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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF PATHOGENS ISOLATED FROM ENDOMETRIAL SAMPLES OF FEMALE INFERTILITY CASES

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ABSTRACT

Many microorganisms seem to be involved in female reproductive failure in various ways. Infection of the lower genital tract seems to have little importance, if not, in the unlikely event of an occlusion. However, such infections, as well as those involving other parts of the male genito-urinary tract, may cause a microbial colonization. Virtually all parts of the female reproductive system may be influenced by infectious agents; some conditions, however, seem to have a greater impact on female fertility. Vaginal infections are of doubtful impact and cases of endometritis, leading to uterine synechiae, are less common than tubal occlusions 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. The aim of the present study was to carry out a microbiological investigation of endometrial samples of females suffering from unexplained infertility. Sample sizes of 168 women were evaluated by standard bacterial culture method and for fungal isolates. Bacterial infections were detected in 42.85% of infertility cases. Among the bacterial infections *Enterobacter spp* was the dominant followed by *E.coli*. The fungal infections were isolated in 65.47% of the infertility cases *Candida* spp were isolated from 5.36% of cases. *Aspergillus* was found only in 0.6% of cases. A combination of *Candida* and *Aspergillus* growth was found in a single case(0.6%). Following the isolation the organisms were sequenced then deposited in NCBI. The restriction sites and secondary structures were studied to confirm the emergence of new sub strains.

INTRODUCTION

One of the most important and underappreciated reproductive health problems in developing countries is the high rate of infertility and childlessness (1). The inability to procreate is frequently considered a personal tragedy and a curse for the couple, impacting on the entire family and even the local community. Negative psychosocial consequences of childlessness are common and often severe (2), (3). Infectious agents can impair various important human functions, including reproduction. Bacteria, fungi, viruses and parasites are able to interfere with the reproductive function in both sexes. Infections of male genito-urinary tract account for about 15% of the case of male infertility. In female, the microbial colonization is certainly involved in cervical, tubal, and peritoneal damage, while Herpes simplex cervicitis is less dangerous. The overall importance of cervical involvement is still under discussion. Tubo-peritoneal damage seems to be the foremost manner in which microorganisms interfere with human fertility. (4)

Apart from Endometrial tuberculosis, the most common infection that seems to be associated with Infertility is urinary tract infections which are caused by bacteria entering the urethra from outside. Of all cases somewhere around 70% is caused by the bacterium *E.coli*. It actually lives naturally in the bowel and is harmless .However when it enters the bladder it multiplies rapidly and causes symptoms within hours. Bacterium usually enters the urethra due to cross contamination from the bowel.

There are reports that some protozoa, helminths and fungi may impair women's reproductive capacity, causing deformities of genital tract, so that conception is impossible, or, if it does occur, normal implantation and development of placenta are impossible. The consequence of fungal infections, such as colpitis and endometritis, caused by *Candida albicans*, leads to infertility (5). The incidence of systemic fungal infections is increasing in patients. Because these infections frequently disseminate, they may affect the function of the pituitary, thyroid, parathyroid, and adrenal glands; the pancreas; and the reproductive organs and manifest into severe infertility problem. (6).

Recently, the use of molecular methods for detection, identification, and characterization of infectious agents in general is gaining importance in clinical microbiology laboratories. Emerging and re-emerging pathogens pose several challenges to diagnosis, treatment, and public health surveillance. Identification of an emerging pathogen by conventional methods is difficult and time-consuming due to the "novel" nature of the agent, requiring a large array of techniques including cell cultures, inoculation of animals, cultivation using artificial media, histopathological evaluation of tissues, serological techniques using surrogate antigens. (7)

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Above all the most convenient and time saving Molecular methods are preferred widely for accurate diagnostic purpose.

MATERIALS AND METHODS

Isolation of Bacterial and Fungal strains

Collection of endometrial specimen

The endometrial biopsy tissue was collected by laparoscopic surgical procedure. 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.

Detection of bacterial and fungal pathogens from the endometrial specimen

For the detection of fungal and bacterial pathogen, the collected samples were subjected to staining techniques and plate culture method.

Morphological investigation

The morphological characters of the microbial isolates collected were studied by the following staining method such as Gram staining. KOH mount, Wet mount technique and Lacto phenol cotton blue staining were done using the endometrial samples.

