MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1 CHEMICALS

Various reagents and chemicals used in the present study were of analytical quality and were procured from BDH (England), E. Merck Ltd., Glaxo Laboratories Ltd. India and Sigma Chemical Co., U.S.A. The media used were from Hi Media Laboratories, India. The elastin and elastin-orcein were obtained from Sigma Chemical Co., U.S.A. and soluble elastin was procured from Fluka Chemical Co., Switzerland.

3.2 MICROORGANISMS

The bacterial isolates used in the present study were taken from cervices of fertile and infertile (primary unexplained infertility) women attending the Obstetrics and Gynaecology Department of Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh. The criteria of fertility was their parity (primiparous and multiparous). The cases of unexplained infertility were the women who were unable to conceive after two years of unprotected coitus and all other factors (tubal factor, ovulatory status, luteal phase defects, cervical factor, antibodies against sperms, laparoscopy, other endocrine or medical disorders) responsible for infertility being ruled out. Before taking the sample, the patient's recent medical history was taken into consideration. The cervical swab samples were taken only from those females who had not had any antibiotic intake for
at least a week. The sample was taken only at that time when there was no clinical incidence of vaginal or cervical infection. Therefore, all the cervical isolates which were obtained were considered to be the normal microflora.

3.2.1 **Collection of cervical samples for microbial isolations**

Samples were taken mainly by the method of Hok et al. (1967). The details are as follows:

The cervix was made visible with the help of a sterilized spaculum. The sample on cotton swab was taken from the endocervical area in duplicate and care was taken not to contaminate the swab with the vaginal microflora. The samples were directly spread by streaking on sheep blood agar and MacConkey's agar plates. The plates were incubated at 37°C for 48h and observed for bacterial growth. The isolates were picked up on the basis of their colony size and morphology and subjected to various tests for identification of their genera and species according to the characteristics laid down in the Bergey's Manual of Determinative Bacteriology. Various isolates so obtained were maintained on nutrient agar and blood agar slants.

3.3 **COLLECTION AND EXAMINATION OF HUMAN SEMEN**

Semen samples from healthy male donors were obtained by masturbation. The ejaculates were collected from the gynaecology O.P.D. of PGIMER, Chandigarh, India. The samples
were protected from extremes of temperature (not less than 20°C and not more than 40°C) during transport to the laboratory. The samples were kept at room temperature for 60 minutes for liquefaction.

3.3.1 Initial macroscopic examination

Colour of the sample was noted. A normal sample has a grey-opalescent appearance and is homogenous.

3.3.1.1 Volume

The volume of the ejaculate was measured in a graduated cylinder or by aspirating the whole sample in a graduated syringe or pipette.

3.3.1.2 Consistency

The consistency was evaluated by introducing a glass rod into the sample and observing the length of the thread that formed on withdrawal of the rod. In normal samples, the length of the thread did not exceed 2 cm.

3.3.1.3 pH

A drop of semen was spread evenly onto the pH paper. After 30 seconds the colour of the impregnated zone was uniform and was compared with the calibration strip to read the pH. The pH in the range of 7.2 to 7.8 was considered normal.

3.3.2 Initial microscopic investigation

During the initial microscopic investigation of the
sample, estimates of motility and concentration of spermatozoa were performed and the presence of cells other than spermatozoa and of agglutination were determined.

A fixed volume of semen (10-15 µl) delivered with a micropipette was placed on a clean glass slide and covered with a coverslip of between 20mm x 20mm to 24mm x 24mm. The volume of semen and the dimensions of the coverslip were so standardized that the analyses were always carried out in a preparation of fixed depth (25-30um). This depth allowed full expression of the rotating movement of normal spermatozoa.

The preparation was examined at a magnification of 400x. An ordinary light microscope was used. The freshly made, wet preparation was left to stabilize for approximately one minute before scanning. Four to six fields were scanned and mean number of spermatozoa in several fields under a 40x objective multiplied by 10^6. According to the WHO laboratory manual (WHO, 1987b) for the examination of human semen and semen - cervical mucus interaction, 40 spermatozoa are observed in a field can be considered equivalent to 40x10^6 spermatozoa/ml. The same criterion was followed for rough estimation of sperm concentration.

3.3.3 Further microscopic examination
3.3.3.1 Sperm viability

Supra-vital staining was done following the method of Eliasson (1977). Mixed one drop (10-15 µl) of semen with one
drop of 0.5% (w/v) eosin solution prepared in physiological saline on a microscope slide and covered it with a coverslip. After one or two minutes, observed the preparation at 40x under bright light. Counted one hundred spermatozoa and differentiated live (unstained) from the dead (stained) cells. This technique provided a check on the accuracy in motility evaluation.

