ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 5, Issue, 05, pp.1123-1126, May, 2013

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

A PHYLOGENETIC ANALYSIS OF *Mycobacterium tuberculosis* COMPLEX FROM ENDOMETRIAL SAMPLES OF FEMALE INFERTILITY CASES

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ARTICLE INFO

Article History:
Received 12th February, 2013
Received in revised form
20th March, 2013
Accepted 24th April, 2013
Published online 12th May, 2013

Key words:
Infertiltiy,
Mycobacterium tuberculosis,
16S rRNA.

ABSTRACT

One of the most important and underappreciated reproductive health problems in developing countries is the high rate of infertility and childlessness. Many microorganisms seem to be involved in male & female reproductive systems. However, such infections, as well as those involving other and cases of endometritis, leading to uterine synechiae, are less common than tubal occlusions parts of the male genito-urinary tract, may cause a microbial colonization of the semen. Some conditions; however, seem to have a greater impact on female fertility. Vaginal infections are of doubtful impact resulting from salpingitis. Adhesions, caused by pelvic inflammatory disease, seem to affect the functional status of the tubes more harshly than that of the uterus. Molecular epidemiologic studies of tuberculosis (TB) have focused largely on utilizing molecular techniques to address short- and long-term epidemiologic questions, such as in outbreak investigations and in assessing the global dissemination of strains, respectively. In essence, molecular epidemiology focuses on the role of genetic and environmental risk factors, at the molecular/cellular or biochemical level, in disease etiology and distribution among populations. This is done primarily by examining the extent of genetic diversity of clinical strains of Mycobacterium tuberculosis**.** The traditional identification of bacteria on the basis of a phenotypic characteristic is generally not as accurate as identification based on genotypic methods. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains, can be routinely used for identification of Mycobacterium, and can lead to the recognition of novel pathogens and non cultured bacteria.

INTRODUCTION

Tuberculosis (TB) is an increasing public health concern worldwide, with about six million new cases a year. It is one of the most important causes of infectious morbidity and mortality. On a global scale, TB has a devastating impact in developing nations with 13 countries accounting for nearly 75% of all cases (1). TB exists in two forms: pulmonary tuberculosis and extrapulmonary tuberculosis. Genital TB is one form of extrapulmonary TB and is not uncommon particularly in communities where pulmonary TB is prevalent, but it is rare in Western societies. Classically, genital tuberculosis has been described as a disease of young women, in 80–90% of patients being first diagnosed between the age of 20 and 40 years (5). Primary genital tuberculosis is extremely rare and genital tract infection is always secondary to tuberculosis elsewhere in the body. The fallopian tube constitutes the initial focus of genital tuberculosis in 90–100% of patients, followed by the uterus (50–60%), the ovaries (20–30%), the cervix (5–15%) and lastly the vagina in 1% of patients (6). This is evidenced by the paucity of literature on this subject in developed countries as compared with less developed countries. Genital TB affects about 12% of patients with pulmonary tuberculosis (2) and represents 15–20% of extrapulmonary tuberculosis (3). Genital TB may be asymptomatic and diagnosis requires a high index of suspicion. Moreover, the disease may masquerade as other gynecological conditions and can go unrecognized (4).

Prevalence of infertility in developing countries

TB is found in 5-10% of women with infertility problems (8) with low rates in Australia (1%) and high rates of up to 19% in India (7).

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Only a limited number of papers report on the prevalence of infertility in developing countries. According to Boivin *et al*. (2007), the 12-month prevalence rate ranges from 6.9 to 9.3% in less developed countries. Substantial geographical differences in the prevalence are noted, and these differences are largely explained by different environmental, cultural and socioeconomic influences. In Sub-Saharan Africa, the prevalence differs widely from 9% in the Gambia (9) and 11.8% in Ghana (10) compared with 21.2% in northwestern Ethiopia (11) and between 20 and 30% in Nigeria (12). Even less data are available from Asia and Latin-America, but a report compiled by the World Health Organization (WHO) indicated that the prevalence of infertility in these regions fell within the globally expected range of 8–12% of couples of reproductive age and was thus lower when compared with African countries (13).

