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RESEARCH ARTICLE

DETECTION OF MYCOBACTERIUM TUBERCULOSIS DNA IN SPUTUM SAMPLES WITH MPB 64 GENE PRIMERS USING POLYMERASE CHAIN REACTION.

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Abstract

Tuberculosis (TB) is preventable and curable, but it can lead to death if no actions are taken. In order to prevent transmission, it is necessary to identify infectious TB patients in a timely manner. The present study was aimed to detect the mycobacterium tuberculosis DNA in sputum samples with MPB 64 gene primers using PCR. Hundred patients were enrolled in the study based on inclusion and exclusion criteria. Sputum samples were collected and processed for the required procedure. Ziehl-Neelsen staining was done and slides were examined under microscope. DNA was extracted from the samples and amplified using PCR. Staining results of both spot and morning samples was compared under microscope with grading.

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Introduction:-

Tuberculosis must have been a scourge since mankind existed as reports of this dreadful disease are recorded centuries back. Tuberculosis is the major public health problem. In 1993, the World Health Organization (WHO) in an unprecedented step declared tuberculosis a global emergency. The fact sheet of global tuberculosis is stunning. World Health Organization (WHO) emphasized both the large death toll from tuberculosis, which kills more people than any other single infections agent, and the curability of the disease (Alcaide et al., 2000).

The family mycobacteriaceae consists of more than 80 different species and more than half of them may be isolated from humans. Most of them are apathogenic, but may cause disease in immunocompromised patients. The pathogenic species, the members of the *M. tuberculosis* complex (MTC) and *M. leprae*, cause tuberculosis and leprosy, respectively. MTC comprise *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. caprae*, *M. microti* and *M. canetti* (Britten, 2005). The natural host of *M. tuberculosis* and *M. africanum* are humans, whereas *M. bovis* can cause disease in a wide range of animals like cattle or goats, as well as in humans (Cegielski et al., 1997).

The emergence of drug resistant strains of *M. tuberculosis* is an increasing problem in developed and developing countries (Klemen et al., 1998; Kolk et al., 2003). Drug resistance tuberculosis has been reported since the early days of introduction of anti TB chemotherapy, but multidrug resistance tuberculosis (MDR-TB) have been areas of growing concern, and are posing a threat to global effort of tuberculosis control. Prevalence of drug resistance tuberculosis (TB) mirrors the functional state and efficacy of tuberculosis control programs and realistic attitude of the community towards implementation of such program (Dunlop and Briles, 1993). Poor tuberculosis (TB) control generates MDR-TB and the misuse of second line drugs generates XDR-TB. More than 4000 cases of MDR-TB emerge every year as a result of poor management of drug sensitive as well as drug resistant tuberculosis (TB).

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The present study was aimed to detect the mycobacterium tuberculosis DNA in sputum samples with MPB 64 gene primers using PCR. The results obtained were compared with Ziehl Neelsen technique for sensitivity and specificity.

Materials and Methods:-

Subjects:-

It is a cross sectional study carried out on 100 patients in the Department of Microbiology, Santosh Medical College, Ghaziabad (U.P). Patients were enrolled based on the following inclusion criteria

1. Patients with chronic cough \geq 2 weeks.
2. Patients with fever \geq 2 weeks.
3. Patients having cough with or without sputum

Patients who refused to give consent to participate were excluded from the study.

Sample Collection:-

A wide mouth, sterile, clean, leak proof screw cap containers were used for sputum collection. Five ml of sputum were collected as per the RNTCP guidelines.

Microbiological Tests:-

New clean unscratched slide was taken. Sample was transferred to the mucopurulent portion of slide with the help of broom stick of 5mm dm (27SWG). Smear of approximate 2 to 3 cm was made and allowed to air dry for 15 minutes. The smear was fixed on the slide by passing it over the flame 3 to 5 times for 3 to 4 seconds each.

Ziehl-Neelsen Staining:-

Heat fixed slide was placed on the staining rack with the smeared slide facing up. Then, the slide was flooded with filtered carbol-fuchsin (1%) for 5 minutes. It was washed briefly in running water to remove excess dye. Slide was flooded with 25 % sulfuric acid (H₂SO₄) solution for 2–3 minutes. Again, it was washed briefly in water, keeping in mind not to let the section dry out. Next the slide was flooded with methylene blue (0.1%) for 30 seconds. Finally, the slide was washed immediately in tap water to remove the stain. Slide was allowed to air dry before it was examined under microscope.

Decontamination and homogenization:-

All sputum specimens submitted to the tuberculosis cultures were subjected to a harsh digestion and decontamination procedure that liquefies the organic debris and eliminates contaminants. Four ml of sputum sample was transferred to a centrifuge tube. Add double the volume of sterile NaOH solution (4%) into the tube. Tighten the caps of the tube and mix it well by hand for 1 minute. The tubes were placed in shaker and incubated at 37 °C for 15 minutes. At the end of 15 minutes, tubes are removed from the incubator and 15 ml of sterile distilled water added. Mix it well and centrifuge at 3000g for 15 minutes. Tubes should be carefully removed from the centrifuge without shaking. The supernatant fluid is discarded slowly into a container with phenol solution (5%). Wash the pellet with sterile distilled water at 3000g for 15 minutes and decant the supernatant. Sediment part was used for PCR.

Polymerase Chain Reaction (PCR):-

The sputum samples obtained after processing with modified petroff's methods were used for DNA extraction. DNA was extracted using commercially available QIAmp DNA mini kit, QIAGEN, Germany with one initial additional step. The preliminary processed materials were kept at 80°C for 10 min for inactivation of possible mycobacterium. The material was then further processed as per standard method. DNA sequences were amplified in the PCR thermal cycles model 2700 (Applied Bio systems). In each independent PCR assay, test results were compared with the results for one positive and one negative control. The positive control includes the DNA of H37Rv and negative control includes the PCR grade water.

