Research Article

Evaluation of polymerase chain reaction in space-occupying lesions of liver reported as granulomatous inflammation/tuberculosis on fine-needle aspiration cytology

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Abstract

Background: Tubercular involvement of the liver is uncommon, but is a serious consideration in differential diagnosis of granulomatous conditions, especially in endemic regions like India. Objective: To assess the role of polymerase chain reaction (PCR) done on archival cytological material in diagnosing tuberculosis (TB) in cases reported as granulomatous inflammation/TB in liver lesions. Materials and Methods: This was a retrospective study including a total of 17 cases of liver space-occupying lesions (SOLs) reported as granulomatous inflammation (n = 12) and TB (n = 5). The smears were retrieved from the archives of the department and were reviewed for the cytometric features. Air-dried smears stained with May–Grünwald–Giemsa (MGG) stain were assessed for the representative material in the form of epithelioid granulomas and giant cells. One/two MGG smears from each case were destained and the material was used for performing PCR for Mycobacterium tuberculosis by amplification of 123 bp fragment of the IS6110 insertion element. Results: The age of the patients ranged from 3 to 61 years. There were 12 females and 5 males. The patients presented with solitary/multiple liver SOLs. DNA could be extracted from 10/17 cases from archival MGG smears. PCR positivity was noted in 8/10 cases (including four acid-fast bacilli smear-positive cases), confirming a diagnosis of TB. Conclusion: Cytomorphology alone may not be sufficient for differentiating various granulomatous lesions reported in liver SOLs. DNA can be extracted from the archival cytological MGG-stained smears. PCR should be carried out if Ziehl–Neelsen staining is negative in granulomatous lesions, especially when material has not been submitted for culture.

Key words: Granulomatous inflammation, liver, polymerase chain reaction, tuberculosis

INTRODUCTION

Tuberculosis (TB) is a major health problem with a global mortality ranging from 1.6 to 2.2 million cases per annum.[1] According to the WHO estimates,[2] India accounts for one-fifth of the global burden of TB. Early
diagnosis is important for the proper management of TB. *Mycobacterium tuberculosis* can involve any organ, and clinical features depend on the organ system involved. Liver involvement in TB, though common both in pulmonary and extrapulmonary TB, is usually clinically silent. In developing countries where the burden of TB is high, there are asymptomatic patients with hepatic TB. Hepatic TB is an important differential diagnosis of granulomatous liver disease. However, due to lack of acquaintance with this condition, many cases are missed or diagnosed inaccurately.

The final diagnosis of hepatic TB rests on histopathologic evidence of caseating granulomas, demonstration of acid-fast bacilli (AFB) on smear or isolation of *Mycobacterium* by culture of biopsy specimen. To establish the specific diagnosis of TB, one has to demonstrate the AFB in the specimen or isolate the *Mycobacterium* by culture. Many a times, direct demonstration of AFB is not possible due to low sensitivity of Ziehl–Neelsen (ZN) staining in extrapulmonary cases. Fine-needle aspiration cytology (FNAC) has shown a varying positivity of AFB, epithelioid granuloma, and caseation necrosis in 0–59%, 80–100%, and 30–83% of cases, respectively.[3,4] Although culture is considered to be a gold standard, it is time-consuming and reported to have a low sensitivity, especially in paucibacillary extrapulmonary conditions.[7]

For unknown reasons, the results of AFB demonstration in liver tissue in studies reported from India have not been rewarding. AFB could also be absent in cases showing caseation necrosis and epithelioid granulomas.[6,8] Thus, diagnosing liver TB still remains a challenge, and the present study was carried out to evaluate the utility of polymerase chain reaction (PCR) targeting the IS6110 on archival samples of ultrasonography-FNAC collected over a period of 6 years in a tertiary care hospital of North India.

**MATERIALS AND METHODS**

This was a retrospective study comprising a total of 17 ultrasound-guided FNAC samples from patients having hepatic space-occupying lesions (SOLs). Samples were collected over a period of 6 years (from January 2005 to January 2011). Approval from the Institute’s Ethics Committee was sought, but informed consent from patients could not be taken as it was a retrospective study. The smears were stained with May–Grünwald–Giemsa (MGG) stain, hematoxylin and eosin stain, ZN stain, and periodic acid-Schiff’s stain. The stained smears were retrieved from the archives of the department and were reviewed for the cytomorphologic features. On cytology, the cases were divided into four categories:

1. Category 1 - Cases with necrotizing inflammation and ZN stain for AFB positive
2. Category 2 - Cases with granulomatous inflammation with/without necrosis, positive for AFB
3. Category 3 - Cases with granulomatous inflammation with/without necrosis, negative for AFB
4. Category 4 - Cases with occasional epithelioid cell collections and/or giant cells, negative for AFB.

