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Original Research Article

Assessment of tubercle bacilli in various organs by staining and polymerase chain reaction technique

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ABSTRACT

Background: Early diagnosis of tuberculosis (TB) by different clinical methods plays a major role in control of TB in early stages. The present study was done with the aim to assess and compare the efficiency of staining techniques and polymerase chain reaction (PCR) for detection of tubercle bacilli in various organs.
Methods: The study included data 14,472 patients of both prospective (during March 2008 to 2009) and retrospective cases (past one year of the study period). For prospective cases the cytological material for the study was collected by fine needle aspiration cytology (FNAC). For retrospective cases of past one year were retrieved from the records. TB suspected cases were evaluated by Ziel-Nelson (ZN), Auramine-Rhodamine (A-R) staining techniques and by PCR.
Results: Total 284 cases were diagnosed with TB in various organs. In them, epithelioid granuloma was seen 227 cases (79.92%) and Langhans giant cells were seen in 18 cases (6.34%). AFB positivity on ZN staining was observed in 161 cases (56.69%) and 9 cases (3.16%) showed positivity for tubercle bacilli on A-R staining. PCR was done in 20 prospective cases and total percentage of positivity by PCR was seen in 18 cases (90%).
Conclusions: The total percentage positivity for detection of tubercule bacilli by PCR assay was found to be more (90%) with high sensitivity and specificity compared to ZN (56.6%) and AR staining techniques (3.16%).

Keywords: Polymerase chain reaction, Staining techniques, Tubercle bacilli

INTRODUCTION

Tuberculosis (TB) is a deadly infectious disease caused by various strains of Mycobacteria, usually Mycobacterium tuberculosis in humans.[1] It usually attacks the lungs (pulmonary TB) but can also affect other parts of the body (extra pulmonary TB).

TB kills around 1.7 million people globally every year. Almost 40% of the patients with active TB remains undiagnosed due to poor sensitivity of old diagnostic methods and increased prevalence of drug resistant TB. In addition, more people in the developed world are contracting tuberculosis because their immune systems are compromised by immunosuppressive drugs, substance abuse, or AIDS.[2] The distribution of tuberculosis is not uniform across the globe; about 80% of the population in many Asian and African countries test positive to tuberculin tests, while only 5-10% of the US population test positive.[1]

Direct microscopic examination of the sputum sample for the detection of acid-fast bacilli (AFB) was the most commonly used microbiological method for diagnosis and initial confirmation of TB.[3]

The main disadvantage of the study was low sensitivity relative to culture. But by the development of fluorescent technique the sensitivity was increased significantly. Other techniques involved are chest radiographic findings

and culture studies. But these methodologies are infective due to limitations of time consuming and low levels of bacterium in the samples.[4]

Polymerase chain reaction (PCR) is a powerful and reliable technique for rapid diagnosis of M. tuberculosis. It reduced the diagnostic problems in both pulmonary and extrapulmonary cases with improved sensitivity and specificity even in smear negative samples, and with sensitivity of 55-95% in culture positives and 100% in both smear- and culture-positive clinical specimens.[5-7]

Comparative studies on different staining techniques and PCR in FNA cytology for the diagnosis of TB in the population of Uttarakhand, are limited. Hence, the present study was designed to assess the presence of tuberculi bacilli in various organs by staining and PCR method.

METHODS

The present study included a total of 14,472 patients who came to the Department of pathology, Himalayan Institute of Hospital trust, University, Swami Ram Nagar, Dehradun over a period of one year during March 2008 to 2009. The population includes prospective cases during the study period and retrospective cases of past one year. All the cases received in cytology and histology section were also included in the study.

For prospective cases the cytological material for the study was collected by fine needle aspiration cytology (FNAC) either by aspiration or non-aspiration method. For retrospective cases of past one year were retrieved from the records. All cases which showed tuberculosis were re-evaluated.

Smears were made from the drop of aspirate with the help of another glass slide which acted as spreader. Minimum one air dried and one wet smear was made. Wet smear was immediately fixed in 95% isopropyl alcohol.

Wet fixed smears were stained by Papanicolaou staining and/or Haematoxylin and Eosin staining method. Dry fixed smears were prepared by air drying and then fixed in methanol.