Isolation of bacteria and fungi from endometrial specimen of female infertility cases

The tissue samples were collected as explained in the sample collection procedure and further transported to the laboratory in a sterile saline container. Further it was taken and mechanically digested in a sterile Petri dish to lyse the tissue to get a good microbial quantity for analysis. Blood, Mac Conkey agar, Robertson's cooked meat media and Sabourad Dextrose agar was prepared and sterilized in autoclave at 121°C for 15mins. A loop full of digested tissue was inoculated to the respective culture media namely blood agar, Mac Conkey agar and Robertson cooked meat medium and the growth was observed after 24hrs incubation at 37°C for bacterial culture. Sabouraud Dextrose agar was used for fungal culture simultaneously and the growth was observed after 2-3 days of incubation. The microbial colonies grown in the culture plates were graded as scanty growth, moderate growth and heavy growth based on the number of colonies as shown in the (Table 1)

Morphological characterization

The bacterial and fungal isolates obtained from the endometrial specimen were morphologically characterized by Gram staining, Lactophenol cotton blue staining method to reconfirm the isolates.

Biochemical characterization of opportunistic pathogens

The biochemical characterization of opportunistic pathogens were carried out (8). The organisms were identified as Gram Negative Bacilli (By Gram staining) were inoculated into peptone water for Indole production, Mannitol motility medium, Simmons citrate agar, Urease agar, Triple sugar iron agar, and Methyl red, Vogus proskuar broth (MR-VP broth). Oxidase test was also performed. The organisms which were identified as Gram positive Cocci were taken for catalase test. The Catalase positive organisms were identified as *Staphylococcus* species. The *Staphylococcus* species were further differentiated as *Staphylococcus aureus* by Coagulase test.

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.

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. Nucleotide sequences were compared with sequences from GenBank using BLAST software. The ends of sequences were trimmed with BioEdit v5.0.9 software (9) and subsequently aligned using the software CLUSTALX v.1.81 (10).

GenBank submission

The 16S rRNA gene sequences were submitted to GenBank database using the BankIt sequence submission tool and accession numbers were obtained.

Phylogenetic analysis of microbial isolates in female infertility cases

The phylograms were constructed using the Neighbor-Joining algorithm (11) and processed using MEGA software, version 2.1 (12). 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.

Secondary structure analysis of opportunistic pathogens from endometrial samples

GeneBee package (13) is primarily devoted to analysis of amino acid sequences and three-dimensional protein structures. The goal is, first, to elucidate information about evolutionary and functional properties of a protein, based on the comparison of its primary sequence with the databanks of known proteins, and, secondly to study the principles of protein folding into the three-dimensional structure that can be used in making conclusions about the protein function. Based on the software development, the secondary structure of 16S rRNA gene sequence of opportunistic pathogens were analysed using online Genebee software (http://www.gene bee.msu.su).

Virtual digestion of opportunistic pathogens from endometrial samples

NEBCUTTER tool (14) takes a DNA sequence and finds the large, non-overlapping open reading frames using the *E. coli* genetic code and the sites for all Type II and Type III commercially available restriction enzymes that cut the sequence just once. By default, only enzymes available from NEB were used, but other sets may be chosen. Just the sequence of interest was submitted and further options appear with the output. The maximum size of the input file is 1 MByte, and the maximum sequence length is 300 KBases. The restriction site analysis of 16S rRNA gene sequences in opportunistic pathogens were evaluated using online NEBCutter programme Version 2 (http://tools.neb.com/ NEBCUTTER2/). The number of restriction sites and GC content of the submitted 16S r RNA gene were recorded.