3.3.3.2 Counting of spermatozoa

The concentration of spermatozoa was determined using the haemocytometer method. In this procedure, 1:20 dilution was made for each well-mixed sample by diluting 50μl of liquefied semen with 950 μl of physiological saline. If the preliminary examination of the semen indicated low concentration of spermatozoa, then the extent to which the sample was diluted was adjusted accordingly. The diluted samples were charged on to the Neubaur haemocytometer. Haemocytometer chamber comprises of 9 squares each having an area of 1mm². The central chamber is divided into 25 smaller squares. The four corner squares are divided into 16 small squares.

The height of the chamber formed after placing the coverslip in place was 0.1mm. The spermatozoa present in all 64 small corner squares were counted under low power. The number was divided by four to obtain the average number in 16 small squares or one big square of area equal to 1mm².
3.3.3.3 Calculation

Volume under 16 small square = 1mm$^2$ x 0.1mm = 0.1mm$^3$

Let the total no. of spermatozoa counted in the above volume = N

i.e. 0.1mm$^3$ contains N spermatozoa
or 1mm$^3$ contains N x 10 spermatozoa
or 1.0cm$^3$ contains N x 10$^3$ spermatozoa
or 1.0ml contains N x 10$^3$ spermatozoa

Total no. of spermatozoa present per ml of the sample = N x 10$^4$ dilution factor.

3.3.4 Normal values of semen variables (WHO standards)

Volume 2.0 ml or more
pH 7.2 - 7.8
Sperm concentration 20x10$^6$ spermatozoa/ml or more
Total sperm count 40x10$^6$ spermatozoa or more
Motility 50% or more with forward progression or
25% or more with rapid linear progression within 60 min after collection.
Morphology 50% of more with normal morphology.
Viability 50% or more live
White blood cells Fewer than 1x10$^6$/ml.

3.3.5 Spermatozoa of animals

Samples of rat spermatozoa were collected from the vas deferens and cauda epididymis in minimum quantity of physiological saline by the method of Rowett (1962). The number of spermatozoa was checked by counting in a haemocytometer and was adjusted to 40x10$^6$ sperms/ml.

45
3.3.6 Characteristics of spermatozoa

3.3.6.1 Motility

Motility of sperms in various samples was determined by the method of Emmens (1947) with slight modifications.

\[
\text{Percent motile sperms} = \frac{\text{No. of motile sperms/field}}{\text{No. of total sperms/field}}
\]

3.3.6.2 Agglutination

In microtitre plate wells, 50ul of saline was added followed by 50 ul of semen samples were made by using an autopipette. The plates were shaken on a microshaker for 30 seconds and incubated at 37°C for 4-6h. A drop of sample was taken from each well and observed under light (phase contrast) microscope for any agglutination of the sperms in any of the dilutions. In normal semen samples, no agglutination of spermatozoa was observed in routine.

In case of agglutination, the type of agglutination was recorded as follows:

i) Head-Head (H-H) agglutination
ii) Head-Tail (H-T) agglutination
iii) Tail-Tail (T-T) agglutination

3.4 EFFECT OF CULTURE SUPERNATANTS ON MOTILITY OF HUMAN SPERMATOZOA

The endocervical isolates from fertile and infertile were grown in brain heart infusion (BHI) under shake conditions (90 rpm) at 37°C. In each case 0.2ml inoculum of an
overnight culture of the isolate was used to inoculate 20ml of a medium in each flask (100ml capacity). The flasks were incubated for 24h and 48h at 37°C. The cultures were centrifuged at 10,000 rpm for 20 min at 4°C and clear supernatant was separated. The supernatants obtained were made cell free by passing through 22 um sterile millipore filters.

Human semen ejaculates that satisfied the WHO criteria of normal standards were selected and the sperm count adjusted to 40x10⁶ ml⁻¹ with sterile physiological saline or PBS (pH 7.2) used as diluent.

Equal volumes of sperm suspension and culture supernatants were mixed and incubated at 37°C for 4h. In the control tube sterile BHI was added instead of supernatant. After 4h of incubation the motility of spermatozoa was observed under the light microscope. Experiment was performed in triplicates and the mean values noted. The test of proportions was applied to check the significant decrease in motility of spermatozoa with respect to control.

3.5 **AGGLUTINATION OF SPERMATOZOA BY BACTERIAL CELLS, SUPERNATANTS AND CELL WASHINGS**

All the isolates were grown in BHI as described earlier for 24h and 48h at 37°C on a rotary shaker. The cells were harvested and given a washing in PBS (pH 7.2) and resuspended in the same buffer to give an absorbance of 0.05
at 600 nm. The cell suspension was mixed with semen sample (40×10^6 sperms ml^-1) and after 4h incubation at 37°C the sperm agglutination was checked. After gentle agitation of the sample, one drop of it was placed on a glass slide covered with a cover slip and observed for agglutination at 400x under a light microscope.

The supernatant obtained after centrifugation of the culture of all isolates was taken and mixed with sperm suspension. The cells were washed once in PBS (pH 7.2) and the supernatant after centrifugation was used as the cell wash. The washed cells were resuspended in PBS. All the three preparations were used to study their capacity to agglutinate spermatozoa.