MGG-stained air-dried smears from the archival material were assessed for the representative material in the form of epithelioid granulomas and giant cells. One to two MGG smears from each case were destained and the material was scraped to extract DNA.

**DNA extraction**

DNA was extracted by commercially available DNA extraction kit according to manufacturer’s instruction (Qiagen).

**Polymerase chain reaction**

Positive and negative control was included in each independent PCR assay. The positive control was the DNA of H37RV, and negative control included PCR grade water. Identification of *M. tuberculosis* was done using a specific pair of primers designed to amplify IS6110 in the *M. tuberculosis* complex, and the expected band size was about 123 bp. The sequence of primers used was ISI: 5’CCTCGGAGCGTGAGCCGTGGC 3’ and IS-II: 5’CTCGTCCAGCGCCGCTTCGG3’, respectively.

A 25 μl reaction contained 10× assay buffer (Bangalore Genei, Bangalore, India), 10 mM dNTPs (Bangalore Genei), 10 pmole of each primer, 2.5 units Taq DNA Polymerase (Bangalore Genei, Bangalore, India), and 5 μl of extracted DNA. Amplification was carried out in a thermal cycler, which involved 35 cycles including denaturation at 95°C for 4 min, annealing of primers at 63°C for 1 min, and primer extension at 72°C for 1 min. The amplification products were separated on 1.5% agarose gel. The samples showing the presence of 123 bp band under ultraviolet transillumination were considered positive. Results were compared with positive (DNA of H37RV strain) and negative (PCR grade water) control. The 100 bp ladder was used as a molecular marker.

**Statistical analysis**

The sensitivity, specificity, positive predictive value, and the negative predictive value were calculated.

**RESULTS**

The age of the patients ranged from 3 to 61 years with a median age of 41 years. Among 17 patient samples, 12 were from males and 5 from females with a male:female ratio = 2.4:1. Patients presented with solitary/multiple liver SOLs. On review of cytology smears, 17 cases were segregated into the following categories as described previously: Category 1: n = 3 (17.64%), Category 2: n = 2 (11.76%), Category 3: n = 10 (58.82%), and
Category 4: n = 2 (11.76%). DNA could be extracted from 10/17 cases from archival MGG smears. PCR positivity was noted in 8/10 cases (including four AFB smear positive cases), confirming a diagnosis of TB [Figure 1 and Table 1].

DISCUSSION

TB can affect the liver in three forms. The most common form is the diffuse involvement of the liver which can be seen along with pulmonary and/or military TB. The second is granulomatous hepatitis and third form presents as focal tuberculoma or an abscess, which is a rare form of clinical manifestation.\(^9\) If diagnosed early then it can be cured with antitubercular treatment. Cumulative mortality for hepatic TB is reported to vary from 15% to 42%.\(^{1,13}\) Pathological and bacteriological diagnosis is necessary to confirm the diagnosis of hepatic TB. However, the definitive diagnosis of liver TB based on histological evidence of granulomatous inflammation with caseation necrosis is seen only in one-third of cases. The conventional methods such as ZN staining for the direct demonstration of mycobacteria are not sensitive methods in extrapulmonary TB as shown in previously published studies.\(^{11‑13}\) The culture is considered to be a gold standard, but it is tedious and time-consuming. PCR for the detection of TB is considered to be having a good diagnostic value with high sensitivity and specificity. IS6110 is the most common region used in PCR due to its multiple copy number. Thus, the present study was planned to evaluate PCR assay for the detection of \textit{M. tuberculosis} in FNAC samples obtained from cases reported as hepatic granulomatous inflammation and/or TB.

In our study, 17 FNAC samples were analyzed. Among 17 cases, 3 (17.64%) showed necrotizing inflammation which were positive for AFB by ZN staining. Granulomatous inflammation with/without necrosis but positive for AFB was seen in 2 samples (11.76%). Ten samples (58.82%) showed granulomatous inflammation with/without necrosis which were found to be negative for AFB. Occasional epithelioid cell collections and/or giant cells were seen in two samples (11.76%) which were negative for AFB. Among the diagnosed granulomatous lesions in FNAC samples of liver, microscopy for AFB was positive in 29.41% samples. The results of ZN staining are similar to the previously published studies. Tai et al.\(^{14}\) in their 15 years of experience have shown a similar positivity of 20% among pathologically proven hepatic TB. Alcantara-Payawal et al.\(^{15}\) have reported an overall positivity of 12% by conventional methods to detect the mycobacteria among patients with hepatic granuloma. Tuncer et al.\(^{16}\) studied 32 biopsy samples of granulomatous hepatitis and all were found to be negative by ZN staining. The low positivity of AFB has been attributed to paucibacillary nature of extrapulmonary TB and poor sensitivity of ZN staining.