Choice of the 16S rRNA Gene as the Gene to Sequence

Widespread use of this gene sequence for bacterial identification and taxonomy followed a body of pioneering work by Woes, (18) who defined important properties. The degree of conservation is assumed to result from the importance of the 16S rRNA as a critical component of cell function. This is in contrast to the genes needed to make enzymes. Mutations in these genes can usually be tolerated more frequently since they may affect structures not as unique and essential as rRNA. Thus, few other genes are as highly conserved as the 16S rRNA gene. Although the absolute rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms (15, 16, and 17). The possibility that this rate of change of 16S rRNA gene may not be identical for all organisms (different taxonomic groups could have different rates of change), the rates could vary at times during evolution, and the rates could be different at different sites throughout

the 16S rRNA gene. There are so-called "hot spots" which show larger numbers of mutations (19); these areas are not the same for all species. Lastly, the 16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria (18) In general, the comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including what we now call the species and subspecies level. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences. The increasing incidence of endometrial tuberculosis leading to infertility problems in developing countries has become a major threat to the reproductive population. In the present study, *Mycobacterium tuberculosis* strains were isolated from the endometrial tissue samples of the subjects who had an undergone laparoscopic surgery due to strong clinical evidence of endometrial tuberculosis. The strains were characterized with regard to phenotypic and genotypic traits. The molecular relatedness among *Mycobacterium* strains was evaluated by 16S rRNA sequencing and to obtain phylogenetic tree to search for emergence of new variant of *Mycobacterium sp.*

MATERIALS AND METHODS

Study area

The female infertility cases in Tiruchirapalli district were evaluated in Ramakrishna Nursing Home at Tiruchirapalli, Tamil Nadu, India. Clinically evident endometrial tuberculosis cases were selected for further studies.

Collection of endometrial specimen

The endometrial biopsy tissue was collected by laparoscopic surgical procedure. A telescope like instrument was inserted into the abdomen through a small incision which gives a complete picture of the endometrium, uterus etc. Then instruments can be threaded through the laparoscope to perform some surgical procedure and finally, the cutterage of the endometrium especially from both corneual ends were collected in a sterile saline container and labeled neatly then it was transported to the laboratory for further analysis.

Morphological examination of Tuberculli Bacilli Using Ziehl – Neelsen staining technique

The endometrial tissue sample was digested and smear was prepared in clean, grease and scratch free new glass slides. The smear was air dried and heat fixed gently by passing over the Bunsen flame. The smear was covered well with strong carbol-fuchsin (1%) for 7minutes and intermittently the bottom of the slide was slightly heated thrice without boiling. Then the slide was washed in running tap water. The primary stain was removed with the addition of 3% acid – alcohol and washed again in running tap water. Finally the slide was counter stained with malachite green for 1-2minutes and washed again in running tap water. The smear was air dried and viewed under 100x of light microscope for the presence of acid fast bacilli against green background.

Identification of *Mycobacterium* using Real time PCR

Real time PCR was carried out for *Mycobacterial* detection and differentiation of *Mycobacterium tuberculosis* – complex (MTC) and *Mycobacterium* species – Other than tuberculosis complex (MOTT).

Isolation of Genomic DNA from clinical specimen of female infertility cases

AccuPrep Genomic DNA extraction kit can rapidly and conventionally extract an average of 6 µg of total DNA from a variety of sources, such as mammalian tissue, whole blood, leucocytes and cultured cells.

Mycobacterial DNA isolation

The tissue sample was added with 20µl proteinase-K and 200µl of tissue lytic buffer provided in the kit. The contents were vortexed gently and kept at 60^0C for 1 to 2 hours, intermittently the tissue containing tube was vortexed once every 10minutes till the complete tissue lysis. Then 200µl binding buffer was added and gently vortexed for mixing. The tube was spinned briefly and incubated at 60^0C water bath for 10 minutes. After completion of incubation 100µl of isopropyl alcohol was added, vortexed for mixing and spinned briefly. Then the whole 520µl tissue lysate was pipetted into DNA binding column. The binding column was spinned at 10,000 rpm for 2minutes. The collections were discarded into sodium hydrochlorite solution.

