

POLYMERASE CHAIN REACTION FOR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN PAPANICOLAOU-STAINED FINE NEEDLE ASPIRATED SMEARS FOR DIAGNOSIS OF CERVICAL TUBERCULOUS LYMPHADENITIS

Chavit Chantranuwat, Thamathorn Assanasen, Shanop Shuangshoti and Pichet Sampatanukul

King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Abstract. A polymerase chain reaction (PCR) protocol for detecting IS6110 repetitive insertion sequence of *Mycobacterium tuberculosis* (MTB) was tested on archival Papanicolaou (Pap)-stained fine needle aspirated (FNA) smears from 24 patients with cervical tuberculous lymphadenopathy and 30 negative controls. The protocol involved protease digestion or phenol-chloroform extraction, and simple or nested PCR, with PCR amplification of human β -globin gene for internal control of DNA quality. Sensitivity of 50% and specificity of 100% were obtained. Sensitivity in smears showing necrosis without granuloma was 70% (7/10), whereas it was 36% (5/14) in smears with presence of granuloma. On the other hand, sensitivity of 18% (4/22) was obtained using FNA acid-fast stain, 25% (1/4) for acid-fast stain in histological section, 50% (2/4) for culture, and 100% (8/8) for PCR of fresh specimens. PCR for MTB detection in Papanicolaou-stained slides is a practical and valuable method when no fresh specimen but only Pap-stained smear is available.

INTRODUCTION

Tuberculosis, an infection of *Mycobacterium tuberculosis* complex (MTB), is a worldwide infection of large magnitude in terms of morbidity and mortality, especially in developing countries. There are three conventional methods of laboratory diagnosis, morphologic examination (cytology and histology), acid-fast stain, and culture for mycobacterium. Each method possesses different advantages and disadvantages. Histopathology of necrotizing or caseating granulomatous inflammation combined with proper clinical manifestation provides higher sensitivity but lower specific-

ity in diagnosing MTB infection than acid-fast stain and culture. Acid-fast stain is the cheapest but has the lowest sensitivity and can not differentiate MTB from non-MTB, such as *Mycobacterium avium* complex. Culture, with better sensitivity, can identify specific species of mycobacterium and anti-TB drug susceptibility test can be performed, but it needs weeks to be accomplished.

In the past decade, advance in molecular techniques, particularly polymerase chain reaction (PCR), have made tests more rapid with high sensitivity and specificity for MTB. The technique has been applied to two major types of specimen; firstly, fresh specimens including clinical sample from respiratory tract, fine needle aspiration (FNA), and fresh tissue biopsy (Kearns *et al*, 1998; Singh *et al*, 2000; Watterson and Drobniowski, 2000), and secondly, formalin-fixed paraffin-embedded tissue biopsy from cervical lymph node and lung (Marchetti *et al*, 1998; Salian *et al*, 1998;

Correspondence: Dr Chavit Chantranuwat, Department of Pathology, Chulalongkorn Memorial Hospital Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand.

Tel: 66 (0) 2256-4235 ext 211; Fax: 66 (0) 2652-4208

E-mail: chavit_ch@hotmail.com

Hirunwiwatkul *et al*, 2002; Park *et al*, 2003). All previous studies revealed higher sensitivity as compared to other conventional laboratory tests with very high specificity reaching 100%. In practice, it is not uncommon to have only formalin-fixed tissue and no fresh tissue available for PCR. In this situation, using paraffin-embedded tissue for PCR becomes routine. PCR on paraffin embedded tissue revealed good agreement with acid-fast stain, culture, and resolution of a case as being tuberculosis (Salian *et al*, 1998).

For cytological specimens, various fresh clinical samples can be used for PCR. However, a system to preserve fresh specimen during transportation, such as freezing in liquid nitrogen or storing in refrigerator at -20°C, is required. These methods are not practical and not available in many places, especially in clinics or remote hospitals in Thailand. Therefore, it would be beneficial if PCR can be performed using routine Papanicolaou (Pap) stained smears. The Pap-stained slides can be sent easily and safely from primary health care providers to tertiary centers where PCR testing is available. Previous data have shown that DNA and even mRNA in archival Pap-stained cytological specimens are well preserved (de Roda Husman *et al*, 1995; Chen *et al*, 1996; Chuaqui *et al*, 1999; Mitteldorf *et al*, 2002). Specimens were successfully used for PCR analysis with high sensitivity and specificity.