TB-PCR is a rapid and useful tool in the diagnosis of hepatic TB. In the literature, the diagnostic sensitivities of TB-PCR in detecting culture-confirmed and clinically diagnosed TB infection are 75.9% and 81.3%, respectively.\(^{17}\) The specificity of TB-PCR in diagnosing TB infection is 100%. In the present study, adequate DNA could be extracted from 58.82% (10/17) samples. PCR was positive in 80% (8/10) of the samples. PCR picked additional 4 cases, which were negative for AFB. There was one case which was positive for AFB but negative for TB-PCR. This may be due to the predominance of necrosis which might have hindered the extraction of DNA. Our results are similar to previously published results. Diaz et al.\(^{17}\) reported that over 50% of hepatic TB patients had positive TB-PCR but Popper et al.\(^{18}\) and Ghossein et al.\(^{19}\) and Tai et al.\(^{14}\) reported that almost all patients with pathologic confirmation of hepatic TB had positive TB-PCR.

Thus, PCR is a rapid method of identifying \textit{M. tuberculosis} to the species level in clinical specimens. In the present study, PCR was positive for TB in 80% (8/10) cases as compared to FNAC with AFB smear positivity in 29.4% (5/17) cases. Our previously published data have also shown a very good sensitivity and specificity at other extrapulmonary tuberculosis sites.\(^{11,20}\) Thus, PCR

\textbf{Table 1: Polymerase chain reaction results in cases with adequate DNA (n=10)}

| Total number of cases | 17 |
|-----------------------|----|
| FNAC diagnosis - granulomatous inflammation | 12 |
| TB (AFB positive) | 5 |
| Adequate DNA extracted | 10/17 cases |
| PCR done on | 10 cases |

| PCR | AFB positive on smear | AFB negative on smear |
|-----|-----------------------|-----------------------|
| PCR positive: 8/10 | 4/10 | 4/10 |
| PCR negative: 2/10 | 1* | 1 |

*DNA could not be extracted from one case with AFB positivity on smear possibly due to predominance of necrosis. FNAC: Fine-needle aspiration cytology, AFB: Acid-fast bacilli, TB: Tuberculosis, PCR: Polymerase chain reaction.
targeting IS6110 can be used as a rapid diagnostic tool in the diagnosis of smear and culture negative cases of tuberculosis. In future, multiplexing by targeting more than one gene can be used to increase the sensitivity of PCR.[12,21] The limitation of the present study is the small sample size, and a large number of samples are required to be analyzed in fresh tissue samples so that PCR can be utilized for routine patient care.

CONCLUSION

PCR can provide strong evidence to the clinicians and pathologists where there is a dilemma about the cause of hepatic granuloma or SOLs of liver. Early initiation of antitubercular treatment can save lives of many patients in whom conventional techniques fail to diagnose the TB.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORSHIP STATEMENT BY ALL AUTHORS

To give appropriate credit to each author of paper, the individual contributions of all authors to the manuscript have been specified.

According to the International Committee of Medical Journal Editors (ICMJE http://www.icmje.org), “author” is generally considered to be someone who has made substantive intellectual contributions to a published study.

Authorship credit is based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors meet conditions 1, 2, and 3.

We declare that each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content.

All authors of this article declare that they qualify for authorship as defined by the ICMJE. Each author has participated sufficiently in the work and takes public responsibility for appropriate portions of the content of this article.

KS, KG, and NG were instrumental in the collection of material, doing PCRs and interpretation of data. We reviewed all smears and follow-up with culture and PCR reports to come to the conclusion. Clinical data and final interpretation were done by NG, KG, KS, and AR. Initial acquisition of data, PubMed search, and modification in the manuscript were done by NG, KG, and KS, respectively. AS and AKD provided the clinical samples and clinical and follow-up details of the patients. Final review and approval were done by AR, NG, and KS.

All authors read and approved the final manuscript.

Each author acknowledges that this final version was read and approved.

ETHICS STATEMENT BY ALL AUTHORS

This study was conducted with approval from the Institutional Review Board of the institution associated with this study as applicable. This study was a retrospective study not directly involving the patients. Authors take responsibility to maintain relevant documentation in this respect.

LIST OF ABBREVIATIONS (In alphabetic order)

AFB – Acid-fast bacilli
Bp - Base pair
FNAC – Fine-needle aspiration cytology
MGG - May–Grünewald–Giemsa
PAS - Periodic acid-Schiff’s
PCR - Polymerase chain reaction
SOLs – Space-occupying lesions
TB – Tuberculosis
USG - Ultrasonography
WHO - World Health Organization
ZN - Ziehl–Neelsen.

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**EDITORIAL/PEER-REVIEW STATEMENT**

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