Table 1 Grading microbial growth in culture media

S. No	Number of individual Colonies / Plate	Growth terminology	Growth grade symbol
1.	1-20	Scanty Growth	+
2.	21-100	Moderate Growth	+ +
3.	More than 100	Heavy Growth	+ + +

RESULTS AND DISCUSSION

The screened infertility patients were subjected to microbiological investigations for the presence of bacteria and fungi. They were isolated, characterized and identified based on morphological, biochemical and molecular diagnostic techniques. In the present study, *Enterococcus faecalis* , *E. coli* , *Enterobacter* sp , *Klebsiella* , *Streptococcus* , *Pseudomonas* , *Acinetobacter* sp , *Staphylococcus* were isolated and identified using culture methods. There was no chlamydial, gonococcal infection and Sexually Transmitted Disease in the female patients. Pelvic inflammation was seen among the study group. Among the fungal pathogens *Candida* and *Aspergillus sp* were isolated and tabulated (Table 2). It is important to insist on prevention, early diagnosis and treatment as the main role to decrease the number of tubal occlusion and incidence of pelvic inflammation. The DNA of the bacterial, fungal and organism were isolated, 16S rRNA gene sequenced and deposited at NCBI and the accession numbers were obtained. (Table 3).

Table 3 GENE bank accession numbers of the deposited opportunistic pathogens

No	*Bacteria*	Accession No
01	*Escherisia coli (ANT 01)*	*HM803167*
02	*Enterobacter* spp *(ANT 02)*	*HM803168*
03	*Kiebsiella pneumonia (ANT 03)*	*HM803169*
04	*Pseudomonas aeruginosa (ANT 04)*	*HM803170*
05	*Staphylococcus aureus (ANT 05)*	*HM803171*
06	*Enterococcus faecalis (ANT 06)*	*HM803172*

The phylogenetic tree analysis (Fig 1), Restriction site analysis (Table 4) and Secondary structure (Fig 2) of 16S rRNA gene of *Escherisia coli* ANT 01 (HM803167), *Enterobacter* sp ANT 02(HM803168*), Klebsiella pneumonia* ANT 03 (HM803169*), Pseudomonas aeruginosa* ANT 04 (HM803170*), Staphylococcus aureus* ANT 05(HM803171*), Enterococcus faecalis* ANT 06, (HM803172*), Mycobacterium* TB complex ANT 07(HM803173*),* were evaluated to find out the genetic variations among the microbial isolates in the endometrial samples of the female infertility cases.

DISCUSSION

Phylogenetic analysis of isolated Pathogens

In the present study, the phylogenetic tree was constructed with *Rhodococcus equi* sequence as an out group organism and added to the alignment profile. Alignments were analysed and phylogenetic relationships among sequences were established using Neighbour-Joining method.

Table 2 Bacterial and Fungal organisms isolated from the endometrial samples of infertility Cases

S.No	Bacteria	No. of isolates
1	*E.coli*	10 (6%)
2	*Enterobacter* spp	7(4.16%)
3	*S.aureus*	4(2.38%)
4	*K.pneumoniae*	2(1.2%)
5	*Enterobacter spp and Pseudomonas aeruginosa*	1(0.5%)
6	*Enterococcus faecalis*	18(10.71%)
7	*E.coli (var)*	8(4.76%)
8	*Pseudomonas aeruginosa*	8(4.76%)
9	*Acinetobacter* spp	5(2.97%)
10	*Streptococcus spp*	8(4.76%)
	Fungi	
11	*Aspergillus flavus*	1(0.6%)
12	*Candida* spp	9(5.36%)
13	*Candida and Aspergillus* spp	1(0.6%)

Table 4 The Restriction sites and GC content of genes 16S rRNA in isolated microbial pathogens from endometrial tissue samples

S. No.	Bacteria	Accession Number	Total nucleotides (bp)	% G+C	Restriction enzymes (Two-sites)	Restriction enzymes (Three-sites)	Restriction enzymes producing polymorphic fragments in common
01	*Escherisia coli* ANT 01	HM803167	1472	55	31	13	
02	*Enterobacter* spp ANT 02	HM803168	1476	55	26	21	
03	*Klebsiella pneumoniae* ANT 03	HM803169	1475	55	27	12	Stu I , xmn I, pcs I
04	*Pseudomonas aeruginosa* ANT 04	HM803170	1467	54	23	12	
05	*Staphylococcus aureus* ANT 05	HM803171	1485	51	19	14	Pm1 I, AF1 I, Tif ,EcoP151, Xmn ,
06	*Enterococcus faecalis* ANT 06	HM803172	1496	54	27	15	Bae G, Hga, Bass S

Trees and genetic distances were based on 100 replicates in order to assess the degree of confidence (in percentage) for each branch on the trees. Heuristic searches were completed with maximum parsimony. Absolute distances and pairwise distances were calculated for all pairwise combinations of operational taxonomic units (OTUs).