Washing

First wash 1: The first wash buffer 500µl was added and centrifuged for 2 minutes at 10,000rpm. The flow through was discarded.

Second wash 2: The second wash buffer 500 µl was added and centrifuged for 2 minutes at 10,000rpm. The flow through was discarded. The binding column was fitted into a new 1.5ml of ependroff tube and centrifuged at 14,000 rpm for 5minutes for the complete removal of residual ethanol used in wash buffers.

Elution of Genomic DNA

The binding column was fitted into a new 1.5ml ependroff tube for DNA collection. Then 50µl warm (60^0C) elution buffer was added and incubated for 5minutes at room temperature. Finally the binding column was spinned at 10,000 rpm for 3minutes to elute the DNA. The eluted DNA was amplified under Real time PCR condition for the diagnosis of Mycobacterial infections and differentiation with the specific primers and probes. The remaining DNA was preserved at -20^0C.

Real time PCR amplification (Fig 1)

The Geno-sen's reagents are ready to use primer system for the detection and differentiation of MTC/MOTT using polymerase chain reaction (PCR) in the Rotor Gene 2000/3000/6000 (Corbett Research).

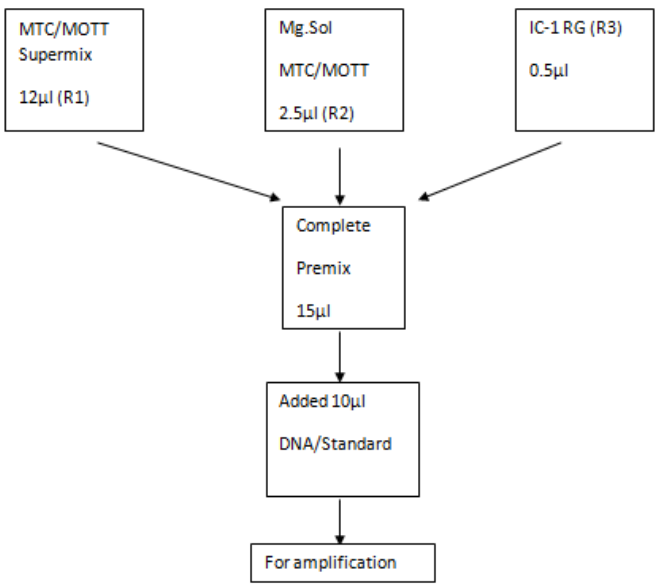

Fig. 1. Real time PCR amplification

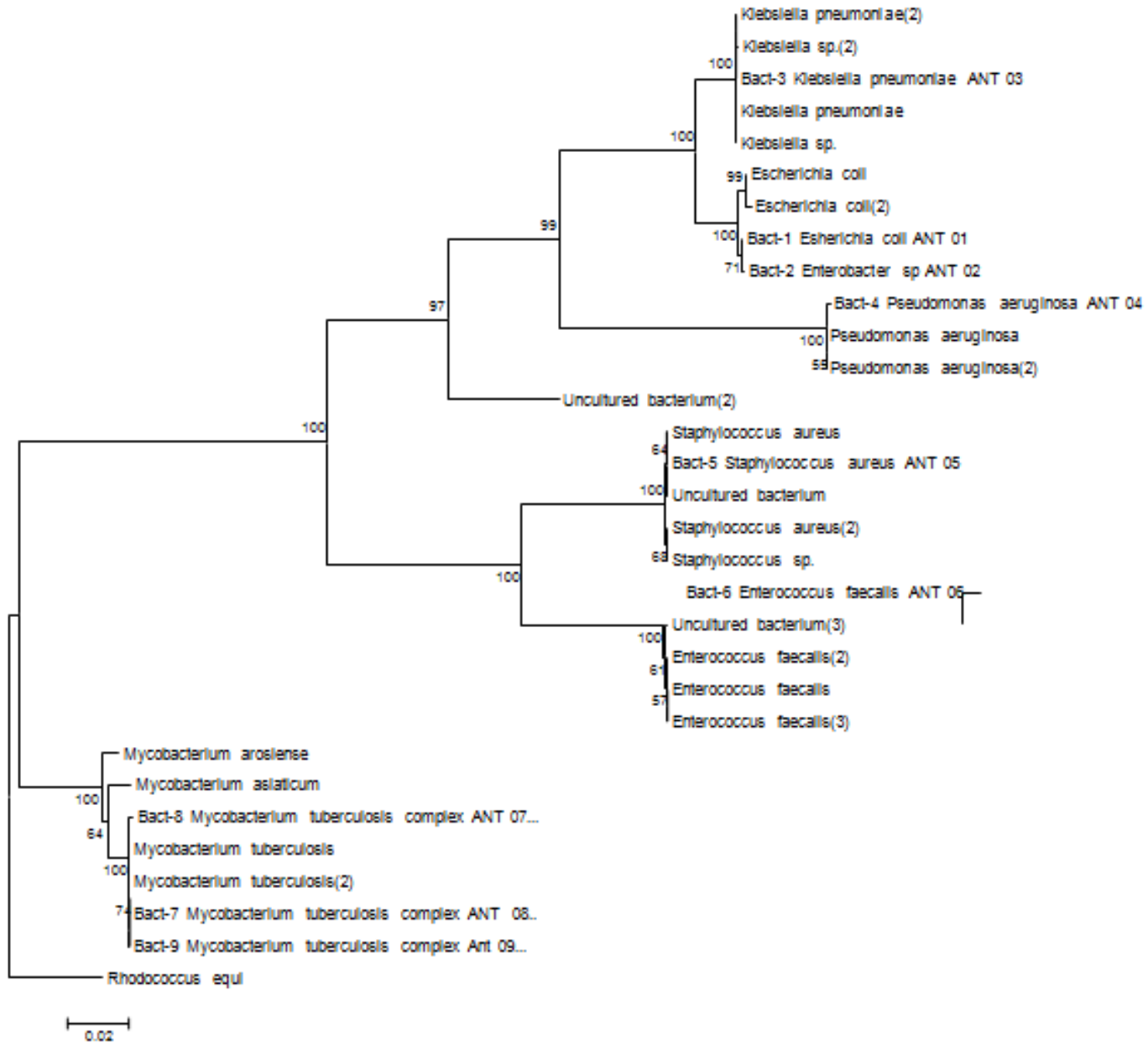

Fig. 2. Phylogenetic tree of *Mycobacterium tuberculosis* from endometrial tissue sample

