June 1998

Detection of mycobacterium tuberculosis in paraffin embedded intestinal tissue specimens by polymerase chain reaction: characterization of IS6110 element negative strains

Tariq Moatter
Aga Khan University, tariq.moatter@aku.edu

Shaper Mirza
Aga Khan University

Mohammad Shahid Siddiqui
Aga Khan University

Irshad Nabi Soomro
Aga Khan University

Detection of Mycobacterium Tuberculosis in Paraffin Embedded Intestinal Tissue Specimens by Polymerase Chain Reaction: Characterization of 1S6110 Element Negative Strains

Pages with reference to book, From 174 To 178

Tariq Moatter,Shaper Mirza,Mohammad Shahid Siddiqui,Irshad Nabi Soomro (Department of Pathology, Aga Khan University Hospital, Karachi.)

Abstract

Tuberculosis is still one of the most widespread infection known to mankind. Although lung is the predominant site of disease, a sizeable population in Pakistan gets intestinal disease. Clinical presentation, radiologic and endoscopic examination provide clues to the diagnosis. However, a definitive diagnosis requires biopsy material with granulomas and/or caseation complemented by acid fast staining and culture. There are many occasions when biopsy material is scanty and even in some intestinal resection cases histologic evaluation fails to confirm or rule out tuberculosis. Therefore, an investigation was conducted to assess the efficacy of PCR in the detection of mycobacterial DNA in paraffin embedded intestinal tissue. In this study 12 histologically confirmed cases of intestinal tuberculosis and 2 cases with non specific inflammation but clinically suspected for abdominal tuberculosis were selected. One case of rectal polyp was included to serve as a negative control. M. tuberculosis DNA was amplified in 8 out of 12 histologically confirmed cases and in 2 cases diagnosed with non specific inflammation. Amplified products were obtained in 6 out of 10 PCR positive specimens with 1S6110 region specific primers while 4 samples were negative, suggesting the absence of insertion sequence 6110 in these strains. However, amplification was obtained in these negative specimens with a second primer pair confirming them as M. tuberculosis complex species. On the basis of this study we conclude that; (1) Processed and paraffin embedded tissue material is suitable for PCR analysis, (2) PCR assay can be used to complement the diagnosis of intestinal tuberculosis especially in situations where a definite conclusion can not be drawn by conventional methods, (3) M. tuberculosis species lacking insertion sequence 6110 element are present in our population. Therefore, several primer pair sets should be included when applying PCR for the detection of mycobacterial DNA (JPMA 48:174, 1998).

Introduction

Tuberculosis is the predominant infectious cause of morbidity and monality worldwide, It is estimated that one third of the world population (approx. 1.8 billion persons) is infected with M. tuberculosis and each year there are 8 million new cases worldwide[1-3]. This is projected to increase to over 10 million cases by the year 2000, an estimated increase of almost 36% over the numbers in 1990. The mortality rate is expected to increase to 3.5 million cases over the same period[4]. The increase in tuberculosis infections has been attributed to the HIV epidemic, poverty and malnutrition and a decline in tuberculosis control efforts[5].

In Pakistan, tuberculosis is not only a major health problem but also a leading cause of death. Literature review illustrates that sporadic studies were done to identify the magnitude and severity of this problem in local community. Data from surveys conducted by the government of Pakistan (1960-61) and World Health Organization (1974-1978) suggested a high frequency of tuberculosis in Pakistani population[6]. Seriousness of tuberculosis problem in Pakistan can bejudged from the Marsh report prepared in 1988, which stated that the number of yearly reported new cases is 1.6 million, including more than 250,000

open cases. Unpublished data from The Aga Khan University Hospital, Karachi, showed a steady increase in the number of M. tuberculosis isolated from suspected cases of pulmonary tuberculosis from 150 in 1990 to 450 in 1993.

Abdominal tuberculosis is a common form of extra pulmonary infections and its clinical manifestations include fever, weight loss, abdominal pain and swelling, elevated erythrocyte sedimentation rate and variable response to tuberculin testing. Tuberculous enteritis may occur in upto 24% of patients with advanced pulmonary tuberculosis, due to swallowing of infectious sputum. Primary tuberculosis of the intestine can follow ingestion of M. Bovis through unpasteunzed milk or milk products. A study carried out in Egypt in 1982, showed M. Bovis infection in 9 out of 20 patients with tuberculous peritonitis[7]. These observations depict that ingestion of milk products contaminated with M. Bovis is a common cause of primary gastrointestinal infection in African countries.