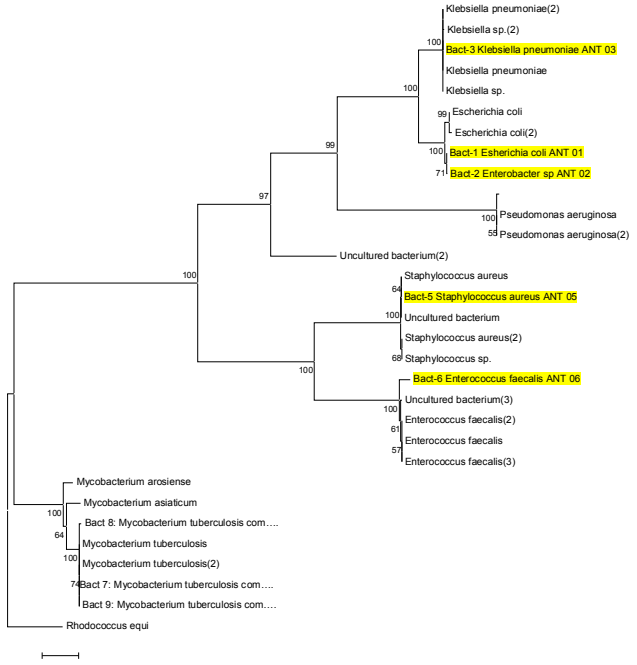

Fig. 1 Phylogenetic tree of *Mycobacterium tuberculosis* from endometrial tissue sample

In the phylogenetic tree the branching pattern of all OTUs were precise. All the enteric bacteria were clustered precisely based on sequence similarity. The bootstrap values on the clades is the confidence level on the branching patterns which shows the divergence or convergence of the enteric community, for example, *Klebsiella pneumoniae, E. coli* and *Pseudomonas aeruginosa* are distantly related to *Mycobacterium* sp. The bootstrap value on the clade which separates *Klebsiella pneumonia* and *E. coli* shows that 100% divergence of these bacteria from a common ancestral point.

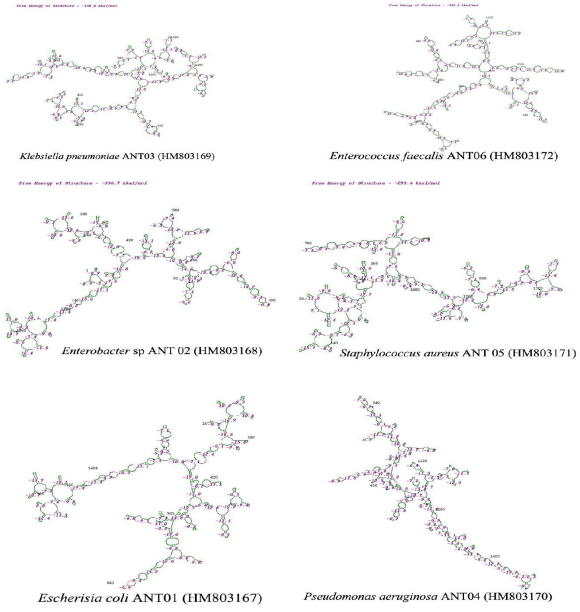

Fig.2

Secondary structures of 16S rRNA gene and their restriction site analysis

In the present study, the restriction analysis confirmed the specificity of the amplified DNA fragments revealing the restriction sites of the Gram positive organism, Gram negative bacteria indicating that the restriction sites were unique to one another. A comparative genome analysis and Restriction pattern and RNA secondary structure prediction has the potential to define the genetic basis of these phenotypes which will be invaluable in the development of urgently needed new vaccines and drugs. A similar type of research was carried out by (15) in which the researcher used different techniques that have been employed to compare the *Mycobacterium* genomes.

