

In vitro cultivation of *Pneumocystis* isolated from infected rat lungs

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ABSTRACT. The studies were undertaken to check the possibility of a long-term *in vitro* cultivation of *Pneumocystis* obtained from immunosuppressed rats using slightly modified method of Merali et al. The growth of *Pneumocystis* in the established axenic cultures was examined by counting the number of cysts in Giemsa and Diff-Quik stained preparations or by estimating the number of DNA copies with a real-time PCR method. Growing organisms were subpassaged at 7-day intervals for at least 6 weeks, however, the highest growth of *Pneumocystis* was usually noted in the primary and the first 3 subcultures, reaching an average of 175-fold increase in the number of cysts and 286-fold increase in the number of DNA copies in primary cultures. The organisms collected from *in vitro* cultures were examined for immunogenic and antigenic properties showing the ability to raise high-titre antisera in rabbits. The immune sera proved very valuable in a Western-blot analysis of *Pneumocystis* antigens and in immunodiagnostic tests, such as dot-ELISA, enabling to detect circulating *Pneumocystis* antigens in bronchoalveolar lavage and serum samples from infected rats. Production of diagnostic antisera is so far the main advantage of the successful *in vitro* cultivation of *Pneumocystis* in axenic media.

Key words: *Pneumocystis*, *in vitro* culture, real-time PCR, Western-blot

Introduction

Infection with *Pneumocystis* is one of the major causes of death among persons with impaired immunity, mostly in HIV-infected persons, often in patients subjected to chemotherapy for malignant diseases or immunosuppressed for organ transplantation, less frequently in malnourished individuals. The usual presentation of the infection is that of pneumonia (PCP).

The diagnosis of PCP has long relied on direct examination of respiratory specimens because of lack of efficient, continuous *in vitro* culture systems, which, apart from diagnostic implications, has hindered studies on *Pneumocystis* transmission, life cycle, biochemistry and immunology. First report on a successful cultivation of *P. carinii* was published in the late 1970s, when Latorre and her colleagues [1] from CDC in Atlanta informed of continuous *in vitro* propagation of the organism on Vero cell line, however, the results have not been confirmed by other investigators. The major breakthrough in the long history of unsuccessful attempts to grow *P. carinii in vitro* came only in 1999, when Merali and his associates [2] from the New York University School of Medicine published a paper on a continuous axenic cultivation of *P. carinii* using acellular medium. This spectacular achievement, however, was questioned by later studies of Larsen and his colleagues [3] from NIH, Bethesda, who developed a quantitative touchdown PCR assay for detecting *P. carinii* in cultures established according to Merali et al. [2], and found that „no organism multiplication occurred during such cultivation".

In view of the above conflicting results it was decided to perform our own studies on *in vitro* culture of *Pneumocystis* using slightly modified method of Merali et al. [2].

Material and methods

Pneumocystis organisms

Pneumocystis organisms for *in vitro* culture were obtained from the lungs of Wistar rats which were immunosuppressed for 8 to 12 weeks by subcutaneous biweekly injections of hydrocortisone acetate at a dose of 0.037 g. The animals were given tetracycline in their drinking water (1mg/ml) to prevent bacterial infections and fed standard granulated food *ad libitum*. At the end of immunosuppressive treatment rats were sacrificed by intraperitoneal injection of Morbital (Biowet, Poland) at the dose of 0.5 ml/animal, the lungs were removed and an intensity of infection was determined by examination of impression smears of cross-sections of the lungs stained with Giemsa and Diff-Quik. Fragments of heavily infected lung tissues, free from bacterial and mycotic contaminants, were selected for *Pneumocystis* isolation.

The experiments on animals were performed according to the approval by the IV Ethics Committee for Animal Experimentation (nos. 15/04,10/05).