3.5.1 **Effect of incubation temperature**

The washed cell suspension of the endocervical isolates causing agglutination were incubated with human spermatozoa for 4h at three different temperatures viz. 37°C, 20°C and 4°C. The degree of agglutination was noted at the end of the incubation time.

3.5.2 **Effect of heat treatment**

The aliquots of bacterial cell suspensions treated at 50, 60, 70, 80, 90 and 100°C for 10 min. The heat treated cells were then incubated with human spermatozoa for 4h at 37°C and observed for agglutination.
3.5.3 Effect of diluents

The cells of endocervical isolates were washed and suspended in different diluents, phosphate buffered saline (pH 7.2), citrate egg yolk solution, Ringer solution, normal saline. These were then used to study the degree of agglutination caused by the different isolates.

3.6 PROTEOLYTIC ACTIVITY
3.6.1 Qualitative assay

Proteolytic activity was determined by the method described by Iida et al. (1982). Protease activity was assessed on 1% milk casein agar plates. The bacteria were inoculated on milk agar and incubated at 37°C for 24-48h. The activity was revealed by the clear zone formation around the inoculum spot.

3.6.2 Quantitative assay
3.6.2.1 Plate assay

For studying the protease activity in culture supernatants, the above method was modified as follows: 25 ml of sterile milk casein agar (containing 1.0% skimmed milk), 1.2% agar powder, 0.1% sodium azide, 0.01 mM CaCl₂ and 0.05M Tris-HCl buffer, pH 8.0) was poured in each sterile plate. 100ul of the supernatants of a bacterial culture or an enzyme preparation was added to a sterile steel cylinder (well) placed on the agar. Proteolytic activity was revealed by the clear zone formation around the well after incubation.
for 24h, 48h and 72h at 37°C. The clear zone was measured and expressed in mm.

3.6.2.2 Colorimetric assay method (Keay and Wildi, 1970)

(i) **Substrate**: One gram of casein was dissolved in a minimal amount of 0.1N NaOH using a magnetic stirrer. The solution was adjusted to pH 7.0 with 0.1N HCl and the volume was made upto 50ml with distilled water. The final volume was made upto 100ml with phosphate buffer (0.05M, pH 7.0). Substrate was stored at 4°C in a refrigerator and discarded after 3 days if not used.

(ii) **Procedure**: The casein solution (1ml) and 1.0ml of cell free supernatant (CFS) were equilibrated for 15 min at 37°C in a water bath, mixed, incubated at 37°C for 30 min. The reaction was terminated by adding 2ml of 0.4M TCA (Trichloro acetic acid) and the mixture was further incubated for 10 min at the same temperature. The precipitated protein was filtered through Whatman no.1 filter paper and the amount of non-protein nitrogen in filtrate was monitored at 280nm. In the control tubes, the substrate was mixed with CFS (pre boiled for 10 min) and treated as above. One unit of protease is the amount of enzyme required to hydrolyse casein in 30 min at 37°C so as give an absorbance of 0.01 at 280 nm.

Protease Units/ml of sample

\[
\frac{(\text{absorbance of test} - \text{absorbance of control})}{\text{ml of sample}} \times 100
\]
3.7 ELASTOLYTIC ACTIVITY

3.7.1 Qualitative screening of microorganisms for elastase activity

3.7.1.1 In solid agar containing insoluble elastin

Various bacterial strains and isolates were screened for their elastase activity by plate method (Sbarra et al., 1960) with slight modifications. Nutrient agar was incorporated with elastin (0.1% w/v) before autoclaving. The medium was autoclaved and 20-25 ml of this was poured into sterile petri plates and allowed to solidify. Care was taken to have the elastin uniformly distributed in the agar and this was achieved by thorough shaking of the agar before pouring into the plates. The plates were incubated overnight at 37°C to exclude the contaminated ones. Different bacterial strains were grown in brain heart infusion for 24h at 37°C. These cultures were streaked on the nutrient elastin-agar plates and incubated at 37°C. Plates were observed for the formation of clear zones up to 5 days. Generally clear zones around elastase positive colonies appeared within 2-4 days. The strains showing clear zone around the colonies were taken as elastase positive.

3.7.1.2 In solid agar containing soluble elastin

For screening the organisms for production of elastase degrading soluble elastin, the method described by Williams et al. (1988) was followed. Nutrient elastin agar
medium was prepared by adding 1% w/v solubilized elastin to nutrient agar. The medium was autoclaved and dispensed in petri plates in 20ml amounts. Plates of the elastin media were heavily spot inoculated with the test organisms from fresh cultures growing on appropriate agar plate media. All isolates were incubated at 37°C for 48h. Bacterial elastase was detected by flooding the elastin plate cultures with an aqueous solution of 30% w/v trichloroacetic acid. This caused an opaque precipitate in the medium in the presence of undigested elastin. Elastase activity was shown by the presence of clear zones beneath and around areas of growth.