In King Chulalongkorn Memorial Hospital, Bangkok, a large number of patients with cervical tuberculous lymphadenitis are encountered. There is a trend towards diagnosing tuberculous lymphadenitis on the basis of FNA cytology with decrement of unnecessary tissue biopsy. To the best of our knowledge, no study of PCR for detection of MTB has been previously conducted in Pap-stained slides of cervical lymph node FNA. There is only one case report diagnosing tuberculosis of the cervix by PCR using Pap-stained cervi-

cal smear (Ferrara *et al*, 1999). The objective of this study was to evaluate sensitivity and specificity of a PCR protocol for detecting MTB on archival Pap-stained FNA smears for the diagnosis of cervical tuberculous lymphadenopathy.

MATERIALS AND METHODS

During April 2001 to January 2002, at King Chulalongkorn Memorial Hospital University, 42 patients were presented with cervical lymphadenopathy, of which FNA revealed granulomatous inflammation and/or necrotic material. Twenty-four cases were assigned to tuberculous lymphadenitis group by demonstrating at least one of the following criteria: 1) positive acid-fast stain in FNA smear, 2) positive acid-fast stain in histologic section, 3) positive PCR for MTB from fresh tissue or aspirated specimen, 4) positive culture for MTB from fresh tissue or aspirated specimen, and 5) lacking the former four criteria, but showing compatible clinical and response to anti-TB drug treatment. Negative control group comprised 30 randomly selected cases of cervical lymphadenopathy unrelated to tuberculosis, as supported by cytological examination, surgical pathology, and clinical data.

The collected clinical data included patient's age, sex, follow-up, and response to treatment. The Pap-stained FNA smeared slides of all cases were reviewed to verify the cytological diagnoses, and their cytological findings were recorded. Acid-fast stain results of the smears were recorded. Surgical specimens were reviewed and acid-fast stain results were recorded if available. In the case where fresh tissue or fresh aspirated specimen was submitted to PCR or culture, the result was recorded.

PCR protocol

PCR protocol was composed of three steps. In step 1, PCR for MTB (0.25 µg of DNA in 25 µl total reaction volume) was performed.

A positive result indicated the case as being positive. If the PCR was negative, then PCR for human β -globin gene was performed. Positive PCR for human β -globin gene indicated that the negative PCR for MTB was a true negative. In the case where PCR for human β -globin gene was negative, the sample was further diluted (1:4 of first dilution) and PCR for human β -globin gene was re-tested (step2). If the PCR was positive, then nested PCR for MTB was performed, and either positive or negative result was accepted. In the case where PCR for human β -globin gene of the second dilution was still negative, phenol-chloroform DNA extraction was performed on the initial digested sample in order to eliminate any inhibitory effect that might be present (step3). The extract was then subjected to nested PCR for MTB and PCR for human β -globin gene (0.25 μ g of DNA in 25 μ l total reaction volume). Positive nested PCR for MTB indicated a positive case. Negative nested PCR for MTB with positive PCR for human β -globin gene indicated a negative case. Negative nested PCR for MTB together with negative PCR for human β -globin gene indicated an inconclusive case.

PCR and nested PCR for IS6110 sequence were selected because IS6110 is a repeated insertion sequence that is present in multiple locations in MTB genome. PCR of the IS6110 sequence showed the highest sensitivity compared to PCR of other gene loci (Marchetti *et al*, 1998). We selected the 30-bp outer primer and the 20-bp inner primer from the fourth PCR procedure in the study of Marchetti *et al* (1998). The sequences of the primers are as follow: outer primers, 5'-CGG GAC CAC CCG CGG CAA AGC CCG CAG GAC-3' (nucleotide 695-724) and 5'-CAT CGT GGA AGC GAC CCG CCA GCC CAG GAT-3' (nucleotide 885-914), to amplify 220 bp fragment; and inner primers, 5'-CCT GCG AGC GTA GGC GTC GG-3' and 5'-CTC GTC CAG CGC CGC TTC GG-3', to amplify 123 bp pro-