Isolation of *Pneumocystis*

Selected fragments of lungs were soaked with NKPC buffer (2.68 mM KCl, 1.47 mM KH_2PO_4, 51.10 mM Na_2HPO_4, 7.43 mM NaH_2PO_4, 62.00 mM NaCl, 0.05 mM $CaCl_2$, 0.05 mM $MgCl_2$) containing 10.00 mM dithiotreitol, penicillin (500 i.u./ml), streptomycin (500 μg/ml), and amphotericin B (2.5 μg/ml), then rubbed through a sieve and filtered through a gauze to remove large particles of host material. The resulting filtrate was treated with 0.85% ammonium chloride to lyse erythrocytes and washed 3 times with NKPC buffer by centrifugation, then the obtained sediment was treated with a solution of 0.04% deoxyribonuclease I, type IV, (Sigma) and 0.2% hyaluronidase (Serva) to get rid of the remaining host cells. In the work of Merali et al. [2] only 0.10% DNase I, type IV was used for *Pneumocystis* isolation. After final washings with NKPC buffer, the sediment was resuspended in a small volume of growth medium and the number of *Pneumocystis* was counted to calculate an infectious inoculum. Identification of the isolated organisms to the species level (*P. carinii* or *P. wakefieldiae)* was not performed.

Culture medium and system of *Pneumocystis* cultivation

The growth medium was prepared essentially as described by Merali et al. [2]. It was based on Minimal Essential Medium with Earle›s salts (Gibco) supplemented with 10% horse serum (Gibco), S-adenosyl-l-methionine sulfate (Calbiochem), putrescine (Sigma), ferric pyrophosphate (Sigma), l--cysteine (Sigma), l-glutamine (Sigma), p-aminobenzoic acid (Sigma), N-acetyl-d-glucosamine (Sigma) and antibiotics (penicillin 500 i.u./ml, streptomycin 500 μg/ml – Sigma and amphotericin B 2.5 μg/ml – Serva). The medium was poured into multiwell culture plates (Costar) holding collagen-coated Transwell 24-mm inserts, 0.4 μm pore size.

Pneumocystis organisms isolated from rat lungs were inoculated into the inserts, then the culture plates were incubated at 31°C with twice-daily exchange of medium in wells. The differences in the culture method described above from that reported by Merali et al. [2] depended on the use of amphotericin B in both NKPC buffer and growth medium, and on the reduction of horse serum supplement from 20% to 10%. Moreover, supplementation of the growth medium with S-adenosyl-l-methionine sulfate (SAM) was performed according to later experiments of Merali et al. [4] in which freshly prepared SAM stock solution (10 mM) was added separately twice daily to the culture wells to obtain a final concentration of 500 μM.

The growth of *Pneumocystis* was checked by counting the number of cysts in Giemsa and Diff-Quik stained preparations. To count the number of cysts, two 10 μl samples of the examined culture fluid were placed on two microscope slides, and each of them was spread over an area of 10 cm^2, then dried, fixed and stained. Twenty oil immersion fields in each preparation were randomly scanned and the mean number of cysts per oil immersion field of the two preparations was established. Total number of cysts in 1.0 ml was calculated by multiplying mean number of cysts per oil immersion field by the number of oil immersion fields in 10 cm^2 and by 10^2. Furthermore, the number of *Pneumocystis* was counted with the aid of real-time PCR method based on detection of *Pneumocystis* mitochondrial large subunit rRNA (mtLSUrRNA) gene, common to both species of rat *Pneumocystis – P. carinii* and *P. wakefieldiae* [5]. Amplification of the mtLSUrRNA gene was performed using primer pAZ102E (5'–GATGGC TGTTTCCAAGCCCA–3') and pAZ102H (5'–GT GTACGTTGCAAAGTACTC–3') [6]. It occurred in the following three-step procedure: denaturation at 95°C for 3 min, 45 cycles of denaturation at 95°C

for 5 s, annealing at 56°C for 10 s and extension at 72°C for 20 s. The transition rate of temperature was set at 20°C for denaturation to annealing and for annealing to extension, and at 10°C for extension to denaturation. Sybr Green (Sigma) and Roche's LightCycler were used for thermocycling and detection. The amplification product was heated to 95°C, annealed at 66°C, and then slowly heated from 66°C to 95°C at 0.2°C/s to obtain the melting curve. Real-time PCR determination of mtLSUrRNA gene copy number in the amplification product was preceded each time by construction of a standard curve obtained with known concentrations of *Pneumocystis* DNA copies. By plotting the crossing points (cycle numbers) against the logarithm of concentration of *Pneumocystis* DNA copies, a linear regression line was obtained. The quantity of DNA copies in each sample was calculated by the software provided with the LightCycler after entering data used to generate the curve.