3.7.2 Quantitative assay
3.7.2.1 Plate assay

Production of elastase by various organisms grown in broth under shake conditions was screened by using the clear supernatant of the centrifuged cultures by the modified plate assay. Various organisms were grown in BHI (20ml) in Erlenmeyer flasks (100ml) at 37°C under shake conditions (90 rpm) for 72h on a rotary shaker. The culture was centrifuged for 10 min at 4°C and 10,000 rpm. Supernatant was decanted off and to this sodium azide (0.1% w/v) was added as preservative. Nutrient agar plates containing insoluble elastin were prepared as described earlier. On this solidified agar plates, steel cylinders were placed and in each cylinder 100ul of supernatant to be tested was added. The plates were left at room temperature for about an hour.
and then incubated at 37°C for 72h. Production of elastase was ratified by the presence of a clear zone around the steel cylinder having the supernatant. Zone diameter was measured in mm as described by Morihara (1964).

3.7.2.2 Colorimetric assay method

This method for the quantitation of elastase was as described by Sachar et al. (1955) with slight modifications. The method used was as follows:

Ten mg of dyed elastin (elastin-orcein) was placed in each screw capped tube (20 ml capacity) and 1ml of Tris-HCl buffer (0.05M, pH 8.0) was added in each tube to suspend the substrate. Enzyme preparation in the form of culture supernatant or in purified form was added in different volumes (50μl-1000μl) and the final volume was made to 3.0 ml with Tris-HCl buffer (0.05M, pH 8.0). The tubes were capped and incubated at 37°C in a shaker water bath. The shaking (40-45 excursions/min) was continued for 4h. At the end of this period the tubes were removed and 2ml of phosphate buffer (0.7M, pH 6.0) was added to the reaction mixture to stop the enzyme reaction with the drop in pH, the colour of the reaction mixture changed from a bluish purple to a reddish purple. The mixture was then centrifuged at 300 rpm for 10 min to remove the undigested elastin-orcein. The absorbance of the supernatant was measured by a spectronic-21 spectrophotometer at 590 nm. The results were converted into specific activity i.e. elastase units per mg protein of the
enzyme used. The elastin activity was defined as the amount of enzyme activity required to solubilize 1mg of elastin in 4h at 37°C under above specified experimental conditions.

3.8 GROWTH OF ORGANISMS AND PRODUCTION OF ELASTASE

3.8.1 Effect of media

*Bacillus subtilis* (isolate no.4) and *Pseudomonas maltophilia* (isolate no.5) were grown in different media viz., synthetic medium, peptone water, nutrient broth (NB), brain heart infusion (BHI) and trypticase soy broth (TSB) under shake conditions (90 rpm) at 37°C. In each case, 0.5ml inoculum of an overnight culture of an organism was used to inoculate 50ml of a medium in a 250ml flask. The flasks were incubated for 72h and the cultures were centrifuged at 10,000 rpm for 10 min at 4°C and clear supernatant was separated. To this supernatant 0.1% sodium azide was added and stored at 4°C if desired. Elastase activity was estimated by the method of Sachar et al. (1955) as described earlier. Protease activity was assayed by using plate method and also by method described in 3.6.2.2.

From the preliminary experiments, BHI for *B. subtilis* and TSB for *P. maltophilia* were found to support maximum production of elastase and total protease by these organisms. In further studies, these organisms were grown in their respective media indicated above. Various parameters were varied to study their effect on elastase production by these organisms. These parameters are given below.
3.8.2 Incubation temperature and shaking or stationary conditions

Effect of incubation temperature (30°C and 37°C) under stationary or shaking conditions (90 rpm) on the production of elastolytic protease by B. subtilis and P. maltophilia in their respective media was studied. The elastolytic activity in the cell free supernatant of 72h culture was assayed colorimetrically as described earlier. Protease activity was assayed by Plate Method.

3.8.3 Aeration

Nine flasks (100ml capacity) were taken for each organism. Different volumes (10, 15, 20, 25, 30, 35, 40, 45, 50ml) of the respective medium were taken and inoculated with 1% inoculum from an overnight culture. All the flasks were kept at 37°C for 72h under shake conditions. Elastase and protease activity of supernatants was assayed.

3.8.4 Incubation time

Ten flasks (250ml) each containing 50ml of the respective medium was inoculated with 0.5ml inoculum from an overnight culture. The flasks were incubated at 37°C under shake condition (90 rpm). At different intervals of incubation viz. 0, 2, 4, 6, 8, 10, 24, 48, 72, 96 and 102 hours, one flask of each organism was withdrawn and dilutions of the culture was made and plated on nutrient agar. The plates were incubated for 24 hours at 37°C and the colony forming units
per ml (CFU ml$^{-1}$) were counted. Rest of the culture was centrifuged at 10,000 rpm for 10 min at 4°C. The corresponding activity was also assayed using the method of Keay and Wildi (1970) as described earlier. Elastolytic activity in each aliquot of cell free supernatant was assayed colorimetrically as described earlier.