The secondary structures of the clinically isolated organisms revealed that the stem (double stranded paired regions) stabilize RNA secondary structures and the number of stems present in each 16S rRNA sequence of the organisms were given in the Fig.2 . The Enteobacteriaceae family members *E. coli, Enterobacter sp. and Klebsiella pneumoniae* have more

or less similar types of loops and number of stems. High degrees of similarity in these sequences were due to their relative evolutionary selection. Finally, *Staphylococcus aureus, Pseudomonas aeruginosa and Enterococcus faecalis* differ quite reasonably in their structures and number of stems and types of loops. This might prove the organisms hail from different families of their phylogenetic ancestors.

Comparison of 16S rRNA sequences from different isolates of same and as well as different family show similarity and variations. Evident that species of the same family display similarity in structural configuration, implying these regions have selective pressure on their phylogenetic determinations. Several common folds were shared among the selected species of the same family for maintaining functional equivalents.

CONCLUSION

The present study clearly signifies that most of the pathogens isolated and molecularly characterized from endometrial samples of Infertility cases were a positive causative agents of Urinary tract infection which is also reported in another study (16) . The findings confirm the possibility of strain variants suggest a future scope for further studies on antigenic determinants, Identification of conserved regions and Drug novel designing to control the virulent strains.

References

1. Bergstrom S (1992). "Reproductive failure as a health priority in the Third World: a review. " East Afr Med J 69: 174–180.
2. Daar AS and M. Z. (2002). "Infertility and social suffering: the case of ART in developing countries. In: Vayena E, Rowe PJ, Griffin PD (eds). ." Current Practices and Controversies in Assisted Reproduction. Geneva, Switzerland: World Health Organization, : 15–21.
3. Leke RJ, Oduma JA, Bassol-Mayagoitia S, Bacha AM, Grigor KM. (1993). "Regional and geographical variations in infertility: effects on environmental, cultural, and socioeconomic factors." Environ Health Perspect 101(Suppl. 2): 73 - 78.
4. Pellati D, Mylonakis I, Bertoloni G, Fiore C, Andrisani A, Ambrosini G, Armanini D. Genital tract infections and infertility. Eur J Obstet Gynecol Reprod Biol. 2008 Sep; 140(1):3-11. doi: 10.1016/j.ejogrb.2008.03.009. Epub 2008 May 23. Review. PubMed PMID: 18456385
5. Kranjcić-Zec I, Dzamić A, Mitrović S, Arsić-Arsenijević V, Radonjić I. (2004) The role of parasites and fungi in secondary infertility; Med Pregl. Jan-Feb;57(1-2):30-2. Review. Serbian. PubMed PMID: 15327187.
6. Michail s. Lionakis, George Samonis, and Dimitrios P. Kontoyiannis; (2008) Endocrine and Metabolic Manifestations of Invasive Fungal Infections and Systemic Antifungal Treatment , Mayo Clin Proc. 83(9):1046-1060
7. Jianlj Dong, Juan P.Olano , Jere W. McBride and David H. Walker (2008) Emerging Pathogens: Challenges and Successes of Molecular Diagnostics; J Mol Diagn . May 10(3): 185-197
8. Cheesbrough M (1985). *Medical Laboratory Manual for Tropical Countries*. Vol. 2. Microbiology. pp. 400-480.
9. Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp., 41, 95-98
10. Thompson, J.D.; Gibson, T.J. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., 24, 4876-82.
11. Saitou, N.; Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4, 406-25.
12. Kumar, S.; Tomura, K.; Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis Software. Bioinformatics Oxford 17(12), 1244-45
13. Brodsky.L.I., Vasiliev A.V., Kalaidzidis Ya.L., Osipov Yu.S., Tatuzov R.L., Feranchuk S.I(1992). GeneBee: the program package for biopolymer structure analysis. Dimacs. 8:127-139.
14. Tamas Vincze, Janos Posfai andRichardJ. Roberts (2003) NEBcutter: a program to cleave DNA with restriction enzymes Nucleic Acids Research, Vol. 31 (13)
15. Brosch, R., Gordon, SV. Buchrieser C, Pym, AS., Garnier, T. & Cole, ST. (2000). "Comparative genomics uncovers large tandem chromosomal duplications in Mycobacterium bovis BCG Pasteur." yeast 17: 111 – 123
16. Langley JM, Hanakowski M, Leblanc JC (2001). Unique epidemiology of nosocomial urinary tract infection in children. Am. J. Infect. Control, 2: 94-98.

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