Before reading the results an analysis of a melting curve of the PCR product was performed to confirm the specificity of the reaction. Nonspecific amplification may result in PCR products that melt at temperatures (T_m) above or below that of the desired product. The amplification was considered specific if only one major peak was apparent at T_m of 79.6°C. [7].

Subcultures were made at 7-day intervals and the remaining organisms were collected and stored at –20°C until they were used for production of immune sera and Western-blot examination.

Immune sera

The immune sera were raised in rabbits which had been negative for anti-*Pneumocystis* antibodies in an immunofluorescent test prior to being used in the experiments. The rabbits were injected subcutaneously with a mixture of equal volumes of 1.5×10^7 cysts of *Pneumocystis* from *in vitro* cultures and Freund's complete adjuvant (Difco). After an interval of 4 weeks the animals were boosted with a dose of 7×10^6 cysts in Freund's incomplete adjuvant (Difco) and bled by ear-vein incision after further 2 weeks; the obtained sera were stored at –20°C. Before being used in an immunofluorescent test, dot-ELISA or Western-blot analysis of *P. carinii* antigens, the immune sera were absorbed with minced pieces of normal rat lungs in a proportion 1:1, initially at 37°C for 1 h, then at 4°C for 24 h.

Immunofluorescent examination of *Pneumocystis*

Pneumocystis from *in vitro* cultures were placed on microscope slides, dried and fixed with methanol–acetone (1:1). Smears prepared from lung samples of normal rats served as control antigens. The slides were incubated with absorbed and non-absorbed rabbit immune sera at 1:10 and 1:20 dilutions, then with fluorescein conjugated swine anti-rabbit globulin (DAKO), and viewed under the fluorescence microscope.

Dot-immunobinding method of circulating soluble *Pneumocystis* antigen demonstration (dot-ELISA)

A modification of the method described by Brooks et al. [8] for detection of soluble *Toxoplasma* antigen was used in the studies. In brief, 5 μl samples of either serum or bronchoalveolar lavage (BAL) from immunosuppressed rats were spotted on strips of nitrocellulose paper (Serva) and allowed to dry. Afterwards, the strips were soaked in 10% solution of foetal bovine serum (FBS – Gibco) in TBS buffer (20 mM Tris-Cl, 150 mM NaCl, pH 7.4) for 1 h, and transferred to a Petri dishes containing a 1:200 solution of rabbit immune serum in 3% FBS. After incubation, the strips were washed three times with 0.05% solution of Tween 20 in TBS, and incubated for 1.5 h in a 1:5000 solution of a peroxidase conjugated goat anti-rabbit antibody (Cappel Labs, Cochranville, USA). Substrate solution contained 4-chloro-1-naphtol and hydrogen peroxide. Results were read visually.

Positive control samples (K^+) were prepared by adding 1 μg proteins of homogenate of *Pneumocystis* from *in vitro* cultures to 5 μl of normal rat serum. Negative controls (K^-) consisted of normal rat serum samples.