3.9 **PURIFICATION OF ELASTASE**

For elastase production, *B. subtilis* isolate no. 4 was grown in BHI for 96h at 37°C and *P. maltophilia* was grown in TSB for 72h at 30°C under shake condition (90 rpm). The supernatant was obtained by centrifugation at 10,000 rpm for 10 min at 4°C. Purification was carried out according to the following scheme:

72h old culture supernatant (*P. maltophilia*) or  
96h old culture supernatant (*B. subtilis*)

Precipitated with ammonium sulphate  
(60% for *B. subtilis* and 70% for *P. maltophilia*)

Centrifuged at 10,000 rpm at 4°C for 20 min.

Pellet  
Supernatant (discarded)

Dissolved in Tris (Hydroxymethyl) aminomethane buffer (0.05M, pH 8.0)

Dialysed against distilled water

Molecular sieving through G-100 using column (1.5cmx40cm) with a flow rate of 24ml/h.

56
The flow rate was adjusted by connecting the column to peristaltic pump and the fractions collected in the fraction collector. The protein concentration was monitored through the uv-cord connected to the recorder (Pharmacia, LKB).

3.10 **EFFECT OF HEAT TREATMENT**

Aliquots of purified elastase were taken in different tubes and heat treatment was done at 50°C, 60°C, 70°C and 80°C. The exposure to a particular temperature was for 10 min and 30 min. The tubes were then kept in ice and elastase activity estimated separately by the colorimetric method described earlier.

3.11 **EFFECT OF pH**

Elastin nutrient agar plates were prepared in Tris-HCl (0.05M) buffer of varying pH viz. 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Steel cylinders were placed on the plates and 100ul elastase from *B. subtilis* and *P. maltophilia* was put in them. After 24, 48 and 72h of incubation at 37°C, the clear zone diameter was noted in mm.

3.12 **EFFECT OF PURIFIED ELASTASE ON MOTILITY OF SPERMATOZOA**

Different amounts of purified elastase viz. 50, 100, 200 and 400ug protein, from *B. subtilis* and *P. maltophilia* was mixed with human and rat spermatozoa (4x10^6 ml^-1) respectively). In case of human spermatozoa, the motility of spermatozoa was checked after every hour till 4h. Rat spermatozoa were comparatively motile for shorter period
and hence the motility was checked every 10 min till the spermatozoa were rendered immotile.

3.13 EFFECT OF ELASTASE ON SPERM ATPase ACTIVITY

The effect of elastase on Na\(^+\), K\(^+\) and Mg\(^{++}\) ATPase activity of human and rat spermatozoa was studied according to Kielley (1955) and Chappel (1963) with slight modifications. The details of the methods were as follows: distilled water washed spermatozoa (1x10\(^8\)/ml) were sonicated at 50 Hz for 10 min at 4°C. The reaction mixture for ATPase activity consisted of 0.2ml Tris-HCl buffer (0.2M, pH 7.6) containing 50, 100, 200 and 400 ug purified elastase, 0.2ml of MgCl\(_2\) (5mM) or NaCl + KCl (5mM each), 0.2ml of ATP (6mg ml\(^{-1}\)) and 0.2ml of sonicated sperm suspension. Control tubes contained no elastase. The mixture was incubated at 37°C for 1h.

After this period of incubation the reaction was stopped by adding 2ml of cold TCA (Trichloroacetic acid, 10%). In the control tubes TCA was added in the beginning to stop the ATPase activity. Inorganic phosphorus (Pi) released was determined according to the method of Fiske and Subbarao (1925) described by Abou-Donia and Dieckert (1974).

3.14 DECAPITATION OF SPERMATOZOA

3.14.1 Effect of cell free supernatant on decapitation of human and rat spermatozoa

* Bacillus subtilis* isolate no.4 and *P. maltophilia* were grown in BHI and TSB respectively. 10 Erlenmeyer flasks
(100 ml capacity) were taken and in each 20 ml sterilised medium was inoculated with 0.2 ml of seed culture. The flasks were kept at 37°C under shake conditions (90 rpm). After every 2, 4, 6, 8, 10, 24, 48, 72, 96 and 120 h, one flask was taken and the culture centrifuged at 10,000 rpm, for 10 min at 4°C. The clear supernatant thus obtained was mixed with equal volumes of human or rat spermatozoa (4 x 10^6 sperms ml^-1) and incubated at 37°C for 4 h. At the end of the incubation period, a drop of the sample from each tube was placed on a glass slide, covered with a coverslip and observed under 400x (light microscope). 100 spermatozoa were counted and the number of decapitated (heads without tail) ones and non-decapitated ones was noted. The percentage cleavage of spermatozoa (decapitation) was calculated as follows:

\[
\% \text{ cleavage} = 100 \times \frac{\text{No. of decapitated spermatozoa}}{\text{No. of decapitated spermatozoa} + \text{Normal spermatozoa}}
\]