Results:-

A total of 100 cases of clinically suspected tuberculosis infection attending OPD/ IPD and DOTs centre at Santosh Medical College / Hospital, Ghaziabad Uttar Pradesh were selected. Clinical profile of selected patients was given in table 1.

Table 1:- Clinical Profile of Selected Patients

S. No	Demographic/ Clinical features		Number (%)
1	Total no of patients		100
2	Sex	Male patients	70 (70%)
		Female patients	30 (30%)
3	Mean age	Male patients	38.15 years
		Female patients	39.77 years
4	Cough	>2 weeks	86 (86%)
		<2 weeks	14 (14%)
5	Fever	>2 weeks	73 (73%)
		<2 weeks	27 (27%)
6	Chest pain		60 (60%)
7	Breathlessness		70 (70%)
8	Hemoptysis		14 (14%)
9	History of contact		45 (45%)
10	Loss of appetite		78 (78%)

Of the total patients (100), the number of male patients was 70 (70%) and female patients were 30 (30%). This data also shows that the average mean age of the male patients were 38.15 yrs and female's patients were 39.77 yrs. Chest pain was observed in 60% of patients and 45% had infection by contact to infected person followed by hemoptysis (14%). The patients also showed loss of appetite (78%).

Sputum smears were made on fresh slide for all the samples. Ziehl-Neelsen staining method was used for staining the slides and examined under microscope. Both the sputum samples (spot and morning) was examined and results shown in table 2 and 3.

Table 2:-Comparison between spot and morning sample examined under microscope

Samples	Positive	Negative
Spot Samples	11(11%)	89(89%)
Morning Samples	14(14%)	86(86%)

Out of 100 samples, during spot sample examination; 11 (11%) smear were positive while on the other hand, in the morning samples, 14 (14%) were smear positive. The samples which were positive in spot sample were also positive for sample collected early in the morning. The isolation rate of *M. tuberculosis* was more in morning samples (14%) as compared to in spot sample (11%).

Table 3:-Ziehl Neelsen Smear Staining Results under Microscopy with Grading

Sample		Smear Positive	Smear Negative	Smear Grading Sputum Positive			
				Scanty	1+	2+	3+
Z.N. Staining	Spot (100)	11 (11%)	89 (89%)	00	04 (36.4%)	05 (45.5%)	02 (18.2%)
	Morning (100)	14 (14%)	86 (86%)	02 (14.3%)	04 (28.6%)	06 (42.8%)	02 (14.3%)

Majority of patient who were positive only in morning samples had sputum smear grade as scanty and 2+.

Discussion:-

Among the communicable diseases, tuberculosis (TB) is the second leading cause of death worldwide. Killing nearly 2 million people each year. It is estimated that about one third of the world population are infected with TB (2 billion people) and about 10% of this figure will progress to disease state most cases are in the under developed countries of the world (Cave et al., 1997).

In the present study, the clinical history of the patients showed that number of male patients 70 (70%) was higher as compared to that of female patients 30 (30%). The global data on tuberculosis prevalence has shown that the prevalence of *M. tuberculosis* is similar in males and females until adolescence; but after that it appears higher in males (Supply et al., 2000). Several studies have explored reasons behind the gender bias in tuberculosis

susceptibility and found that fear and stigma associated with TB makes greater impact on women than on men (Cave et al., 1991). The analysis of epidemiological data was done which also reflects gender to have an impact on the disease and its control (Crafton, 1959). It seems, therefore that the observed differences in disease prevalence in males and females may or may not have any direct biological basis; however, they are strongly associated with malpractice of health care policies by care providers or care seekers. The social and economic impact of Tuberculosis (TB) which claims lives of more than 400,000 people every year is devastating, especially as it affects the economically most productive age group (Lienhardt et al., 2010). Furthermore, in virtually all countries, fewer female than male tuberculosis cases are notified (Grosset, 1980).

Our result showed that 86% of patient had coughed more than two weeks. Earlier study done in 2002 in a different setting showed 47% increase in sputum positive cases among chest symptomatic with >2 weeks cough (Mitchison, 1979). A study done in 2006 reported an increased yield of 16% smear positive cases when the screening criterion used was cough of >2 weeks (44 of 622 with >2 weeks vs. 37 of 275 with > 3 weeks) (Daniel, 2006). Also most of the patients showed chest pain and loss of appetite followed by hemoptysis and history of contact. This data also helped in the diagnosis of TB infection.

In the present study, the smear positive rate was approximately higher for men than for women suggesting that the sex differences reflect biological phenomena rather than lower access to TB diagnosis for women. These may include not only true differences in TB incidence, but also differences in the bacillary load of sputum specimen and thereby in the sensitivity of smear examination (Gutierrez et al., 2005). This result correlates with the study in which workers reported the majority in older ages (after 70 yrs) (Chauhan et al., 2006).

For developing countries, the smear microscopy to detect acid fast bacilli (AFB) in clinical specimens by Z.N. staining is likely to remain the only cost effective tool for diagnosis patients with TB and to monitor the progress of treatment. The overall sensitivity of the smear has been reported to range from 22% to 80% (Niederweis, 2003).

An important factor influencing sensitivity is the minimum amount of sputum submitted to the laboratory. In a long-term study, the sensitivity of a concentrated smear from >5 ml of sputum was significantly greater than the sensitivity of a smear processed regardless of volume. Ever since sputum smear examination was made the specific tool for diagnosing tuberculosis, Scientists have investigated the additional case yield from examination of multiple successive sputum specimens (Chambers et al., 1995).

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