Western-blot analysis of *Pneumocystis* antigen

The method used in the present studies was a modification of that described by Towbin et al. [9]. Initially, antigen samples of *Pneumocystis* from rat lungs and from *in vitro* cultures were suspended in 2 ml of phosphate buffered saline containing 1 mM PMSF (phenylmethylsulphonyl fluoride) and disrupted by repeated freezing and thawing. After clearing by centrifugation the supernatants containing 30 μg proteins/15 μl were mixed with an equal volume of Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8), then heated at 100°C for 5 min. Proteins were resolved

Table 1. Influence of inoculum size on the growth rate of *Pneumocystis* in primary axenic cultures

No. of cysts in inoculum (ml^{-1})	9.0×10^4	9.5×10^4	1.0×10^5	2.0×10^5	3.0×10^5	4.0×10^5	5.0×10^5	5.5×10^5	6.0×10^5	6.5×10^5
No. of cysts in culture (ml^{-1})*	2.4±0.6 $\times10^6$	3.6±1.0 $\times10^6$	1.6±0.4 $\times10^7$	3.7±0.7 $\times10^7$	7.7±0.8 $\times10^7$	8.5±0.8 $\times10^7$	8.5±0.8 $\times10^7$	6.9±0.9 $\times10^7$	1.4±0.4 $\times10^7$	5.2±2.6 $\times10^6$
Growth rate (x-fold increase)*	27±7	38±10	160±39	185±57	257±28	213±18	170±15	125±16	23±6	8±4

* data represent the mean calculated from 10 independent experiments

by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 4% stacking gel, 12% resolving gel), and transferred to nitrocellulose sheets using Trans-Blot SD transfer cell (Bio-Rad). The sheets were blocked by incubation in 10% solution of FBS in TBS, washed with 0.05% Tween 20 in TBS and incubated with a 1:200 dilution of immune rabbit sera for 16 h at 4°C, then in a 1:1500 dilution of a peroxidase conjugated swine anti-rabbit IgG (Cappel). The reactions were developed using 4-chloro-1-naphtol with addition of hydrogen peroxide as a substrate. Each SDS-PAGE gel was loaded with a prestained molecular weight marker (Precision Plus Protein Standards, dual color, Bio-Rad) for molecular weight determination on gels and monitoring transfer efficiency on blots.

Results

Preliminary studies demonstrated that the growth of *Pneumocystis* in cultures was dependent on the number of organisms in an infecting inoculum. A peak of a 257-fold increase in cysts was achieved with an inoculum size of 3×10^5 organisms; higher or lower inoculum sizes produced less growth, especially inocula using less than 10^5 organisms or above 5.5×10^5 (Table 1). On the basis of the above findings infecting doses approximating 3×10^5 cysts/ml^{-1} were used in all subsequent studies.

When the numbers of *Pneumocystis* DNA copies were measured with a real-time PCR method, a 286-fold increase was found as a mean of 10 independent experiments with 7-day old primary cultures initiated at various times using different inoculum sources, which corresponded to the mean number of 2.0×10^9 DNA copies per ml of a culture (Table 2). Because prolongation of the culture time beyond day 7 did not improve the results, subpassages were made at weekly intervals.

High growth of organisms was usually noted in the primary and the first three subcultures, reaching in primary cultures an average of 175-fold increase in the number of cysts and the above mentioned 286-fold-increase in DNA copies. After 28 days of

Table 2. Growth rate of *Pneumocystis* in successive axenic subcultures

Subculture no. (length of cultivation in days)	primary (7 days)	1 (14 days)	2 (21days)	3 (28 days)	4 (35 days)	5 (42 days)
No. of cysts in inoculum (ml^{-1})	$3.1\pm1.4\times10^5$	$2.6\pm1.4\times10^5$	$2.2\pm0.9\times10^5$	$3.0\pm0.9\times10^5$	$2.5\pm0.7\times10^5$	$2.4\pm0.9\times10^5$
No. of cysts in culture (ml^{-1})	$5.3\pm3.3\times10^7$	$4.3\pm3.2\times10^7$	$3.6\pm2.2\times10^7$	$4.3\pm2.5\times10^7$	$1.7\pm0.8\times10^7$	$1.0\pm1.0\times10^7$
Growth rate (x-fold increase)	175±45	163±58	150±42	143±65	64±18	37±31
No. of DNA copies in inoculum (ml^{-1})	$8.1\pm8.1\times10^6$	$1.3\pm0.8\times10^7$	$1.1\pm1.1\times10^7$	$2.1\pm2.6\times10^7$	$1.3\pm0.9\times10^7$	$3.4\pm3.3\times10^7$
No. of DNA copies in culture (ml^{-1})	$2.0\pm1.7\times10^9$	$3.5\pm2.9\times10^9$	$2.2\pm2.5\times10^9$	$3.4\pm3.5\times10^9$	$1.7\pm1.5\times10^9$	$2.7\pm2.8\times10^9$
x-fold increase in DNA copies	286±144	258±105	231±74	215±77	146±70	97±49