3.14.2 Incubation time

The culture supernatants of *B. subtilis* and *P. maltophilia* cultures were checked for the decapitation after different time of incubation. Equal volumes of supernatant and spermatozoa (human or rat) were incubated at 37°C. After 1, 2, 3 and 4 h respectively, a drop of the sample was observed under microscope for decapitation.
3.14.3 Effect of pH on decapitation

Buffers of pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 were prepared and 100ul of each buffer was incubated at 37°C for 4h with 100ul of rat spermatozoa (20x10^6 sperms ml^{-1}). Percentage of spermatozoa cleaved by each buffer was calculated. The pH of supernatant of *Bacillus subtilis* and *P. maltophilia* was noted and a portion of the supernatant was autoclaved to inactivate the protease, elastase (decapitating factor). The pH was again noted. The supernatants as well as the heat inactivated supernatants were mixed with equal volume of rat spermatozoa and decapitation was studied.

3.14.4 Effect of heat treatment

Cell free culture supernatant from 96h BHI culture was distributed in different test tubes in small aliquots. Water baths were set at 50°C, 60°C and 70°C and at these temperatures, the supernatant was treated for 10 min and 30 min. After heat treatment, the test tubes were kept in ice bath. Control sample was kept without any heat treatment. Elastase activity of all the samples was determined and also the effect of all samples on spermatozoal decapitation.

At 56°C, the enzyme from *Bacillus subtilis* was treated for different time intervals viz. 10, 20, 30, 40, 50 and 60 min. The effect of all the samples on cleavage of rat spermatozoa was then studied.
3.14.5 Effect of cell free supernatant of different ages on decapitation of spermatozoa

0.2ml of cell free supernatant from broth culture of different ages 48, 72, 96 and 120h was serially diluted in 0.2ml of PSB (pH 7.2) and incubated with 0.2ml of rat or human spermatozoa suspension (40x10^6 ml^-1) at 37°C. After 4h, the decapitation caused by each dilution was checked. The supernatant causing decapitation at maximum dilution was considered to have the maximum amount of decapitating factor. For further experimentation, the culture was grown for 96h and 72h for *Bacillus subtilis* and *P. maltophilia* respectively as this corresponded with maximum production of decapitating factor.

3.14.6 Ammonium sulphate precipitation

*Bacillus subtilis* was grown in BHI for 96h and *P. maltophilia* in TSB for 72h, at 37°C under shake conditions. The cultures were centrifuged (10,000g, 10 min at 4°C) and to the clear supernatant, varying amounts of (NH₄)₂SO₄ was added. Five flasks containing 100ml supernatant each were subjected to ammonium sulphate precipitation so as to get 60, 70, 80, 90 and 100% saturation. The flasks were kept at 4°C overnight and the next day precipitates were collected by centrifugation (10,000rpm, 20 min at 4°C). It was then dissolved in minimum amount of Tris-HCl (0.05M, pH 8.0) buffer and dialysed against distilled water. Protein
concentration was determined (Lowry et al., 1951) and adjusted to 2mg ml\(^{-1}\).

Serial two-fold dilutions of 0.2ml of enzyme precipitate were made in PBS (pH 7.2) and mixed with 0.2ml of rat spermatozoa suspension. After 4h of incubation at 37\(^{\circ}\)C decapitation was checked in all the samples.

3.14.7 **Purification of decapitating factor on G-100 column**

Sephadex G-100 was packed with a bed volume of 94 cm\(^3\) and equilibrated with Tris-HCl (0.05M, pH 8.0). 4mg protein of 60\% (NH\(_4\))\(_2\) SO\(_4\) precipitate of *B. subtilis* isolate no.4 was loaded on the column and eluted with Tris-HCl (0.05M, pH 8.0) buffer with a flow rate of 24ml/h. 3ml fractions were collected and protease and elastase activity in each fraction was determined. Protease was assayed by plate assay method and elastase by colorimetric method. 100ul of each fraction was taken and its effect on decapitation of human and rat spermatozoa was studied separately.

In case of *P. maltophilia*, 70\% ammonium sulphate precipitate of 72h old culture supernatant was used. Similar set of experiments were performed as above.

3.15 **DECAPITATION BY PURIFIED ELASTASE**

50 ug of purified elastase from *B. subtilis* and *P. maltophilia* was serially diluted in 100 ul Tris-HCl (0.05M, pH 8.0) to give various dilutions, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 etc. 100ul of rat spermatozoa suspension
(40x10^6 sperms ml^{-1}) was added to each dilution and
decapitation caused in 4h was studied.