duct. The length of the amplified product is the shortest among those in previous studies reviewed (Marchetti *et al*, 1998; Singh *et al*, 2000; Hirunwiwatkul *et al*, 2002; Park *et al*, 2003). Selecting shorter DNA sequence for amplification is to avoid false negative results due to possible fragmentation of genomic DNA in the archival specimen (Greer *et al*, 1991). In this study, the inner primers were used for PCR, while both inner and outer primers were used for nested PCR. PCR for human β -globin gene was performed simultaneously as internal control to evaluate the quality of the DNA and to detect inhibitor effect that might be present. Every batch of PCR has negative and positive controls. The negative control was distilled water and the positive control was DNA extracted from Pap-stained aspirated smears of cases known to have positive MTB culture.

All the Pap-stained smears were de-stained using 1% HCl solution to remove hematoxylin and aluminum sulfate (Chen *et al*, 1996). Each smear was then scraped using new clean razor blade. The specimens were digested with proteinase K (1.82 mg/ml) in digestion buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl₂, 0.1 mg/ml gelatine, 0.45% Nonidet P40, and 0.45% Tween 20) at 53°C for 16 hours and the reaction was terminated at 95°C. The extracts were then measured for DNA and kept at -20°C for PCR tests.

Phenol-chloroform extraction was performed when the second dilution PCR for human β -globin gene revealed no product. DNA in the digested sample was purified by phenol-chloroform-isoamyl alcohol (25:24:1 v/v) extraction, precipitated by ethanol, and dissolved in 20 μ l of molecular biology grade water.

PCR procedure for human β -globin gene was adopted from that of Greer *et al* (1991). The total reaction volume was 25 μ l and composed of 0.2 mM dNTP each, 1.5 mM MgCl₂,

0.625 unit of Taq DNA polymerase (Invitrogen®), 2.5 µl of 10x Taq polymerase buffer without MgCl₂ (supplied with the enzyme), PC04 and GH20 primers (0.1 mM each), and 2 µl of DNA sample (0.25 µg DNA). PCR cycles included an initial denaturation step at 94°C for 5 minutes followed by 40 cycles of the followings three steps, 1) 94°C for 45 seconds, 2) 60°C for 30 seconds, and 3) 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

PCR procedure for MTB consisted of a total reaction volume of 25 µl containing 0.2 mM dNTP each, 2.0 mM MgCl₂, 0.625 unit of Taq DNA polymerase (Qiagen®), 2.5 µl of 10x Taq polymerase buffer (supplied with the enzyme), outer primers or inner primers (0.1 µM each), and 2 µl of DNA sample (0.25 µg). For outer primers, PCR cycles included an initial denaturation at 94°C for 5 minutes, followed by 40 cycles of the followings three steps, 1) 94°C for 45 seconds, 2) 63°C for 30 seconds, and 3) 72°C for 1 minute, and a final extension at 72°C for 10 minutes. For inner primers, PCR cycles included an initial denaturation at 94°C for 5 minutes, followed by 5 cycles of the followings three steps, 1) 94°C for 45 seconds, 2) 65°C to 61°C (decreasing 1°C per cycle) for 30 seconds, and 3) 72°C for 1 minute, followed by 35 cycles of the followings three steps, 1) 94°C for 45 seconds, 2) 60°C for 30 seconds, and 3) 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

For analysis of the amplified products, 10 µl of each reaction solution was electrophoresed in 2% agarose gel containing ethidium bromide (1 µg/ml) and visualized under UV transillumination.

RESULTS

Typical results of PCR are shown in Fig 1. Characteristics and PCR results of study and control groups are shown in Tables 1 and 2. The patients in study group were mainly young adults. The slightly older average age

in control group was due to the presence of 5 relatively older patients with metastatic carcinomas. Almost of the FNA in the study group revealed necrosis (22/24, 92%) and nearly half (10/24, 42%) showed necrosis without pre-

Table 1
Characteristics and PCR results of tuberculous lymphadenitis group (n=24).

