SUMMARY AND CONCLUSIONS
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1. Out of the total 50 endocervical isolates obtained, 30 were from 11 cases of unexplained infertility and 20 from 9 cases of fertiles.

2. *Micrococcus luteus*, other *Micrococcus* sp., *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus faecalis* were the common microorganisms present both in infertile and fertile cases. *B.subtilis*, *P.maltophilia*, *E.coli*, *M.agilis* and *Kluyveromyces* sp. were the organisms which were found only in infertile cases while *M.sedentarius* and *Planococcus* sp. could be isolated only from the fertiles. *B.subtilis* was the most prevalent microorganism as it was isolated from 4 out of 11 cases of unexplained infertility. However, from this data it cannot be concluded that *B.subtilis* is a specific organism found in unexplained cases of infertility as it was absent in rest of the 7 infertile cases.

3. The effect of cell free supernatant, washed cells and cell washings from 24 and 48h old BHI cultures showed that 10-25% of the isolates from fertiles and 40-60% from infertiles produced significant (p<0.05) decrease in motility of human spermatozoa in 4h at 37°C. The difference in the effect in both cases was found to be statistically significant (p<0.05-
p<0.001) which is suggestive of the fact that normal microflora in the cervices of women suffering from unexplained infertility could be responsible for the infertility. Heat treated supernatants (100°C for 10 min) failed to inhibit sperm motility suggesting the presence of heat labile proteins that were responsible for decrease in spermatozoal motility.

4. Eleven isolates from the fertiles and infertiles (B.subtilis, E.coli, S.faecalis, Micrococcus sp., S.aureus, M.sedentarius) were capable of agglutinating human spermatozoa. Only washed cells could agglutinate spermatozoa while culture supernatant and cell washings failed to do so. It seems that there are certain receptors on the bacterial cell surface that are responsible for agglutination. The degree of agglutination was maximum at 37°C. The agglutination was inhibited on heat treatment (100°C for 10 min) implying the presence of heat labile proteins that might be playing a role in agglutination.

5. Therefore, the production of enzymes like proteases (caseinolytic) and elastases (degrading soluble and insoluble elastin), by the endocervical isolates was studied. The number of isolates producing proteases (P), elastase degrading insoluble elastin (E) and elastase degrading soluble elastin (S.E.) were 14, 5
and 9 respectively out of the 30 isolates from infertiles. It was 4, 1 and 4 respectively out of 20 isolates from fertiles.

6. The endocervical isolates were grouped into 5 groups on the basis of protease and elastase production ($P^+E^+S.E.^+, P^+E^-S.E.^+, P^+E^-S.E.^-, P^-E^-S.E.^+, P^-E^-S.E.^-$) and on association with their effect on motility and agglutination of human spermatozoa, it was found that the group $P^+E^+S.E.^+$ was showing maximum effect (100%), $P^-E^-S.E.^+$ group seemed to have no effect while 60% of the non-elastolytic protease producers ($P^+E^-S.E.^-$) showed significant decrease in sperm motility with their culture supernatants. Factors other than proteases and elastases also seemed to play some role in infertility as 33.3% of the $P^-E^-S.E.^-$ group inhibited sperm motility. But the enzyme elastase (degrading insoluble elastin) was found to be most important as all the isolates that produced this enzyme showed 100% inhibition of sperm motility and 83.3% of these isolates agglutinated the spermatozoa.

The human spermatozoa has been known, to contain a protein 'nexin' which has chemical composition similar to the elastin. This is one of the proteins responsible for flagellar (tail) movement. Elastase
could be acting on this protein and thus inhibiting sperm motility.

7. *B. subtilis* (isolate no.4) produced maximum elastase and protease after 96h of incubation at 37°C in BHI under shake conditions while *P. maltophilia* produced maximum elastase protease after 72h in TSB under shake conditions at 37°C. Though the protease activity could be detected at 8h of growth in both the organisms, elastase was released only in the late log or stationary phase of growth.

8. Purification of elastase was done by ammonium sulphate precipitation followed by molecular sieving through Sephadex G-100. 19.6 folds and 22.43 folds purification of elastase was obtained from *B. subtilis* and *P. maltophilia* respectively. The molecular weight of *B. subtilis* and *P. maltophilia* elastase was approximately 25,000 and 24,000 respectively. The elastases were inactivated within 10 minutes of exposure at 70°C and were stable at pH 6.5-9.0 with a maximum activity at pH 8.5 in case of *B. subtilis* and 7.5 in case of *P. maltophilia*.