The specific master mix contains reagents and enzymes for the specific amplification of MTC /MOTT and for the direct detection of the specific amplicon in fluorescence channel cycling A.FAM (MTC), Cy5 (MOTT and MTC) of the Rotor Gene 2000/3000/6000 and the inhibition control gene on cycling A.Joe External positive control (MTC/MOTT) were used for the confirmation of PCR assay working. As per the following scheme mentioned below Real time PCR MTC/MOTT primers were added to the extracted DNA, standards and negative control Real time PCR cycling profile Initial activation of the hot start enzyme 95°C for 10minutes.

Cycling for Amplification 45 cycles amplification holds

1. Denaturation profile 95^0C for 15 sec
2. Annealing cycling profile 55^0C for 20 sec
3. Extension cycling profile 72^0C for 15 sec

Gene sequencing of amplified product

The amplified PCR products were purified using Nucleo spin and Accuprep genomic DNA extraction kits as recommended by the manufacturer. Sequencing Primer for amplification of 16S rRNA gene was chosen from a panel of forward and reverse primers provided already.

Primer Sequence

518 F – $CCA_g\,CA_gCC_{gg}C_{gg}TAATAC_g$
800 R – $TACCA_{ggg}TATCTAATCC$

These forward and reverse primers were used as sequencing primer to amplify 16S rRNA gene. The sequences of the PCR product was determined using Applied Biosystem Big Dye terminator cycle sequence V 3.1 kit as on AB model 3730 XL automated DNA sequences system USA according to the manufactures instructions. The 16S rRNA gene sequence of MTC and MOTT were determined from the homosapiens host.

Submission of Gene sequences in NCBI

The 16S rRNA gene sequences of MTC were submitted in NCBI to obtain the accession number.