*all data represent the mean calculated from 10 independent experiments

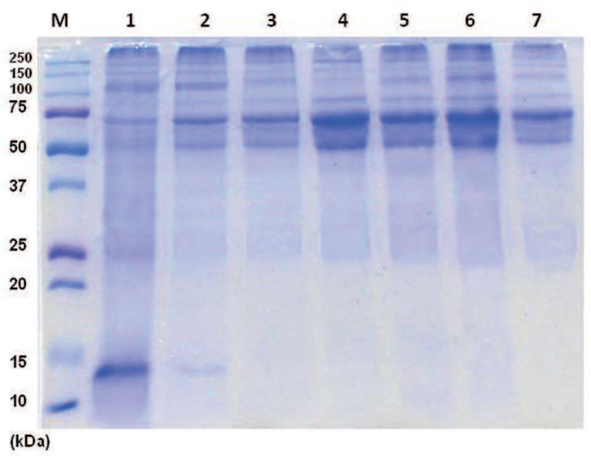

Fig. 1. Coomassie brilliant blue stained SDS-PAGE gel showing fractionation of *Pneumocystis* antigen from *in vitro* cultures.
Lane M – molecular weight standard; 1 – antigen from infectious inoculum; 2 – primary culture (7th day); 3 – 1st subculture; 4 – 2nd subculture; 5 – 3rd subculture; 6 – 4th subculture; 7 – 5th subculture.

the culture the rate of growth dropped down to the level of 37 – 64-fold increase in the number of cysts/ml or 97 – 146-fold increase in the number of DNA copies/ml (Table 2). The studies demonstrated a possibility of maintaining *Pneumocystis* in cultures for at least 6 weeks, however, some of the growing organisms showed morphological changes beginning from 28 to day 35 of the culture.

Pneumocystis collected from *in vitro* cultures in the amount of 8.9×10^9 cysts was used to raise immune sera in rabbits. The immune sera after being absorbed with rat lung tissues did not react with rat lung antigens in the immunofluorescent antibody test, but showed an intense fluorescence

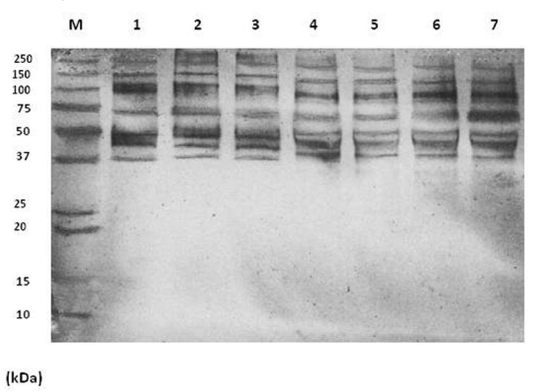

Fig. 2. Western blot analysis of *Pneumocystis* antigen from *in vitro* cultures.
Lane M – molecular weight standard; 1 – antigen from infectious inoculum; 2 – primary culture (7th day); 3 – 1st subculture; 4 – 2nd subculture; 5 – 3rd subculture; 6 – 4th subculture; 7 – 5th subculture.

with *Pneumocystis* from *in vitro* cultures. Preparations of *Pneumocystis* which was isolated from rat lungs for infectious inocula, apart from reaction with rabbit immune sera, produced non-specific fluorescence with fluorescein-conjugated anti-rat globulins.