Tuberculosis of the gastrointestinal tract can mimic a variety of other abdominal disorders. Unless a high index of suspicion is maintained, the diagnosis can easily be missed or delayed, resulting in substantial increased morbdity and mortality[8]. The ileocecal area and jejuno-ileum are the most common sites of tuberculosis involvement of the intestinal tract. A definitive diagnosis of intestinal tuberculosis requires histopathological evaluation, acid fast bacilli staining and culture of involved tissue. Biopsy confirmation depends on the presence of epithelioid granulomas with caseation necrosis. In earlier stages of the disease, prior to the development of a well established hypersensitivity state, necrosis is absent and it is necessary to exclude Crohn's disease, sarcoidosis and other causes of granulomatous inflammation. Caseation necrosis is itself non- specific and may occur in fungal infections or syphilitic gummas. Demonstration of acid fast bacilli in biopsied tissue is therefore essential for a confident diagnosis. However, the acid fast staining is frequently negative[9] and intestinal biopsy culture for M. tuberculous takes about 2-6 weeks. Therefore rapid and sensitive tools for the diagnosis of intestinal tuberculosis are needed to complement the histopathological diagnosis. PCR has the potential to overcome many of these difficulties by the virtue of its sensitivity, specificity and rapidity. Several groups have described PCR as the most rapid method of identifying M. tuberculosis from clinical specimen. De Lassence et al[10] amplified a region within 1S61[10] insertion sequence and demonstrated that one mycobacterial genome could be detected in a sputum sample. Other studies have indicated that PCR is more sensitive than culture with up to one third of culture negative samples from patients with clinical tuberculosis registering as PCR positive[11]. Noordhoek et at reported that the sensitivity and specificity of their PCR assay for the detection of mycobacterial DNA in clinical samples was 92% and 99% respectively[12]. In our pathology laboratory tissue specimen from patients suspected of intestinal tuberculosis are usually received in formalin, we therefore sought to examine the usefulness of PCR in the detection M. tuberculosis DNA in these specimen.

Patients and Methods

We examined 14 paraffin embedded intestinal tissues blocks by PCR. The specimens were originally received in formalin from patients with clinical history of abdominal symptoms and radiologic evidence suggesting tuberculosis. After performing gross examination ulcerated and narrowed regions of the specimen were removed and embedded in paraffin. The 5 uM sections of the embedded tissue were stained with haematoxylin & Eosin and examined under the microscope for histologic features of tuberculosis, which included granulomas with areas of caseation. One case of rectal polyp was included as a negative control. Histologically the rectal polyp specimen revealed polypoid pieces of tissue with sparse colonic glands.

DNA isolation from paraffin embedded blocks

The tissue sections were scrapped, collected in 1.5 ml Eppendorf vials and paraffin was removed. The

DNA was isolated according to the method of Van Embden[13]. Briefly, the tissue sections were homogenized in TE buffer (10 mM Tris pH 7.4,0.2 mM EDTA). The samples were heated to 80°C and then cooled to room temperature followed by lysozyme addition and overnight incubation at 37°C. Next day the samples were digested with proteinase K and CTAB/NaCI solution was added. After 15 min incubation at room temperature chloroform:isoaniyl alcohol (24:1) was added followed by centrifugation at 12000 g for 10 min. DNA present in the aqueous phase was precipitated with isopropanol and pelleted in a microfuge. The DNA pellet was resuspended in autoclaved water and quantified by spectrophotometer at 260 nm. PCR procedure was performed as described by Cormican et al, 1995 and Kolk et al, 1993. Three primer pairs were synthesized by using an Applied Biosystem DNA synthesizer; primer pair TB11 (5'-ACCAACGATGGTGTGTCCAT-3') and TB12 (5'-C'1TGTCGAACCGCATACCCT-3') was genus specific, it amplified geoomic DNA region (hsp-65 gene) common in all mycobaterial species and gave a product of 439 base pair, primer pair (MP64-1 (5'-TCCGCTGCCAGTCGGCTITCC-3') and MP64- 2 (5'-GTCCTCGCGAGTCTAGGCCA-3') which amplified a 240 base pair sequence (MPB64 gene) exclusively from members of the M. tuberculosis complex (M. tuberculosis, M. Bovis, M. Bovis BCG) [14]. Primer pair INS- l(5'-CGTGAGGGCATCGAGGTGGC-3') and INS-2 (5'- GCGTAGGCGTCGGTGACAAA-3') Specific for sequences in the insertion sequence IS6 110, present in members of M. tuberculosis complex and gave a predicted amplified product of 245 bp[15].

Polymerase Chain Reaction

The PCR was performed in a thermocycler (Perkin Elmer 9600 or Perkin Elmer 2400). DNA(1 ug) was added to PCR mixture which was composed of PCRbuffer(10 rnMTris pH 7.4, 50mM KCI and 1.5 mM MgCl2) 200uM of each dNTPs, 0.5 U Taq Polymerase, 200 ng of primer in a final volume of 100 ul. After initial denaturation at 94°C, 40 cycles of amplification were performed at 94°C 2 min. 65°C for 2 rain and extension at 72°C for 2 min13 .

