the present study show that oral administration of dexamethasone induces immune suppression slightly better than does subcutaneous administration. Microscopy was positive only in lungs of rats orally administered dexamethasone. PCR was positive in lungs and oral swabs of rats prior to the administration of dexamethasone. After the administration of dexamethasone, the *P. carinii* MSG gene was detected in oral swabs, lungs, spleen, kidney and first-time in nasal swabs. Nasal swab PCR was negative before immune suppression in healthy rats, showing that colonization has not occurred. PCR was positive in nasal swabs during the second and sixth weeks of oral and subcutaneous administration of dexamethasone, respectively. In a patient without any symptoms of PCP the presence of *P. carinii* in nasopharyngeal aspirate, oropharyngeal wash, oral swab, induced sputum or BAL, and absence in nasal swab, may support the clinician's decision that colonization has or has not occurred. Overall, detection of *P. carinii* first-time in nasal swabs of rats by PCR demonstrated that nasal sampling can be used for the diagnosis of *P. carinii* pneumonia. Therefore, non-invasive nasal swab sampling emerges

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as a better solution for determining the colonization of *P. carinii* in the upper respiratory tract.

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Conflict of interest

None declared.

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