Phylogenetic analysis of microbial isolates in female infertility cases

The NJ method is a simplified version of the minimum evolution (ME) method which uses distance measures to correct for multiple hits at the same sites, and chooses a topology showing the smallest value of the sum of all branches as an estimate of the correct tree. However, the construction of an ME tree is time-consuming because, in principle, the *S* values for all topologies have to be evaluated and the number of possible topologies (unrooted trees) rapidly increases with the number of taxa. With the NJ method, the *S* value is not computed for all or many topologies. The examination of different topologies is imbedded in the algorithm, so that only one final tree is produced. This method does not require the assumption of a constant rate of evolution so it produces an unrooted tree. However, for ease of inspection, *MEGA* displays NJ trees in a manner similar to rooted trees. In the construction of the NJ tree, MEGA may request to specify the distance estimation method, subset of sites to include, and whether to conduct a test of the inferred tree through an Analysis Preferences dialog box was carried out.

RESULT AND DISCUSSION

16S rRNA sequencing and similar molecular identification methods have the additional advantage of reducing the time required to identify slow-growing bacteria such as *Mycobacterium*, which may take 6–8 weeks to grow in culture sufficiently for phenotypic tests to be performed. The following *Mycobacterium tuberculosis* organisms were isolated and amplified using Real time PCR , gene sequenced and their 16S rRNA were deposited in NCBI and accession Numbers were obtained (Table 2).

Table 1. Isolated Mycobacterial pathogens from endometrial tissue

Organisms isolated	Real time -PCR
MTC	25 (14.88%)
MOTT	7 (4.16%)

Table 2. GENE bank accession numbers of the deposited opportunistic pathogens

S.No	ORGANISMS	NCBI Accession No
1.	Mycobacterium TB complex (ANT07)	HM803173
2.	Mycobacteria TB complex (ANT 08)	HM803174
3.	Mycobacteria TB complex (ANT 09)	HM803175

Phylogenetic Investigation

The extra information obtained by a genetic approach to taxonomic studies allows the reconstruction of the phylogenetic tree of related systematic entities. Highly conserved regions present in particular genomic regions are the ideal target for such studies since the number, type, and position of mutations appearing through evolution represent a sensitive metric of phylogenetic distances. Some perplexity arises, however, at least for non taxonomists, when phylogenetic trees based on different highly conserved regions grossly differ or when different clustering emerge from the analysis of the same genetic sequences performed with various mathematic algorithms. The phylogenetic trees reported here (Fig. 2) were developed by submitting to the BLAST program. The 16S rRNA sequences present in publicly available databases for all the established species of the genus Mycobacterium. The three major phylogenetic groups within the genus Mycobacterium on the basis of 16S rRNA sequences is almost homogeneous and certainly related to M. tuberculosis of already exisisting group. The present study reports no new variant strain of Mycobacterium tuberculosis emerging recently. This study is a first of its kind to be carried out in southern part of India to study the phylogenetic relationship of the organism (Mycobacterium) isolated from the endometrial tissue sample. There is much more research needed to be carried out in this area to probe the exact pathogenesis of the organisms from pulmonary site to the extra pulmonary site which would provide future scope to further research.

Conclusion

It is remarkable and equally serendipitous that 16S rRNA gene sequence would have almost the exact amount of variability to define a species or at least provide a clinically useful distinction among bacterial strains. Identifying bacterial isolates in the clinical laboratory by sequence instead of phenotype can improve clinical microbiology by better identifying poorly described, rarely isolated or biochemically aberrant strains. 16S rRNA gene sequences allow bacterial identification that is more robust, reproducible, and accurate than that obtained by phenotypic testing. The test results are less subjective. 16S rRNA gene sequence analysis can lead to the discovery of novel pathogens. 16S rRNA gene sequence analysis can identify noncultured bacteria, allowing independence from growth conditions. On a population scale, accurate identification has greatly improved our understanding of the epidemiology of clinical syndromes, and hence has improved empirical treatment of these. Our understanding of all these aspects is further improved by the discovery of novel genera and species of bacteria, which has been greatly facilitated by using 16S rRNA sequencing.

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