9. 100 ug of purified *B. subtilis* elastase and 50 ug of *P. maltophilia* elastase inhibited the motility of human spermatozoa completely in 4h. At 200 ug or above concentrations, the motility was zero in 5 min.
100 µg of elastase inhibited the rat sperm motility in 15-30 min.

10. Purified elastase inhibited the activity of Na\(^+\)K\(^+\) and Mg\(^{2+}\) ATPase of human and rat spermatozoa significantly (p<0.001). The inhibition was concentration dependent. This shows that elastase might be inhibiting the sperm motility by interfering the energy producing mechanism.

11. Decapititation of human and rat spermatozoa occurred with supernatants of isolates producing elastase degrading insoluble elastin. On purification of the decapititating factor was found to be elastase. Decapititation was not observed with non-elastolytic protease and soluble elastin degrading elastase. When elastase was inhibited using elastatinal or antibodies against elastase, it failed to decapitate rat and human spermatozoa.

12. 1.5 µg of *B.subtilis* elastase and 0.78 µg of *P.maltophilia* elastase was the minimum quantity of elastase required to decapitate 2x10\(^6\) rat spermatozoa.

13. Scanning electron microscopic studies on the effect of purified elastase on human and rat spermatozoa revealed that the rat spermatozoa were more susceptible than human spermatozoa for decapitation. The head and tail of rat sperms cleaved within 1h of
incubation whereas in case of human spermatozoa only 14-17% could be cleaved even after 4h of elastase treatment. The breakage was at the neck. Loosening and disruption of the membrane around the neck and acrosomal and postacrosomal region was observed in both rat and human spermatozoa. The membrane over the tail was not damaged while it showed slight loosening at the middlepiece region. In rat spermatozoa, though the disruption of plasma membrane was observed in all heads, the extent of damage varied. The extent of damage to spermatozoa increased with increasing time of treatment with elastase. Elastase degrading only soluble elastin and non-elastolytic protease did not affect the spermatozoa. It neither caused decapitation nor did it bring about any change in the surface membrane over the head and tail.

14. In the reproductive tract of males and females, there are proteinases and various activators and inhibitors. These enzymes are required in optimal concentrations and any imbalance in the enzyme - enzyme inhibitor concentration has an adverse effect on fertilization. In female infertility (unexplained cases), one or more factor(s) may be responsible for its causation. One of the factor observed in the present study is the proteolytic enzyme elastase. But elastase could also be isolated from fertiles. The
question arises why this isolate (M. sedentarius) did not cause infertility, one of the reasons could be that it produced lesser amount of elastase than endocervical isolates from infertiles in vitro or there might be some inhibitors present in such fertile women. The cervical washings from human females showed that in infertiles, the washings had elastase activity which enhanced the activity of purified microbial elastase in vitro. The washings from the fertiles showed no such activity while in some cases it inhibited the elastase to some extent. The cervical washings from infertiles that showed elastase activity were detrimental for the spermatozoal motility. The sperm motility increased as the quantity of cervical mucus in the reaction mixture was decreased. Elastase inhibitors were also found in the intrauterine fluid of fertile women and not in infertiles. All these results showed that inhibitors of microbial elastase play a crucial part in determining the fertility status of a woman harbouring elastase producing microorganisms in her cervix.

Male factor could also be an additional factor in the unexplained infertility in females. It was observed that there are certain factors in the seminal plasma that either enhance or inhibit the activity of
microbial elastase since the seminal plasma comes in direct contact with the cervical mucus, the survival of the spermatozoa in the female genital tract depends on the presence of elastase inhibitor in the seminal plasma. Though *in vitro*, inhibition of sperm motility by endocervical isolates from fertiles was observed in the present study, it seems that *in vivo* some other factors are involved in the ultimate outcome of the fertility status of a woman.

When the elastase positive *P. maltophilia* and *B. subtilis* (isolated no.4), isolated from infertile human females were established in the genital tracts of female rats, the trait to produce elastase remained unchanged when tested after their reisolation. Such animals when segregated after mating showed a loss of fertility in about 80-100% of animals compared to the control group where 86.7-96.3% animals were fertile. The results indicate towards the possibility that an imbalance of vaginal microflora ecology with elastase positive organisms might be one of the reasons for unexplained infertility in man and animals.

The animals that became infertile after elastase producer was established in their genital tract were treated with tetracycline (200ug daily) given locally for 7 days. After this treatment the elastase
producers were found to be eradicated from the genital tract. Fertility was resumed in 62.5-66.66% of the animals.

To confirm whether the infertility was due to the elastase produced by these isolates, the purified elastase was instilled in the genital tract of the female rats during the mating period. All the rats became infertile proving that elastase is an important factor in the causation of infertility.