Analysis of PCR products

Specific products were separated by agarose gel electrophoresis, detected with ethidium bromide staining and photographed. Performance of amplification reaction was monitored by testing one sample without DNA and one sample with 100 ng of M. tuberculosis DNA. Results were considered valid only if all controls were correct.

Results

Clinical and Histologic Findings

Twelve cases demonstrating histological features of multiple discrete and confluent granulomas characterized by aggregates of epitheloid cells, langhan's type giant cells and foci of necrosis (Figure 1)

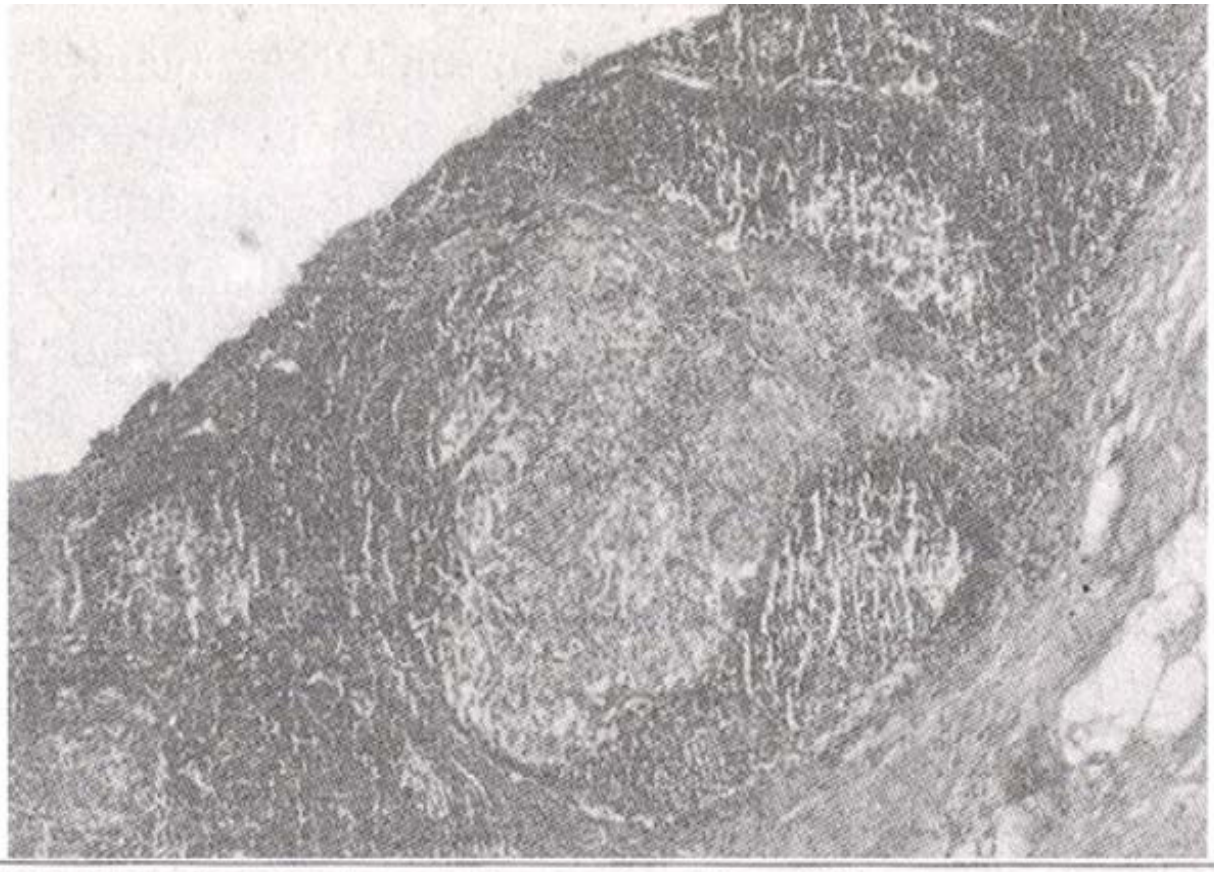

Figure 1. Histologic section demonstrating several well formed tuberculous granulomas. Note the presence of epitheloid cells surrounded by lymphocytes. The center of a large granuloma also shows signs of necrosis (Haematoxylin & Eosin stain X200)

were selected from our files. Two cases with non specific chronic inflammation but clinically suspected for abdominal tuberculosis were also included in this study. In addition, one case of rectal polyp was chosen as a negative control. A total of!! biopsies were takenfrom small intestinal region while 3 biopsies were from large intestine area (Table I).

Table. Patient's characteristics and PCR findings.