SDS-PAGE resolved antigens of *Pneumocystis* from infectious inocula showed 8 antigenic fractions estimated at 200, 150, 120, 90, 70, 50, 25, and 13 kDa, whereas the antigens of *Pneumocystis* collected from 5 consecutive *in vitro* subcultures demonstrated the presence of only 6 of the above fractions, all in the range of 200–50 kDa (Fig. 1). Fractions having molecular weights of 13 and 25 kDa were present in trace amounts in antigens prepared from primary cultures, but were virtually absent from subculture preparations.

Western-blot analysis of the antigenic preparations confirmed the presence of 6 fractions in the range of 200–50 kDa in both kinds of the examined preparations. Furthermore, the analysis showed an absence of 25 and 13 kDa bands, and revealed 2 additional ones at 45 and 37 kDa (Fig. 2)

Dot-ELISA examination of serum and BAL samples collected from immunosuppressed rats demonstrated soluble *Pneumocystis* antigens. Serum samples were positive for the antigens in week 11 and 12 of immunosuppression, whereas BAL samples were positive in the period between 6 and 12 weeks of immunosuppression (Fig. 3).

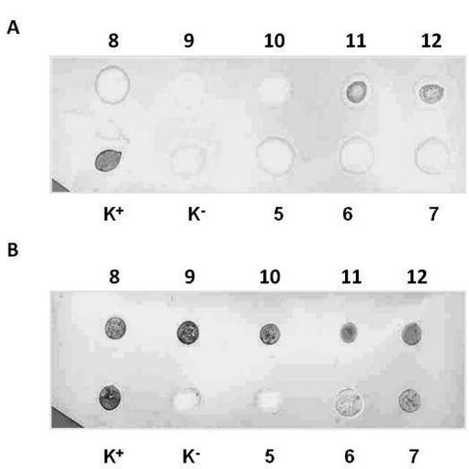

Fig. 3. Results of dot-ELISA examination for circulating *Pneumocystis* antigen of serum (A) and bronchoalveolar lavage (B) samples from immunosuppressed rats.
K^+ – positive control sample; K^- – negative control sample; 5–12 numbers denote weeks of immunosuppressive treatment of rats at which samples were taken for examination.

Discussion

The results of the studies described above demonstrated the possibility of a long-term cultivation of rat *Pneumocystis* in axenic cultures. In contradistinction to the negative opinion of Larsen and his colleagues [3] on the uselessness of Merali's method for *Pneumocystis* multiplication, the results of the present studies proved very optimistic testifying to the value and usefulness of the questioned method. Moreover, successful attempts at axenic cultivation of *Pneumocystis* according to a modified Merali's method have also been reported by Chinese workers [10] who received 5 continuously and axenically cultured isolates from bronchoalveolar lavage fluid of infected rats.

Pneumocystis collected during the phase of intensive *in vitro* multiplication, i.e., up to day 28 of culture, appeared morphologically indistinguishable from the organisms occurring in rat lungs, as observed by ordinary light microscopy of Giemsa and Diff-Quik-stained preparations. Furthermore, no differences between *in vivo* and *in vitro* grown organisms have been demonstrated by SDS-PAGE and Western-blot methods, because 2 additional bands present in the pattern of the resolved antigen of *Pneumocystis* from infectious inoculum were identified as contaminants of rat origin. Most of the main bands, i.e. those estimated at 120 kDa, 90 kDa, and 50 kDa were described in earlier studies [11,12]. The 120 kDa fraction belongs to a 90–140 kDa major surface glycoprotein complex termed „MSG" or „gp120" which is a group of related glycoproteins encoded by a multigene family, and capable of antigenic variation [13], whereas the 50 kDa fraction is the major circulating antigen in *Pneumocystis*-infected rats [11].

