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Evaluation of molecular tests in diagnosis of osteoarticular tuberculosis

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Abstract

Osteoarticular tuberculosis is a relatively uncommon type of extrapulmonary tuberculosis. It is an important cause of mortality and morbidity and accounts for approximately 10-15% of all extrapulmonary forms of tuberculosis. The diagnosis is difficult and hence often late. The disability resulting from osteoarticular tuberculosis is directly related to the time of detection of disease and initiation of treatment.

A prospective study of 36 patients suspected with osteoarticular tuberculosis was done from August 2010 to December 2012 was done at St. Stephens hospital Delhi. For the purpose of this study, a diagnosis of osteoarticular TB was based on a combination of suggestive clinical features, in conjunction with typical radiological findings associated with osteoarticular TB. The specimens were subjected to ZN staining, Real Time PCR, Bac-T alert culture & Accuprobe. The sensitivity of real time PCR was 100%, specificity was 58.8%, positive predictive value was 73%, negative predictive value was 100%, efficiency was 80.5% considering culture as gold standard. As shown by the study, each diagnostic test including Real time PCR has its own disadvantages and shortcomings and does not provide 100% accuracy in diagnosing osteoarticular tuberculosis, therefore, strong clinical suspicion and correlation along with radiological and laboratory evidence is a must in establishing a diagnosis.

Keywords: Osteoarticular TB, Real time PCR, Bac-T alert culture

1. Introduction

Infection with *Mycobacterium tuberculosis* remains a major health problem worldwide. The global incidence, as estimated by the World Health Organization (WHO), is said to have increased by 0.4% per annum [1, 2]. India has also seen a significant rise in incidence. India is the highest TUBERCULOSIS burden country accounting for one fifth (21%) of the global incidence (Global annual incidence estimate is 9.4 million cases out of which it is estimated that 2 million cases are from India) [42]. India is 17th among 22 High Burden Countries in terms of TB incidence rate. (Source: WHO global TB report 2010). Osteoarticular tuberculosis is a relatively uncommon type of extrapulmonary tuberculosis. It is an important cause of mortality and morbidity and accounts for approximately 10-15% of all extrapulmonary forms of tuberculosis.

The diagnosis is difficult and hence often late. The disability resulting from osteoarticular tuberculosis is directly related to the time of detection of disease and initiation of treatment. Thus with early diagnosis deformity can be prevented. The WHO estimates that of the 14 million patients worldwide with active TB, approximately 3% will have skeletal infection [15]. Of these, approximately 25% to 60% will have the infectious focus in the spine [16]. Spinal TB produces an indolent and slow-growing infection [17] and is characteristically paucibacillary [18]. For this reason, diagnosis by demonstration of the micro-organisms is often problematic [19]. Despite the diagnostic pitfalls, early accurate identification of the organism and determination of antibiotic sensitivity is essential, as early appropriate treatment is associated with improved outcome and reduced mortality [20].

2. Materials & methods

2.1 Patient population

All patients coming to the orthopedic department at St. Stephen's hospital with suspected tuberculosis of bones and joints from August 2010 to December 2012 were included in this study.

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2.2 Methodology & Technique

In patients of suspected tuberculosis, tissue/pus/fluid specimens were gathered at the time of surgical intervention or aspiration of pus or core biopsy of the lesions. These may be surgical decompression for the spine, incision and drainage of large abscesses, and when indicated a CT guided core biopsy was done. The specimens were subjected to following tests:

2.2.1 ZN Staining

Samples received in tuberculosis laboratory was centrifuged at 2500-3000 rpm for 10 min. and from the pellet slides were prepared, air dried, heat fixed and stained with auramine O and heated underneath till vapour arises and allowed to stand for 5 minutes. Decolourised with 25% sulphuric acid was done for 2-4 minutes and counterstaining with methyleneblue for 30 seconds. Washed with water, air dried and observed under 100x oil immersion. Acid fast bacilli appeared as dark red rods against blue background under 100x oil immersion.