Case No.	Sex	Age	Site of involvement	TB confirmed by Histology	PCR
1	F	22	S.intestine	No	Yes
2	F	43	L.intestine	Yes	Yes
3	M	20	S.intestine	Yes	Yes
4	F	29	S.intestine	No	Yes
5	M	24	S.intestine	Yes	Yes
6	F	36	S.intestine	Yes	Yes
7	F	37	S.intestine	Yes	Yes
8	F	26	S.intestine	Yes	Yes
9	F	33	S.intestine	Yes	Yes
10	M	26	L.intestine	Yes	Yes
11	F	25	S.intestine	Yes	No
12	M	30	S.intestine	Yes	No
13	F	23	S.intestine	Yes	No
14	F	20	L.intestine	Yes	No

The clinical presentation of seven patients included sign and symptoms of recurrent subacute orcomplete intestinal obstniction. Most of these had ileocecal disease with stricture and/or ulceration at the site of involvement. One patient had disease restricted to small intestine. Two patients showed perforation predominantly involving small intestine. Two patients had lymphadenopathy associated with abdominal symptoms. One patient had pelvic abscess with abdominal symptoms. One patient had mass in the ileocecal region which on examination revealed constricted area in the tenninal ileum. In addition, proximal part of caecum was thickened. Each patient exhibited clinical histoiy of abdominal tubereulosis. Intestinal tuberculosis was diagnosed on the basis of histologic examination in 12 cases which demonstrated multiple granulomas and caseation. Two specimens had histological features compatible with non specific inflammation. The histological analysis of these cases showed lymphoid aggregates present inthe muscle layeralong with fibrosis. Out of 14 patients 5 were male and 9 were females, the male to female ratio was 1:8. The patients ranged from 20 to 43 (mean, 28) years of age.

Polymerase Chain Reaction Assay

Polymerase Chain analysis demonstrated amplification of M. tuberculosis DNA in 8 out of 12 histologically proven cases of intestinal tuberculosis. Two cases with features compatible to chronic non-specific inflammation also demonstrated strong signals for M. tuberculosis DNA. In these

specimen, specific bands of 245 base pairwith INS-I and INS-2 pair and 240 base pair with MP64- 1 and MP64-2 primer set were obtained. Four specimens were negative with all primerpairs.