Final part of the work was devoted to the studies on the immunogenic properties of *Pneumocystis* collected during *in vitro* cultivation. To achieve this end the collected organisms were tested for the capacity to induce an antibody response. The response was easily provoked in rabbits by injecting them with a mixture of *Pneumocystis* and adjuvant. The immune sera obtained from these rabbits proved very valuable in a Western-blot analysis of *Pneumocystis* antigens and in an immunodiagnostic tests, enabling to detect circulating *Pneumocystis* antigens in BAL and serum samples of infected rats. In agreement with some previous studies [14], during the course of induced infections circulating antigens were detected earlier in the BAL samples than in rat sera.

The described above production of diagnostic antisera is so far the main advantage of this successful *in vitro* cultivation of *Pneumocystis* in axenic media. Future studies should concentrate on the use of the cultures for testing new anti-*Pneumocystis* drugs.

References

[1] Latorre C., Sulzer A.J., Norman L.G. 1977. Serial propagation of *Pneumocystis carinii* in cell line culture. *Applied and Environmental Microbiology* 33: 1204-1206.

[2] Merali S., Frevert U., Williams J.H., Chin K., Bryan R., Clarkson A.B. 1999. Continuous axenic cultivation of *Pneumocystis carinii. Proceedings of the National Academy of Sciences USA* 96: 2402-2407.

[3] Larsen H.H., Kovacs J.A., Stock F.M., Vestereng V.H., Lundgren B., Fischer S.H., Gill V.J. 2002. Development of rapid real-time PCR assay for quantitation of *Pneumocystis carinii f. sp. carinii*. *Journal of Clinical Microbiolgy* 40: 2989–2993.

[4] Merali S., Vargas M., Franklin M., Clarkson A.B. 2000. S-adenosylmethionine and *Pneumocystis carinii. Journal of Biological Chemistry* 275: 14958-14963.

[5] Cushion M.T., Keely S.P., Stringer J.S. 2004. Molecular and phenotypic description of *Pneumocystis wakefieldiae* sp. nov., a new species in rats. *Mycologia* 96: 429-488.

[6] Wakefield A.E., Pixley F.J., Banerji S., Sinclair K., Miller R.F., Moxon E.R., Hopkin J.M. 1990. Detection of *Pneumocystis carinii* with DNA amplification. *Lancet* 336: 451-453.

[7] Kaiser K., Rabodonirina M., Picot S. 2001. Real time quantitative PCR and RT-PCR for analysis of *Pneumocystis carinii hominis*. *Journal of Microbiological Methods* 45: 113-118.

[8] Brooks R.G., Sharma S.D., Remington J.S. 1985. Detection of *Toxoplasma gondii* antigens by a dot-immunobinding technique. *Journal of Clinical Microbiolgy* 21: 113-116.

[9] Towbin H., Staehelin T., Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proceedings of the National Academy of Sciences USA* 76: 4350-4354.

[10] Huang M.J., An Y.J., Li S.Z., Lu S.Q., Guo Z.Z. 2007. Continuous axenic cultivation of *Pneumocystis carinii* isolated from the bronchoalveolar lavage fluid of infected rat. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*. (*Chinese Journal of Parasitology and Parasitic Diseases*) 25: 129-132.

[11] Ishimaru T., Shimono N., Sawae Y., Niho Y. 1992. Purification of *Pneumocystis carinii* trophozoites and identification of their circulating antigens. *Journal of Clinical Microbiology* 30: 3263-3267.

[12] Pascale J.M., Shaw M.M., Bartlett M.S., Smith J.S., Mclaughlin G.L. 1996. Western blot analysis of rat *Pneumocystis carinii* antigens using convalescent sera and rabbit anti-gp 120. *Journal of Eukaryotic Microbiology* 43: 30S.

[13] Theus S.A., Smulian A.G., Steele P., Linke M.J., Walzer P.D. 1998. Immunization with the major surface glycoprotein of *Pneumocystis carinii* elicits a protective response. *Vaccine* 16: 1149-1157.

[14] McNabb S.J.N., Graves D.C., Kosanke S.D., Moyer M.J., Ivey M.H. 1988. *Pneumocystis carinii* antigen detection in rat serum and lung lavage. *Journal of Clinical Microbiolgy* 26: 1763-1771.

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