These were stained with Leishman stain. In all those cases where tuberculosis was suspected ZN staining was done. Fluorescent stain (A-R) was done on those cases where AFB was not seen on ZN staining and findings were recorded.

Positivity of tubercule bacilli in prospective cases were confirmed by PCR. Fresh tissue was taken in saline in cases where tuberculosis was suspected. DNA extraction from tissue samples were performed using commercial kits with the extracted DNA, PCR for TB was done using mycobacterium tuberculosis specific primer.

The steps involved in PCR include denaturation, primer annealing and elongation; which were carried out using a Techne Thermal cycler. Post-PCR analysis of the amplified DNA was undertaken by agarose gel electrophoresis. Interpretation and analysis of data was done and carried out using standard statistical tests of significance.

RESULTS

In the present study, out of 14,472 cases, inflammatory and tubercular lesions were noticed in 4338 cases. In them a total of 284 (6.55%) cases were diagnosed with tuberculosis. In most of the cases (n=155; 55.55%) reticuloendothelial system was more affected with TB followed by bone and joint (n=33; 22%), GIT (n=29; 3.47%), CNS (n=2; 6.06%), endocrine (n=2; 1.66%) and hepatobiliary (n=2; 0.45%).

Table 1: Morphological variations in tubercular lesions.

Organs	EG	EG+ LGS	EG+LGS+N	EG+N	Only CN	AFB on ZN staining	Tubercule bacilli on AR
Retiuloendothelial system	04	03	61	57	30	108	05
Gastrointestinal tract	-	02	20	06	01	08	01
Lung	-	-	03	01	07	11	-
Urogenital tract	-	-	07	01	-	01	-
Female genital tract	01	06	02	-	-	2	01
Central nervous system	-	-	01	01	-	01	-
Breast	-	-	04	-	01	01	-
Hepatobiliary	-	01	-	01	-	-	-
Endocrine	-	-	-	-	02	02	-
Soft tissue	-	-	10	02	06	10	01
Skin	-	06	02	02	-	03	01
Bone and Joints	-	-	19	04	10	14	-
Total	05 (1.76%)	18 (6.34%)	129 (45.42%)	75 (26.40)	57 (20.10%)	161 (56.69%)	09 (3.17%)

EG-epitheloid granuloma, LGS- Langhans giant cells, N-necrosis, CN-caseaous necrosis, AR-Auramine-Rodamine, ZN- Ziel Nelson stain

Table 2: Morphological features in AFB positive cases.

Organs	Occasional EG only	EG+CN	EG+LGS+CN	N
Reticuloendothelial system	03	41	34	30
Gastrointestinal system	00	03	05	00
Lung	03	02	03	03
Urogenital tract	00	00	01	00
Female genital tract	02	00	00	00
Central nervous system	00	00	01	00
Breast	00	00	00	01
Hepatobiliary	00	00	00	00
Endocrine	00	00	00	02
Soft tissue	01	02	04	03
Skin	01	01	01	00
Bone and joints	00	02	05	07
Total (161)	10 (6.21%)	51 (31.68%)	54 (33.54%)	46 (28.57%)

EG-epitheloid granuloma, LGS- Langhans giant cells, N-necrosis, CN-caseaous necrosis

Table 3: Positivity of AFB by ZN and A-R staining in different organs.

Organs	Total cases of tuberculosis	AFB positive by ZN Stain	Tubercle bacilli positive by A-R stain	Total % of positivity for tubercle bacilli
Reticuloendothelial system	155	108	05	113 (66.47%)
Gastrointestinal system	29	08	01	09 (5.29%)
Lung	11	11	-	11 (6.47%)
Urogenital tract	08	01	-	01 (0.58%)
Female genital tract	09	02	01	03 (1.76%)
Central nervous system	02	01	-	01(0.58%)
Breast	05	01	-	01(0.58%)
Hepatobiliary	02	-	-	-
Endocrine	02	02	-	02 (1.18%)
Soft tissue	18	10	01	11 (6.47%)
Skin	10	03	01	04 (2.35%)
Bone and joints	33	14	-	14 (8.23%)
Total	284	161(56.69%)	09 (3.16%)	170 (59.85%)

AR-Auramine-Rodamine, ZN- Ziel Nelson staing

Table 4: Positivity of tubercle bacilli by PCR.