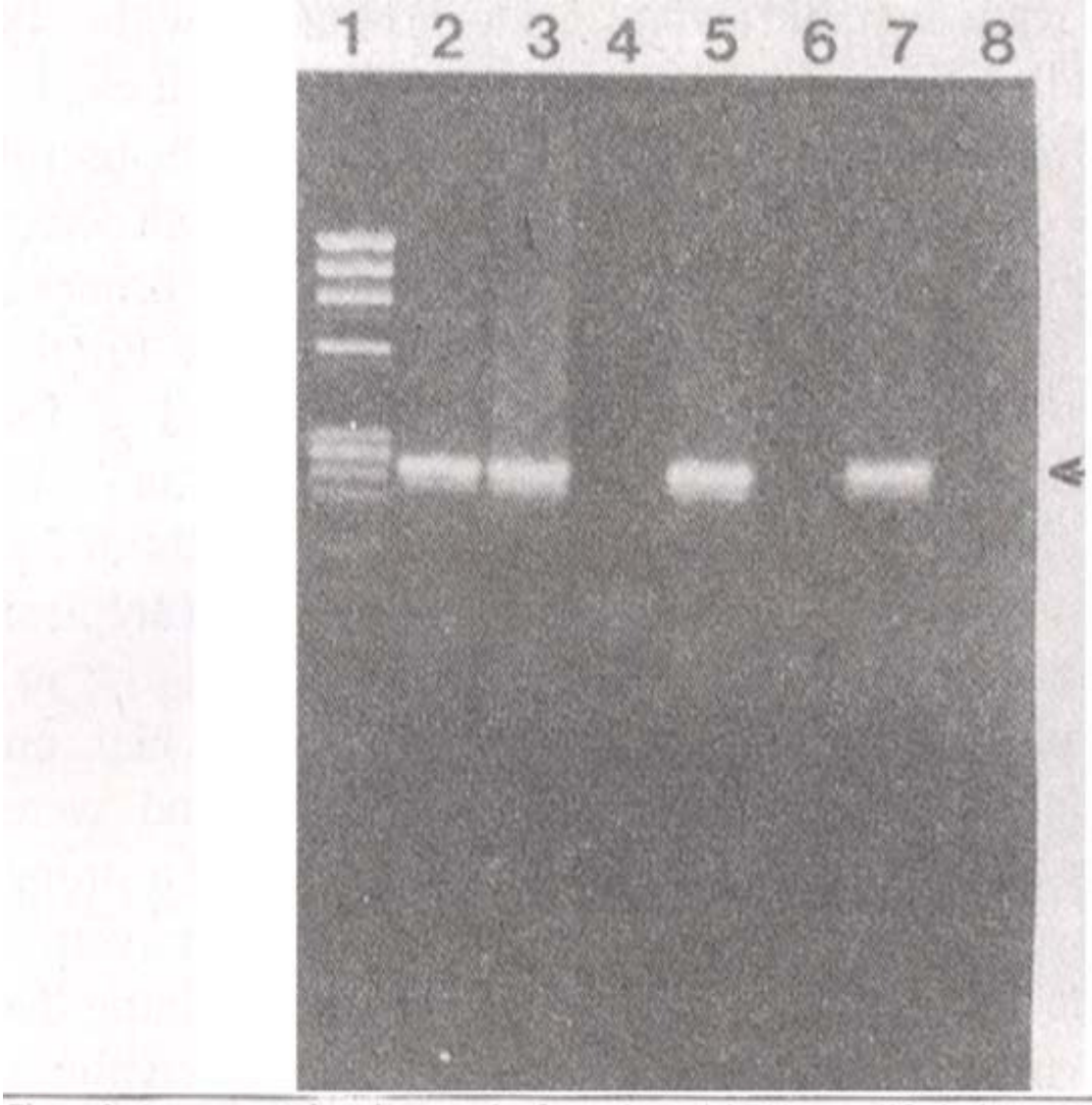

Figure 2. A representative photograph of mycobacterial DNA amplified with IS6110 specific primers.INS-1 and INS-2 generated a 245 base pair fragment. Thermal cycling included 40 cycles of 2 min at 94°C, 2 min at 65°C and 2 min at 72°C. Lane 1, fX174 DNA markers, Lanes 2,3 &5, PCR positive specimens; Lane 4, specimen negative with IS6110 but positive with other primer pairs; Lane 6, negative control DNA; Lane 7, Positive control DNA; Lane 8, blank.

Figure 2 shows a representative photograph ofan agarose gel illustrating the amplified products generated with IS6 110 sequence specific primer from mycobacterial DNA in selected cases. Out of 10 PCR positive specimens, amplified products were obtained with primer pair INS- 1 and JNS-2 in 6 samples suggesting the presence of insertion sequence 6110. However, in 4 tissue samples 1S6 110 was absent since no amplification was obtained with INS primer pair. PCR amplification was obtained in IS6 110 negative specimen with a second primer pair, specific for M. tuberculosis complex (MP64-l and MP64-2) but complementary to a different region on bacterial genome (Figure 3).

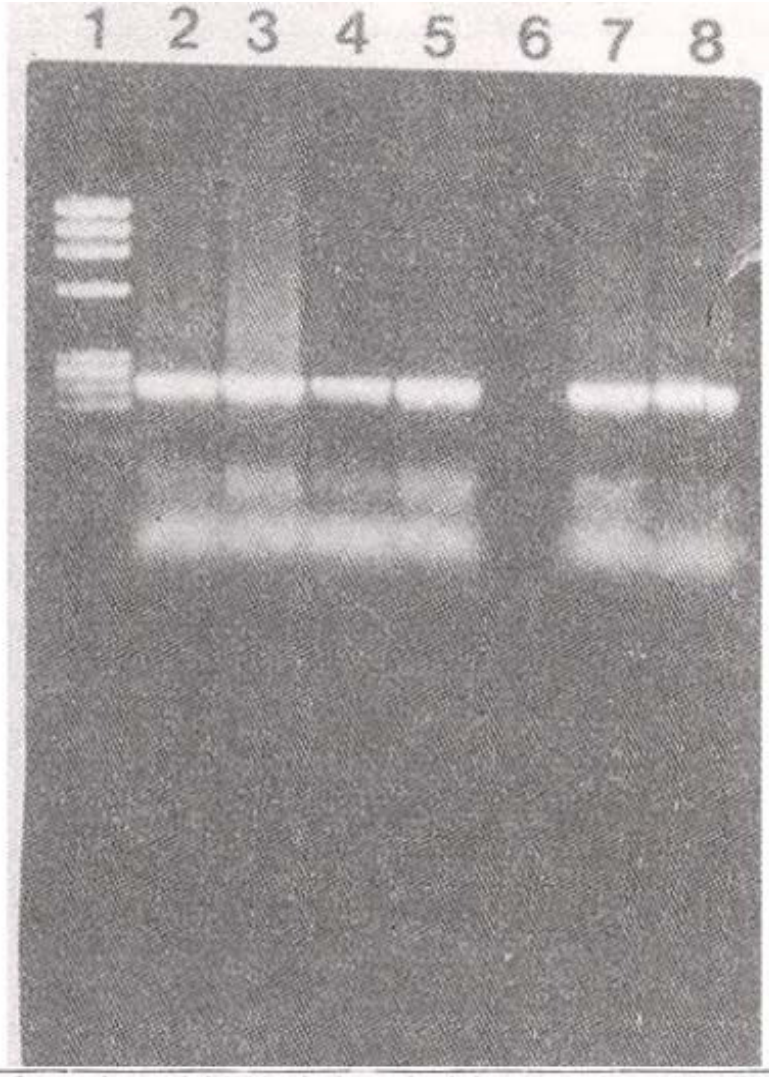

Figure 3. Detection of mycobacterial DNA in intestinal tissue sections. The DNA obtained from intestinal tissue sections was amplified with specific primers. The PCR products were distinguished on an agarose gel and stained with ethidium. Lane 1, fX174 DNA markers; Lanes 2-5, PCR positive specimens; Lane 6, negative control DNA; Lanes 7-8, positive control DNA. The arrow points to 240 base pair product.