Male: Female	8:16 (1:2)
Average age	33 years (25 to 52 years)
FNA findings (n=24)	
granuloma	14/24 (58%)
without necrosis	2
with necrosis	12
no granuloma	10/24 (42%)
acute/ suppurative	2
necrotizing/ caseous	8
acid-fast positive	4/22 (18%)
Biopsy findings (n=7)	
granulomatous, necrotizing	6
organizing inflammation	1
acid-fast positive	1/4 (25%)
MTB PCR, fresh specimen (n=8)	
tissue PCR positive	1/1 (100%)
FNA PCR positive	7/7 (100%)
Culture (n=4)	
positive for MTB	2/4 (50%)
Follow-up (n=21)	
improved after anti-TB drugs	21/21 (100%)
PCR of Pap stained slides (n=24)	
	step 1 step 2 step 3 total
granuloma (n=14)	
positive	4 0 1 5
negative	6 0 1 7
inconclusive	0 0 2 2
no granuloma (n=10)	
positive	7 0 0 7
negative	3 0 0 3
inconclusive	0 0 0 0

Table 2
 Characteristics and PCR results of control group (n=30).

Male: Female	10:20 (1:2)			
Average age	40.1 years (16 to 65 years)			
FNA findings (n=30)				
Necrotizing granulomatous lymphadenitis				
- resolved as Kikuchi-Fujimoto lymphadenitis				1
- resolved as SLE related lymphadenopathy				1
reactive hyperplasia				23
metastatic carcinoma				5
Biopsy findings (n=2)				
necrotizing histiocytic lymphadenitis				2
acid-fast positive				0/2
PCR, Pap stained slides (n=30)				
	step1	step2	step3	total
positive	0	0	0	0
negative	16	6	3	25
inconclusive	0	0	5	5

sence of granulomatous fragment. Of 21 cases with follow-up in the study group, all showed clinical improvement after anti-TB drug administration. The negative control group was composed of 23 cases of reactive hyperplasia, 5 cases of malignancy, 1 case of SLE related lymphadenitis, and 1 case of Kikuchi-Fujimoto lymphadenitis.

Based on the study group, an overall sensitivity of the PCR protocol on archival Pap-stained aspirated smears for diagnosis of tuberculous lymphadenitis was 50% (12/24). The overall sensitivity of PCR was in the middle range, compared to acid-fast stain of FNA smears (18%, 4/22), acid-fast stain of histological sections (25%, 1/4), culture (50%, 2/4), and nested PCR of fresh specimen (100%, 8/8). It is noted that the sensitivity for FNA that contain no granuloma but mainly necrosis and caseous material, or suppurative inflammation was higher (70%, 7/10) than that of FNA showing granuloma with or without necrosis (36%, 5/14). Since there was no positive PCR in

negative control group, specificity was 100%.

DISCUSSION

Previous studies on sensitivity and specificity of PCR for MTB using processed specimens were mainly based on paraffin-embedded tissue (Marchetti *et al*, 1998; Salian *et al*, 1998; Hirunwiwatkul *et al*, 2002; Park *et al*, 2003). A recent study successfully performed PCR for MTB using Ziehl-Neelsen acid-fast stained sputum smears (Tansuphasiri *et al*, 2004). The current study is the first to evaluate the value of PCR for diagnosing MTB infection using Pap-stained smears. Data from paraffin-embedded tissue studies revealed high and varying sensitivities and specificities. The best results (when multiple methods were tested in study) ranged from 78 to 100% and 88 to 100%, for sensitivity and specificity, respectively. The cause of this variation is attributed to various factors including inclusion criteria, gold standard for study group, speci-