2.2.2 Real time PCR

Real time PCR was performed by mycobacterium tuberculosis Real time PCR kits provided by Genome diagnostics pvt ltd. The system used was corbett Research 6000, Australia. The technique utilises taq-man principle. During PCR, forward and reverse primers hybridize to a specific sequence product which is targeting 16s r RNA segment of *M. tuberculosis* genome. A taq-man probe which is contained in the same reaction mixture and which consists of an oligonucleotide labelled with a 5' reporter dye, 3'quencher dye, hybridize to the target sequence with in the PCR product. Taq polymerase with its 5'-3'exonuclease activity cleaves the probe. The reporter dye and quencher dye is seperated on cleavage, resulting in an increase in flourescence for the reporter. Thus the increase in flourescence is directly proportional to the target ampliication during and which consists of an oligonucleotide labelled with a 5' reporter dye, 3'quencher dye, hybridize to the target sequence with in the PCR product. Taq polymerase with its 5'-3'exonuclease activity cleaves the probe. The reporter dye and quencher dye is seperated on cleavage, resulting in an increase in flourescence for the reporter. Thus the increase in flourescence is directly proportional to the target ampliication during PCR. The primers complimentary for target sequences was used and PCR was run.

2.2.3 Bac-T alert 3D Automation method

Full volume of sample received by the laboratory was transferred in a centrifuged tube and centrifuged at 2500-3000 rpm and from the pellet 0.5 ml was inoculated in the MP plastic bact alert bottle containing 10 ml Middle brook's 7H9 broth supplemented with bovine serum albumin, catalase and pancreatic digest of casein. 0.5 ml of reconstitution fluid was also added to the bottle which contains oleic acid, glycerol, bovine serum albumin, and amaranth dye. The bottle was then inserted in the bac t alert 3D system which is maintained at 37 °C for 8 weeks. The day bottle flashes positive is recorded and confirmed by ZN smear. This is a microbial detection system and a culture media with suitable nutritional and environmental conditions to recover mycobacterial species commonly isolated from patient's specimen other than blood.

It employs a colorimetric sensor and reflected light to monitor the production of CO₂ dissolved in the culture medium. If micro organisms are present in a test sample CO₂ is produced as the organism metabolizes the substrates in the culture medium. When growth of the microorganisms produces CO₂, the color of the gas permeable sensor at the bottom of each culture bottle changes from lighter green to yellow. The lighter color results in an increase of reflectance units monitored by the system. Bottle reflectance is monitored and recorded by the instrument every ten minutes.at the time of detection, approximately colony forming units (CFUs)/ml are 10⁶ – 10⁷.

2.2.4 Confirmation of the isolate by Accuprobe

The accuprobe mycobacterium tuberculosis complex culture identification test is a rapid DNA probe test which utilizes the technique of nucleic acid hybridization for the identification of mycobacterium tuberculosis (TUBERCULOSIS complex) isolated from culture. The ACCUPROBE SYSTEM uses a single stranded DNA probe with a chemiluminiscent label that is complementary to the ribosomal RNA of the target organisms. After the ribosomal RNA is released from the organism, the labelled DNA Probe combines with the target organism's ribosomal RNA to form a stable DNA RNA hybrid. The selection reagent allows for the differentiation of non hybridized and hybridized probe. The labelled DNA: RNA hybrids are measured in a GENPROBE luminometer. A positive result is a luminometer reading equal to greater the cut off value. A value below this cutoff value is a negative result. Cut off value -30,000 RLU.

3. Results

A total of 36 patients were included in this prospective study. Out of which 17 (47%) cases belonged to extraspinal category while 19 cases belonged to spinal (53%) category. Among the patients of extraspinal tuberculosis, regional distribution was almost uniform. Hip (18%) and elbow (18%) were most commonly involved joints. Long bones of leg and arm (18%) were also commonly involved. Interestingly a majority (23%) of patients had only soft tissue involvement without bony involvement. Among the spinal tuberculosis patients, cervical (11%), dorsal (58%), dorsolumbar (5%), lumbar (16%) and 10% of patients were found to have skip lesions. Skip involvement was considered only when two different type of vertebrae were involved. One skip involvement was between cervical and dorsal vertebrae while the other one was between cervical and dorsal vertebrae. Out of the 17 cases suspected to be having extraspinal TB, 3(18%) showed positivity on smear examination, Real time PCR was positive in 11 (65%) cases, 7(41%) cases showed positivity on Bac-t culture. In patients suspected to have spinal tuberculosis, 3 (16%) showed positivity on smear examination, 15 (79%) were Real time per positive and Bac-t alert culture was positive in 12 (63%) cases. Overall smear positive cases were 6(16%), Real time pcr was positive in 26 cases (72%), Bac-t alert culture was positive in 19 (53%) cases in all cases suspected to be having osteoarticular tuberculosis. Although accuprobe was used in all cases but was not included in comparison as it lacked specificity and only confirmed the presence of mycobacteria in tissue/fluid samples.