Our results showed specific 439 bp band with mycobacterial genus specific primers in all samples positive with other primer sets (Figure 4).

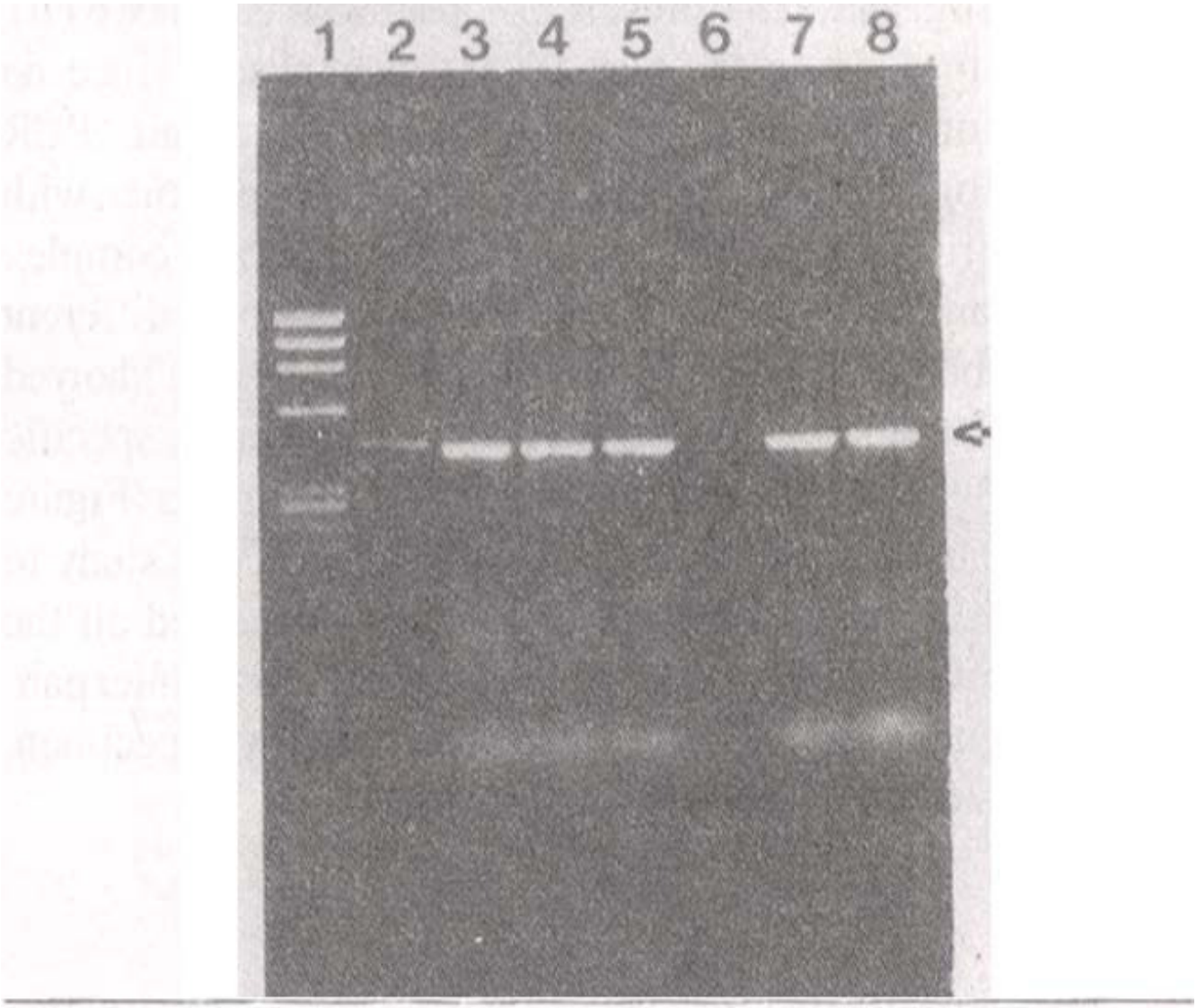

Figure 4. Analysis of PCR products by agarose gel electrophoresis. A 439 base pair fragment was obtained with TB11 and TB12. Mycobacterial DNA isolated from intestinal tissue sections was amplified with genus specific primers, separated on agarose gel and stained with ethidium bromide.Lane 1, fX174 DNA markers; Lanes 2-5, PCR positive specimens; Lane 6, negative cotnrol DNA, Lane 7-8, positive control DNA.