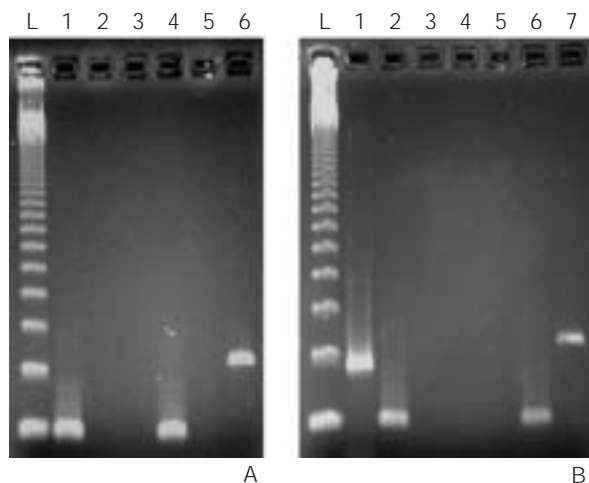


Fig 1—Examples of PCR results. PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. A) PCR for MTB in step1: lane 1, positive control; lane 2, distilled water; lane 3, negative control; lanes 4 and 5, positive and negative samples, respectively; lane 6, β -globin gene PCR product. B), nested PCR for MTB in tep 2: lanes 1 and 2, 1st and 2nd rounds PCR product of positive control; lanes 3 and 4, 1st and 2nd rounds PCR product of negative control; lanes 5 and 6, 1st and 2nd rounds PCR product of positive sample; lane 7, β -globin gene PCR product. Lane L, 123 base pair DNA ladder (Invitrogen, California, USA).

men factors, and internal variation of the PCR method. DNA target for PCR, extraction method, and DNA concentration in the reaction solution were all shown to affect the sensitivity and specificity (de Roda Husman *et al*, 1995; Marchetti *et al*, 1998; Tansuphasiri *et al*, 2004). Study using clinical diagnosis of tuberculosis as gold standard resulted in lower sensitivity than those using laboratory tests, such as positive culture or acid-fast stain as gold standard (Salian *et al*, 1998; Park *et al*, 2003; Tansuphasiri *et al*, 2004). The storage age of specimen could also affect the sensitivity, possibly more significantly in tropical areas, where storage temperature is higher (Hirunwiwatkul *et al*, 2002; Tansuphasiri *et al*, 2004).

Our protocol was developed in order to balance all factors considered important including sensitivity, specificity, time spent, ease of work, and cost of the test. Although possessing lower sensitivity than nested PCR, normal PCR was the chosen method in the first step because it reduced the cost, technical complexity, and risk for contamination. Simple digestion with protease was the method of choice for DNA extraction since it was easier to perform and showed good efficacy as compared to GTC/silica bead and freeze-thaw method (de Roda Husman *et al*, 1995). Although phenol-chloroform extraction gave better DNA quality for the molecular test, it is time consuming and involves more complex tasks, which is less convenient in a laboratory with high workload and few personnel. With the protocol described herein, 67% (36/54) of the cases were judged as being either as positive or negative in step 1. As much as 92% (11/12) of the PCR positive cases were identified in the first step, with only one remaining positive case identified in the third step. Identification in the first step reduces the time used and cost of the tests.

The overall 50% sensitivity of the PCR in the current study is relatively low, compared to studies using paraffin sections and fresh tissue samples. The sensitivity might be improved if the negative PCR samples in the first step were subsequently subjected to nested PCR or the nested PCR was performed in the first step. Other factors that also affect the sensitivity include 1) amount of cytologic material in the smears, 2) cytologic features, 3) inhibitory substances in Pap stain, and 4) age of specimen. Of six smears containing scant cytologic material, four are inconclusive by the PCR. Very low DNA content was also present in these cases. The results show a possible trend towards higher sensitivity in smears containing more necrosis than smears with less necrosis and more granulomata. The smears with less necrosis or containing only granulo-

matous fragments might contain fewer organisms. A study using paraffin embedded tissue showed histopathologic findings of necrosis, langhan cell, and well-formed granuloma to be associated with more to less positive PCR results (Park *et al*, 2003). Of 54 cases, inhibitory effects from the Pap smears were noted in 5 (9%) cases in which human β -globin gene could only be amplified by phenol-chloroform extraction in the third step. This suggests that the simple acid decolorization might not be able to remove inhibitors in a small proportion of cases. We also noted that the measured DNA contents in some specimens were in the range for PCR but amplification of human β globin gene was still negative. Lastly, the storage age of the specimen has been shown to influence the PCR results. (Hirunwiwatkul *et al*, 2002; Tansuphasiri *et al*, 2004). Our specimens were from April 2001 to Jan 2002, 3 to 4 years of storage before the PCR tests were performed in early 2005.