3.1 Positivity of diagnostic methods with their clinical correlation.

No. of Cases	AFB No. (%Age)	Real Time PCR No. (%Age)	BAC-T Alert Culture No. (%Age)
Total (36)	6(17%)	26(72%)	19(53%)
Extraspinal Cases (17)	3(18%)	11(65%)	7(41%)
Spinal Cases (19)	3(16%)	15(79%)	12(63%)

3.1.1 Comparison of Real time PCR with ZN staining

	Real Time PCR Positive	Real Time PCR Negative	Positivity (% Age)
Smear Positive (6)	6	0	100%
Smear Negative (30)	20	10	67%

Real time pcr was positive in all 6(100%) cases showing positivity with smear examination. Even in 30 smear negative cases real time pcr could pick 20 (67%) as positive.

3.1.2 Comparison of Real time PCR with Bac- alert culture

	Real Time PCR Positive	Real Time PCR Negative	Positivity (% AGE)
BAC-T Alert Positive (19)	19	0	100%
BAC-T Alert Negative (17)	7	10	41%

Considering culture as gold standard, 19 cases were true positives, 7 were false negatives, 10 were true negatives and no false negative were seen with Real time PCR.

	BAC T Alert Culture Positive	BAC T Alert Culture Negative
Real Time PCR Positive	19 (true positive)	7 (false positive)
Real Time PCR Negative	0 (false negative)	10 (true negative)

Modality	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)	Efficiency
Real Time PCR	100%	58.8%	73%	100%	80.5%

The sensitivity of real time PCR was 100%, specificity was 58.8%, positive predictive value was 73%, negative predictive value was 100%, efficiency was 80.5% considering culture as gold standard.

4. Discussion

Osteoarticular tuberculosis is an important cause of morbidity and mortality in tuberculosis endemic countries like India^[22]. The spine is the site most commonly affected with tuberculosis, followed by the hip and the knee^[31, 32]. The confirmation of infection with *M. tuberculosis* in osteoarticular tuberculosis remains a diagnostic dilemma despite advances in radiological and laboratory testing. In fact, radiological findings are often so very similar for TB and various malignancies, that some authors advocate the use of microbiological or histological confirmation in all cases. Confirmation with culture can take as long as eight weeks with solid media culture, and alternative testing platforms are therefore surely needed.

Recently, various forms of serological testing including the interferon-gamma assay have been suggested for diagnosis. This has very limited utility in a high prevalence setting, as most individuals will show some degree of reactivity, irrespective of disease activity. Molecular testing seems to be

more promising and is performed by amplifying and detecting nucleic acids specific to the micro-organism in question. These assays are often capable of delivering results within 24 hours. They also promise superior sensitivities and specificities, depending on number and actual sites targeted for amplification, as well as clinical sites sampled and the HIV-1 status of the patient. Use of molecular methods has the added advantage of improved laboratory safety, as live, infectious organisms are not amplified by culture. Histology is still considered to be the gold standard for diagnosis by some authors, but requires good sampling techniques, and poor quality biopsies often have poor diagnostic utility. In this setting, molecular testing on tissue samples may render superior results, as very little genetic material is needed to be amplified and detected.

3.2 Sensitivity of various diagnostic methods & their clinical correlation

Diagnostic Modality	Sensitivity
Real Time PCR (26/36)	72%
Smear Examination (6/36)	17%
BAC-T Alert Culture (19/36)	53%
Imaging (20/36)	55.5%

Overall positivity of real time PCR was 72%. Smear examination showed a positivity of 17%, while that of Bac-t alert culture was 53% and radiological imaging (Xrays) was 55.5%.