The genus specific primers were included in this study to serve as an additional control, because we expected all the mycobacterial species to be PCR positive with this primer pair. DNA obtained from negative control, rectal polyp specimen, was negative with all primers pairs.

Discussion

We investigated the role of PCR in complementing the diagnosis of intestinal tuberculosis on the basis of mycobactenal DNA detection in paraffin embedded intestinal tissues. Sometimes it is difficult to establish the diagnosis of intestinal tuberculosis which requires demonstration of acid fast bacilli in biopsy specimen, culture and histopathologic evaluation. Identification of M. tuberculosis in tissue sections by acid fast stains usually gives unsatisfactory results because Ziehl-Nelson staining lacks sensitivity and requires 10^4 organism ml-^{1} or greater for detection[16]. Culture of M. tuberculosis usually

takes 4 to 6 weeks but this may not always be successful and may also be limited by the number of organisms within tuberculous lesion. The clinical, radiological and endoscopic picture is sometimes confused with Crohn's disease and other non-secific inflammatory bowel diseases. PCR has been reported to be highly sensitive and specinc and could circumvent many of these problems.

The sensitivity of our PCR assay on DNA obtained from paraffin embedded tissue was 71%, without monitoring for inhibition. The given sensitivity of PCR is comparable to the results of Popper et al, who have suggested that PCR is more sensitive in the detection of niycobacteria in formalin fixed paraffin embedded lung tissue than acid fast stain[17]~. Several other studies have described the feasibility of using a PCR test in the routine clinical Iaboratory and reported sensitivity in the range of 80-92%. Clarridge et al used PCR assay on sputum samples fmm patients suspected of tuberculosis by PCR and reported 86% sensitivity[18]. Forbes et al achieved a sensitivity of 87% for their PCR[19] assay. Similarly Noordhoek et al performed PCR assay on 1957 clinical specimen and the sensitivity of their PCR assay was 92%[12]. The sensitivity of M. tuberculosis DNA detection in formalin fixed tissue was slightly lower than reported for fresh samples. As expected, PCR assay was vet rapid and results were obtained within 48 hour. In addition it was not limited by sample size, it can be used on tiny biopsy specimens and therefore would be useful on material obtained with endoscopy. By PCR one can detect mycobacteria at species level by employing specific primers. Cormican et al developed a multiplex PCR assay for the detection of 14 mycobacterial species and used it for identification of most clinical isolates of mycobacteria[14]. We have used three sets of primers to identify members of M. tuberculosis complex and to differentiate them from atypical my cobacteria.

The present report documents two cases, which on histologic examination showed no granuloma and were classified as non specific chronic inflammation, but a strong signal of mycobacterial DNA was detected. This observation was reproducible with two other primer pairs excluding the possibility of a false positive result. Such observations underline the need for more sensitive assays like PCR in the differential diagnose of intestinal tuberculosis with conditions like Crohn's disease, Yersinia infecion, GI histoplasmosis and periappendiceal abscess. In our practice of surgical pathology it is conunon to attend cases of non-specific chronic inflammation affecting gastrointestinal tract. In addition, there are occasions when we find caseating granulornas in lymph nodes and non-specific inflammation in the bowel. These cases must be differentiated from other conditions such as inflammatory bowel disease and in such conditions PCR could provide a pertinent tool to supplement histologic diagnosis.

For mycobacterial strain differentiation insertion element 1S6 110 has been studied most intensively. This 1,335 bp sequence is related to the IS3 family of Iss and has been found exclusively in M. tuberculosis complex group of mycobacteria. It is an excellent genetic marker for tracing individual M. tubereulosis strains during micro epidemics, nosocomial infections and the clonal dissemination of multiple dmg resistant strains[20]. Hermans et al have shown that about 85% of the M. tuerculosis isolates that they have investigated contained one or two copies of IS6 110 in the DR-containing region of the chromosome[21]. Since the presence of 1S6 110 is restricted to M. tuberculosis complex species it makes this element a widely used target for amplification and detection of genetic diversity among M. tuberculosis isolates. However, our study reports that DNA extracted from 4 out of 10 M. tuberculosis strains failed to amplify with INSI and INS2 primers suggesting that these strains were lacking IS6 110 element. On the basis of amplification with two other sets of mycobacterial specific primer pairs, these strains were shown to be genuine M. tuberculosis strains. To date, all the strains of European origin contain one or mote copies of IS6 110 whereas reports from South East Asian region documents the existence of IS6 110 negative strains. For instance, Soolingen et a1[20], while comparing various markers of M. tuberculosis strain differentiation identified one strain which was negative for IS6 110. This strain originated in India, and by all standards it was proved to be a real M. tuberculosis strain. In addition, identification of seven M. Tuberculosis strain negative for IS6 110 element were reported

from patients in other regions of South East Asia[22]. We suggest that if such strains would occur frequently in our region, one should be careful to use 1S6 110 DNA as a target for amplification by PCR for detection of M. tuebrculosis in clinical sample.
Further studies must be carried Out to find the prevalence of 1S6110 negative strains in local population. In conclusion, PCR is an important tool for the sensitive and rapid identification of M. tuerculosis in formalin fixed tissue sections. It has the potential to complement the histologic diagnosis of intestinal tuberculosis specially in those cases where patient's clinical picture is suggestive of tuberculosis but histologic examination fails to confirm it.