In summary, the study documented 50% overall sensitivity and 100% specificity of a PCR protocol for the detection of MTB in archival Pap-stained aspirated smears from patients with cervical lymphadenopathy. It has a medium-range sensitivity and should be used in conjunction with other tests for better sensitivity rather than to be used alone. Apart from speed of the test, using Pap-stained slide for PCR has several additional appealing properties, including convenience and safety of specimen transportation, and an ability to make cytological evaluation in the same slide prior to making decision for PCR. When there is no fresh specimen or paraffin embedded tissue available, this PCR method can be a valuable option to be considered when dealing with necrotizing and granulomatous lymphadenitis.

ACKNOWLEDGEMENTS

The study was supported by Ratchadapiseksompotch Fund, Faculty of Medicine,

King Chulalongkorn Memorial Hospital Chulalongkorn University, Bangkok, Thailand. The study was reviewed and approved by King Chulalongkorn Memorial Hospital ethics committee.

REFERENCES

- Chen JT, Lane MA, Clark DP. Inhibitors of the polymerase chain reaction in Papanicolaou stain. Removal with a simple destaining procedure. *Acta Cytol* 1996; 40: 873-7.
- Chuaqui R, Cole K, Cuello M, Silva M, Quintana ME, Emmert-Buck MR. Analysis of mRNA quality in freshly prepared and archival Papanicolaou samples. *Acta Cytol* 1999; 43: 831-6.
- de Roda Husman AM, Snijders PJ, Stel HV, van den Brule AJ, Meijer CJ, Walboomers JM. Processing of long-stored archival cervical smears for human papillomavirus detection by the polymerase chain reaction. *Br J Cancer* 1995; 72: 412-7.
- Ferrara G, Cannone M, Guadagnino A, Nappi O, Barberis MC. Nested polymerase chain reaction on vaginal smears of tuberculous cervicitis. A case report. *Acta Cytol* 1999; 43: 308-12.
- Greer CE, Peterson SL, Kiviat NB, Manos MM. PCR amplification from paraffin-embedded tissues: Effects of fixative and fixation time. *Am J Clin Pathol* 1991; 95: 117-24.
- Hirunwiwatkul P, Tumwasorn S, Chantranuwat C, Sirichai U. A comparative study of diagnostic tests for tuberculous lymphadenitis: polymerase chain reaction vs. histopathology and clinical diagnosis. *J Med Assoc Thai* 2002; 85: 320-6.
- Kearns AM, Freeman R, Steward M, Magee JG. A rapid polymerase chain reaction technique for detecting *M. tuberculosis* in variety of clinical specimens. *J Clin Pathol* 1998; 51: 922-24.
- Marchetti G, Gori A, Catozzi L, *et al*. Evaluation of PCR in detection of *Mycobacterium tuberculosis* from formalin-fixed, paraffin-embedded tissues: comparison of four amplification assays. *J Clin Microbiol* 1998; 36: 1512-7.
- Mitteldorf CA, Leite KR, Darini E, Carvalho CM, Camara-Lopes LH. Optimal recovery of DNA

- for polymerase chain reaction-based assays from fine needle aspirates. A comparison of four preparation types. *Acta Cytol* 2002; 46: 1117-22.
- Park DY, Kim JY, Choi KU, *et al.* Comparison of polymerase chain reaction with histopathologic features for diagnosis of tuberculosis in formalin fixed, paraffin embedded histologic specimens. *Arch Pathol Lab Med* 2003; 127: 326-30.
- Salian NV, Rish JA, Eisenach KD, Cave MD, Bates JH. Polymerase chain reaction to detect *Mycobacterium tuberculosis* in histologic specimens. *Am J Respir Crit Care Med* 1998; 158: 1150-5.
- Singh KK, Muralidhar M, Kumar A, *et al.* Comparison of in house polymerase chain reaction with conventional techniques for the detection of *Mycobacterium tuberculosis* DNA in granulomatous lymphadenopathy. *J Clin Pathol* 2000; 53: 355-61.
- Tansuphasiri U, Boonrat P, Rienthong S. Direct identification of *Mycobacterium tuberculosis* from sputum on Ziehl-Neelsen acid fast stained slides by use of silica-based filter combined with polymerase chain reaction assay. *J Med Assoc Thai* 2004; 87: 180-9.
- Watterson SA, Drobniowski FA. Modern laboratory diagnosis of mycobacterial infections. *J Clin Pathol* 2000; 53: 727-32.