3.16 EFFECT OF NON-ELASTOLYTIC PROTEASES AND ELASTOLYTIC
PROTEASES ON RAT AND HUMAN SPERMATOZOA

Few endocervical isolates from fertile and infertile
were selected according to the following criteria:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Protease</th>
<th>Elastase degrading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Insoluble elastin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soluble elastin</td>
</tr>
<tr>
<td>B. subtilis (isolate no.1 to 4)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P. maltophilia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M. sedentarius</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. epidermidis (isolate 10,11)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M. luteus (isolate no.16)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Kluyveromyces sp.</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>S. faecalis (isolate no.22)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>M. luteus (isolate no.42)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All these cultures were grown in BHI for 72h at 37^\circ C
under shake conditions. The supernatant (100\mu l) was mixed
with equal volume of spermatozoa suspension and observed for
decapitation.
3.17 SCANNING ELECTRON MICROSCOPY

Processing of samples was done according to the method described by Hafez and Kanagawa (1973) with slight modification.

The normal human semen samples were taken and the washed sperm suspension (40x10^6 sperm ml^-1) was prepared. Rat spermatozoa were taken from epididymis and vas deferens in PBS (pH 7.2). The concentration of spermatozoa was adjusted to 40x10^6 ml^-1.

200μl of sperm suspension was incubated with 200μg of elastase from P. maltophilia and B. subtilis isolate no.4 for different time intervals till 4h. The spermatozoa were then settled by centrifugation at 500 rpm for 5 min. To each tube 4ml of 2.5% phosphate buffered glutaraldehyde was added and the sperms mixed gently with the help of a pasteur pipette. After 30 min the samples were centrifuged for 5 min at 500 rpm and washed twice in phosphate buffered saline. Control was prepared every time that contained only the sperm suspension and incubated for different time periods along with the other tubes before fixation was done.

One drop of the fixed and washed spermatozoa was placed on a silver painted adhesive tape mounted on brass slugs and air dried. 100Å thick gold coating was done in the Jeol Fine Coat Ion sputter (JFC-1100).

The specimens were observed in a Jeol Scanning Microscope (JSM-6100, Japan) and operated at 20KV.
3.18 INHIBITOR STUDY

3.18.1 Production of protease inhibitor by endocervical isolates

All the endocervical isolates from fertile and infertile women were grown in BHI for 48h at 37°C. The cell free supernatant was collected seperately. To check the production of inhibitor against elastolytic protease, 50ul supernatant from *B. subtilis* and *P. maltophilia* each was mixed with 50 µl of supernatant from all the isolates. This was then put in steel cylinders placed over milk agar plates as described earlier. Clear zone was measured after 24h. In the control, 50 µl of supernatant from *B. subtilis* and *P. maltophilia* cultures was mixed with 50 ul of Tris-HCl buffer (0.05M, pH 8.0) before checking its protease activity.

3.18.2 Effect of cervical mucus on elastase activity

The cervical mucus from fertile and infertile women (unexplained cases) was aspirated with a canula applied to a syringe during the luteal phase of menstrual cycle. The mucus was taken out in an eppendorf tube in minimum amount of sterile physiological saline. The sample were made homogenous by continuous vortexing for some time.

50ug elastase was taken in each tube and mixed with 50, 100 or 200µl of the cervical mucus homogenate. The elastase activity of the mixture was estimated by colorimetric method using elastin-orcein as substrate.
The luteal phase cervical mucus from women with unexplained infertility was taken in small amount of sterile saline. 25μl of this was taken to determine its protein content (Lowry et al., 1951). 25μl was diluted serially in Tris-HCl (0.05M, pH 8.0) buffer to give dilutions 1:1 and 1:2. The cervical mucus samples were mixed with elastase to study their effect on elastase activity. The elastase activity was measured as described earlier.

The corresponding effect of dilution of cervical mucus on motility of human spermatozoa was also studied using equal amounts of the elastase cervical mucus mixture and spermatozoa suspension (40x10^6 ml^-1). Motility was checked after 1h of incubation.

3.18.3 Elastase inhibitor in intra uterine fluid of fertile and infertile women

Uterine fluid was obtained from 4 fertile and 4 infertile women during their mid cycle according to the method of Casslen (1986). The fluid was aspirated in a pediatric feeding tube applied to a syringe aspirator. The fluid was flushed out of the tube with small amount of sterile physiological saline. All the samples were pooled. The effect of varying amounts of uterine fluid (50μl, 75μl and 100μl) on fixed amount (25μg) of elastase was studied by the colorimetric assay method.
3.18.4 Effect of seminal plasma on elastase activity

3.18.4.1 Preparation of seminal plasma (Jecht and Poon, 1975)

Semen was obtained from healthy donors with proven fertility as well as from infertile men. The evaluation of the samples was done according to WHO standards and were labelled normal or abnormal. The sperm free seminal plasma (supernatant) was obtained by centrifugation at 40,000g for 30 min at 4°C. The seminal plasma was frozen till used.