References

1. Rodrigus LC. Smith PG. Tuberculosis in developing countries and methods for its control. Trans. R. Soc. Trop. Med. Hyg., 1990,84.739-744.
2. Blooms BR, Murray CJL. Tuberculosis. Commentary on a re- emergent killer Science, 1992:257:1055-1064.
3. Centers for Disease control. Estimates of future global tuberculosis morbidity and mortality. MMWR., 1993,42:961-964.
4. Schuster M. Mycobacterial disease: A historical and epidemiologic perspective Clin. Dermatol., 1995;13:1-2.
5. John D, Porter H, Keith Pet al. The re-emergence of tuberculosis. Annu. Rev. Public Health, 1994,15:303-323.
6. Tuberculosis control and research strategies for the 1 990s. Memorandum from a WHO meeting. Bull. WHO., 1992;70:17-21.
7. Davis MRQ. Abdominal tuberculosis in children. J Surg., 1982,20:7-18.
8. Marshall JB. Tuberculosis of the gastrointestinal tract and peritoneum. Am. J. Gastroenterol., 1993;88:989-999.
9. Morgante PE, Gandara MA, Sterle E The endoscopic diagnosis of colonic tuberculosis. Gastrountest. Endosc., 1989;35:115-1188
10. DeLassence A, Lecossier D, Piere C et al. Detection of mycobacterial DNA in pleural fluid from patients with pleurisy by means of the polymerase chain reaction. Comparison of two protocols. Thorax,., 1992,47:265-269.
11. Joly V. Hance AJ, Yeni P et al. Mycobacterium tuberculosis infections. Curr. Opin. Infect. Dis. 1993.6:171-178.
12. Noordhoek GT, Kaan JA, Mulder S et al. Routine application of the polymerase chain reaction for detection of Mycoabcterium tuberculosis in clinical samples. J. Clin. Pathol., 1995;48:810-814.
13. Van-Embden JDA. Cave MD, Crawford JT et al Strains identification of M. Tuberculosis by DNA finger printing. Recommendations for a standardized methodology, J. Clin. Microbiol, 1993;31:406-409.
14. Cormican M, Glennon M, Riain U et al. Multiplex PCR for identifying mycobacterial isolates J. Clin. Pathol., 1995,48:203-205.
15. Kolk AHJ, Schuitema ARJ, Kuiper S et al Detection of Mycobacterium tuberculosis in clinical samples by using polymerase chain reaction 3. Clin. Microbiol., 1992;30:2567- 2575
16. Drobniewski FA, Kent RJ, Stoker NG et al. Molecular biology in the diagnosis and epidemiology oftubercülosis. 3. Hosp. Infect., 1994;28:249-263.
17. Popper HH, Winter E, Hofler G. DNA of Mycobacterium tuberculosis in forrnalin fixed, paraffin-embedded tissue in tuberculosis and sarcoidosis detection by polymerasechain reaction. Am. J Clin. Pathol., 1993; 101:738-741.
18. Clarridge JE, Shawar RM, Shinnick TM et al Large scale use of polymerase chain reaction for

detection of Mycobacterium tuberculosis in a routine Mycobacteriology laboratory. J. Clin. Microbiol., 1993;31:2049-2056.
19. Forbes BA, Hicks KES. Direct detection of Mycobacterium tuberculosis in respiratory specimens in a clinical laboratory by polymerase chain reaction. J Clin Microbiol., 1993;31:1688- 1694.
20. Van-Soolingen, D., Petra, E.W , Haas, Dc-Peter, W. et al. Comparision of various repetitive DNA elements as genetic markers for strains differentiation and epidemiology of Mycobacterium tuberculosis. J. Clin. Microbiol., 1993;31:1987-1995.
21. Hermans PWM, Van.Soolingen D, Dale JW. Insertion clement lS986 from Mycobactcrium tuberculosis A useful tool diagnosis and epidemiology of tuebrculosis, 3. Clin. Microbiol., 1990;28:205 1-2058,
22. Das S, Chan SL, Allen BW et al. Application of DNA fingerprinting with 1S986 to sequential mycobacterial isolates obtained from pulmonary tuberculosis patients in Hong Kong before, during after short course of chemotherapy. Tubercle. Lung Dis., 1993;74:47-51.