Organs	Total cases of tuberculosis	Tubercle bacilli positive by PCR	Tubercle bacilli negative by PCR
Reticuloendothelial system	155	4	-
Gastrointestinal system	29	1	-
Lung	11	-	-
Urogenital tract	08	1	-
Female genital tract	09	3	-
Central nervous system	02	-	-
Breast	05	3	-
Hepatobiliary	02	-	-
Endocrine	02	-	-
Soft tissue	18	1	-
Skin	10	2	-
Bone and joints	33	5	2
Total	284	18 (90%)	-

The patients were aged between 2-80 years. Majority of the patients (n=86; 30.28%) were between the group of 21-30 years. Histopathological findings of all tuberculosis cases were categorized into different morphological variations as given in Table 1.

Out of 284 cases with tuberculosis, epithelioid granuloma was seen 227 cases (79.92%) and Langhans giant cells were seen in 18 cases (6.34%). In another 129 cases (45.42%) LGC and necrosis was seen and 57 cases (20.10%) showed only necrosis.

AFB positivity on ZN staining was observed in 161 cases (56.69%) and 9 cases (3.16%) showed positivity for tubercle bacilli on Auramine-Rhodamine staining. Maximum number of cases of necrotizing granulomas were seen in RES 118 cases (41.54%) followed by GIT (n=26; 9.15%), bone and joints (n=23; 8.09%) and then soft tissue (n=12; 4.22%).

Morphological features in 161 AFB positive cases was given in Table 2. Epithelioid granuloma with necrosis and Langhans giant cells was seen in 54 cases (33.54%). Epithelioid granuloma with necrosis but without Langhans giant cells was seen in 51 cases (31.68%). Occasional epithelioid granuloma was seen in 10 cases (6.21%). In 46 cases (28.57%) only necrosis was seen.

Table 3 shows positivity of AFB by ZN staining and Auramine-Rhodamine staining in different organs. Total percent of positivity for tuberculi bacilli was demonstrated in 170 cases (59.85%). Of these 113 cases (66.47%) involved for RES, 14 cases (8.23%) for bone and joint and 11 cases (6.47%) in both lung and soft tissue.

Table 4 shows positivity of tubercle bacilli by PCR in different organs. PCR was done in 20 cases with positive results in 18 (90%) cases showing a high specificity.

DISCUSSION

In this study, out of 155 cases of reticuloendothelial system, epithelioid granuloma with necrosis and Langhans giant was present in 63 of the cases, epithelioid granuloma with necrosis was present in 59 of the cases and only necrosis was present in 35 of the cases. Out of these 69.67% cases and 3.22% cases were AFB positive on ZN stain and on A-R staining respectively. Thus, in a total of 72% of cases tubercle bacilli could be demonstrated. These observations were similar to the findings of Jha et al, and Hirachand et al.[8,9]

In the present study out of 11 cases of pulmonary tuberculosis, epithelioid granuloma with necrosis and langhans giant cells was present in 4 of the cases, epithelioid granuloma with necrosis was present in 4 of the cases and only necrosis was present in 2 of the cases. All the cases were AFB positive on ZN staining. This is probably due to the fact that of small size (only 11cases of pulmonary tuberculosis were included in this study) as compared to other studies where study sample size was 202 to 650. In a similar study by Chakma et al, out of total 635 cases of pulmonary tuberculosis; 450 cases were AFB positive on ZN staining. 35 similar observations were also seen in the studies of Tan et al, and Ariel et al.[10-12]

On histopatholigical study, out of 29 cases of GIT TB, epithelioid granuloma, Langhans cells and necrosis was present in 20 cases. Epithelioid granuloma and langhans giant cells without necrosis was present in 2 cases, Epithelioid granuloma with necrosis was present in 6 cases, and only necrosis was present in one case. AFB was seen with ZN stain in 8 cases and with A-R stain in one case. In a similar study of 59 cases of GIT tuberculosis by Singhal et al, ileocaecal region was found involved in 40% and peritoneum in 32% cases. Caseating granuloma and AFB was demonstrated in 72% cases.[13]

In the present study urogenital system proved to be the 7th commonest extrapulmonary site to be involved by tuberculosis and accounted for 3.3% cases (n=8). Epithelioid granuloma, Langhans cells and necrosis was present in 7 cases, epithelioid granuloma and necrosis was present in one case. One case was AFB positive. On contrary to this finding, in a study by Trsca et al, AFB was observed in all the cases by ZN stain in two cases and with A-R stain in one case.[14]