3.3 Positivity of Real time PCR considering culture as gold standard.

3.4 Calculation of sensinty, specificity, predictive values and efficiency considering culture as gold standard

The diagnostic test of choice should not only provide accurate results, but should also do so in a timely manner, to ensure the early initiation of appropriate therapy⁴⁶. Z-N smear examination and traditional culture (Lowenstein jenson media) methods are also not very sensitive and often show low positive or negative results. The diagnosis of tuberculosis is perforce based on histopathological examination which calls for professional expertise. It also takes about 2-3 weeks for the report to come back, depending upon the type of

sample. The presence of classical caseating tubercle granulomas is a must for establishing a diagnosis of tuberculosis. But the tubercle may be absent in, many samples. The dilemma, then, is between the clinical suspicion and the confirmatory evidence. Real time PCR is now an established method of diagnosing tuberculosis in a rapid manner. It can detect tubercular bacilli, even if they are present in extremely low quantities as low as 10 femtogram. Further, the diagnosis can be established within 24 hours. If the diagnosis of tuberculosis can be made rapidly, the timely institution of antitubercular treatment can prevent further joint damage and disability.

Analysing the results, it is apparent that the positivity of Real time PCR is quite high in spinal samples as compared to extraspinal samples i.e. 79% & 65% respectively as seen with clinical correlation. The lower positivity of the extraspinal cases can be explained due to the dilution of tubercle bacilli in non spinal samples. Bone samples from the spine have higher concentrations of the bacilli and consequently, yield higher sensitivity [8]. The overall positivity of Real time PCR was 72% in accordance with clinical correlation. And, considering culture as gold standard sensitivity of real time PCR test came out to be 100%, specificity 58.8%, positive and negative predictive values as 73% and 100% respectively. The efficiency of the same was 80.5%. Various other studies have reported sensitivity of PCR ranging from 61% to 83% [7, 34, 35]. Real time PCR showed much higher positivity when compared with other two tests ($p < 0.05$) i.e. ZN stained AFB smear examination and Bac-t alert culture, which had a positivity of 17% and 53% respectively or even with radiology, which showed 55.5% positivity. As osteoarticular tuberculosis is a deep seated infection and is a paucibacillary disease, the culture sensitivity of disease is therefore, low. Beside best efforts to get specimen in a tertiary care center and sending it to a well equipped microbiological laboratory, it is a matter of concern as we can't diagnose primary and secondary resistance. The low sensitivity of culture (53%) in our study can be attributed to distance between the sample collection site and the testing laboratory. This can be improved by immediate storage and transfer of samples (samples should be stored at -20°C and should get processed ideally within 1 hour of collection, samples should be kept with in ice packs while transfer). Ideally the volume of sample should be more than 2 ml to demonstrate mycobacterium tuberculosis in a sample. By ensuring better handling and early processing of samples better results can be achieved by culture method.

In comparison with Real time PCR, diagnosis of osteoarticular tuberculosis by Bac -t culture is much more easily available and more reliable and more accurate diagnostic modality especially in a set up like in our country. However Real time PCR can be done in all those cases where Bac-t alert culture is negative and patient seems to be non responder and repeated cultures show no growth. In such cases Real time PCR can be done if it shows positivity and DNA sequencing may help pick drug resistant strains. Real time PCR test was able to give a positive result in less than 24 hours as compared to average time of 15-30 days taken by culture. However, as shown by the study, each diagnostic test including Real time PCR has its own disadvantages and shortcomings and does not provide 100% accuracy in diagnosing osteoarticular tuberculosis, therefore, strong clinical suspicion and correlation along with radiological and laboratory evidence is a must in establishing a diagnosis.

5. Conclusion

Real time PCR has its own deficiencies and fallacies and can't be completely relied upon as a single diagnostic modality. Establishing the diagnosis of tuberculosis beyond doubt is very important when considering the cost and duration of treatment and the effects of delayed treatment. Moreover, it has its economic and psychosocial implications in the developing world. Better results of Real time PCR can be achieved and shortcomings minimized by the adequate training of personnel in molecular methods and preventing laboratory induced contamination. Hence, it would be rational approach to combine clinical presentation with Real Time PCR to solve the diagnostic dilemma in patients presenting with osteoarticular tuberculosis.

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