3.18.4.2 Study of elastase inhibitor

10mg portions by elastin-orcein were distributed in different tubes and 1ml Tris-HCl (0.5M, pH 8.0) was added to suspend the substrate. 100μl of purified elastase was added to all tubes. 100 μl of seminal plasma was also added. The volume was made up to 3ml with Tris-HCl buffer. After 4h of incubation with continuous shaking at 37°C, the reaction was stopped and elastase activity estimated. The test was done in triplicates and standard deviation as well as t-value was calculated to check significant increase or decrease in elastase activity with respect to control.

3.19 Effect of elastase producing organisms on reproduction

Reproduction studies were performed using outbred healthy adult rats (Sprague Dawley) weighing 150-200 gm.

3.19.1 Selection of human endocervical isolates

The selection was done on the basis of the following properties:
<table>
<thead>
<tr>
<th>Group No. &amp; microorganism</th>
<th>Properties of the isolate</th>
<th>Instilled in</th>
<th>Protease</th>
<th>Inhibition</th>
<th>Sperm agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elastase</td>
<td>Genital tract</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I  No organism</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II S. aureus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(isolate no.32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III P. maltophilia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(isolate no.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV B. subtilis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>(isolate no.4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V  Micrococcus sp.</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(isolate no.21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3.19.2 Effect of selected isolates on rat spermatozoa

All the four strains (S. aureus, P. maltophilia, B. subtilis, Micrococcus sp.) were grown in BHI for 48h at 37°C. In each case 0.2ml inoculum of an overnight culture was used to inoculate 20ml medium in a 100ml Erlenmeyer flask. Equal volumes of broth culture and rat spermatozoa were incubated at 37°C for 20 min and the motility and agglutination of spermatozoa was examined.

### 2.19.3 Screening of female rat genital tract microflora for protease and elastase production

Sterile cotton swabs moistened with physiological saline on very thin wooden sticks were used for collecting the samples of the genital mucous of animals. The swab samples were directly spread on nutrient agar plates. The
plates were incubated at 37°C for 24-48h and observed for growth. All the isolates obtained were checked for the production of protease and elastase as described earlier.

3.19.4 Establishment of selected human endocervical isolates in genital tract of female rats

The animals free from elastase positive organisms were taken in groups of 15 rats each. *S.aureus*, *P.malotophilia*, *B.subtilis* and *Micrococcus* sp. were established in groups II, III, IV and V respectively. 24h old culture (0.1ml) of the organisms was directly instilled daily in the genital tract with the help of an autopipette fitted with a microtip (50-200μl). This process was repeated for 10-15 days or till the organisms were established in the tract.

3.19.5 Reisolation of organisms

To check if the organisms got established in the rats, reisolation was done. After 15 days the rats were kept for 3 days without giving any organism. On the 4th day swabs were again taken from the genital tract and directly spread on nutrient agar plates as described earlier. The established organisms were observed for the production of protease, elastase and other characteristics. *P. maltophilia* could be easily picked up because of the yellow pigment it produced.

3.19.6 Effect on fertility

All the female rats were kept mating with the adult male rats (200-230gm) in a ratio of 1:2 for 8 days. After
this time period the rats were separated and observed for reproduction till another 20-30 days.

3.19.7 Sensitivity of elastase positive organisms to antibiotics

Various isolates from the genital tract of animals were screened to check for the established organisms. These were checked for their sensitivity to antibiotics. Combined Microbial sensitivity discs (Combi-discs) (Span Diagnostics, India) were used to check the sensitivity (Brown et al., 1982 and Reeves et al., 1978).

0.1ml of overnight culture was spread on sterile nutrient agar plates. The plates were kept at room temperature for 5-10 min. One combi-disc was removed from the respective container under sterile conditions with the help of a flamed forceps and carefully placed on the surface of the medium. The disc was lightly pressed with the forceps to make complete contact with the surface of the medium. The plates were allowed to stand at room temperature for 30 min (prediffusion time) and later incubated at 37°C for 16-18h. The diameter of the zone of inhibition was recorded to the nearest millimeter. If only isolated colonies grew instead of confluent growth, the inoculum was too light and the test was repeated. The interpretation of the resistance of sensitivity of the organism to a particular antibiotic was done according to the zone size interpretative chart available with the combi-discs.

70
The minimum inhibitory concentration of the antibiotic to which the organism was sensitive was determined and taken as MIC.

3.19.8 Treatment of experimental animals with antibiotics

Animals that turned infertile were given tetracycline (1000μg/ml) locally by instilling 0.1ml in the genital tract. This treatment was done twice daily for 7 days. A gap of 3 days was given and the genital tract microflora was screened for elastase producers.

3.19.9 Reversion of fertility

The treated animals were again kept for mating to check if the infertiles have become fertile by the removal of elastase producer.

3.20 Effect of purified elastase on reproduction

The effect of purified elastase from P. maltophilia and B. subtilis on reproduction was studied in rats (10 animals in each group) by instilling 100μg of purified elastase in the genital tract of animals daily for 7 days during the mating period. The rats were segregated and observed for 20 to 30 days for reproduction.