Of these 33 cases of bone and joint tuberculosis, epithelioid granuloma with necrosis and Langhans giant was present in 9 of the cases, epithelioid granuloma with necrosis was present in 5 cases, and only necrosis was present in 19 cases. 42.4 % (n=14) cases were AFB positive on ZN stain. These observations were in accordance with the findings of Massod et al, and Francis et al.[15,16]

In the present study female genital tract, was the 6th most common site involved in extrapulmonary tuberculosis and accounted for 3.2% cases (n=9). Of them 3 cases were found to be AFB positive on ZN staining (n=2) and by A-R stain (n=1). Similar findings were also noted by Mondal et al.[17]

In this study out of 284 cases of CNS, 2 were tubercular. On histopathological study epithelioid granuloma, langhans cells and necrosis was seen in one case, epithelioid granuloma with necrosis was seen in one case and AFB was present in ZN stain in one case. In a similar study, Liu et al, detected M. tuberculosis genome in 19 of the 21 (90.5 %) patients with clinically suspected tubercular meningitis.[18]

In this study out of 851 case of breast lump 5 were tubercular. On histopathological study epithelioid granuloma, Langhans giant cells, and necrosis was present in 4 cases. AFB were seen with ZN stain in one case. This was in accordance with the findings of Puneet et al. In his study, out of 1016 patients of breast lump, 42 patients had tuberculosis. Out of these 42 cases of TB, 18 (42.85%) cases shows epithelioid cells, lymphocytes and 13 AFB positive, 3 (7.14%) cases shows epithelioid cells, giant cells and 2 AFB positive, 4 (9.52%) cases shows

epithelioid cells, giant cells and necrosis and 3 AFB positive, 15 (35.71%) epithelioid cells, necrosis and 9 AFB positive. Two (4.76%) cases shows necrosis, polymorphs, lymphocytes and 1 AFB positive.[19]

In this study out of 713 cases of endocrine 2 were tubercular. On morphological study only, necrosis was seen in both cases. AFB were seen on ZN stain in both cases. This was similar to the findings of Barnes et al.[20]

In the present study, out of total 433 cases of skin TB was present in 0.02% (n=10) cases, epithelioid granuloma and Langhans cells were present in 6 cases, epithelioid granuloma, Langhans cells and necrosis were present in 2 cases, epithelioid granuloma and necrosis was present in one case, AFB was positive in 3 cases and A-R was positive in one case. Similarly, prevalence of tuberculosis reported in other studies of Patra et al (n=104; 0.26%).[21]

Of these 33 cases of bone and joint tuberculosis, epithelioid granuloma with necrosis and langhans giant was present in 9 of the cases, epithelioid granuloma with necrosis was present in 5 cases, and only necrosis was present in 19 cases. AFB positive cases on ZN stain were 14 (42.4%). In a study by Masood et al AFB were demonstrated in 64% cases on ZN staining.[15]

The overall positivity of AFB in this study by ZN stain was 56.69% which is variable in different organs in different studies and is found to range from 40-60 %. A-R staining was done in 50 cases of which 9 were positive 18% Variable percentage of positivity has been observed in various studies ranging from 70-80%.

Previous studies have concluded that Auramine-Rhodamine staining is better method of microscopy for demonstration of AFB.[22] The low positivity rate in the present study could be due to a technical or observer error.

PCR was done in 20 cases in which 18 were positive for Mycobacterium tuberculosis showing a positivity of 90% (n=18). No false positive or false negative cases were observed, making it a very sensitive method of detection of tubercle bacilli. All other studies in literature have observed similar findings.[23,24]

CONCLUSION

The findings of the study concluded that morphological pattern of tuberculosis was similar in all the organs with either epithelioid granulomas with or without caseous necrosis. Total number of cases that showed positivity for AFB on ZN staining was 161 (56.6%) and AR staining was 9 (3.16%). Comparatively, ZN stain was found to be a very sensitive, easy, quick and simple method for detection of AFB. Polymerase chain reaction was done in 20 cases of which 18 (90%) were positive rendering this method as very sensitive and specific method for detection of tubercle bacilli than staining methods.

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