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Original Article

Microbial Flora of Semen and Its Impact on Sperm Parameters

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Abstract

Various microorganisms have been found to colonise the male genital tract which may play an important role in altering seminal parameters and thereby reducing male fertilizing potential. A total of 35 semen samples, obtained from PGIMER, Sector-12, Chandigarh, India were subjected to routine semen analysis according to WHO guidelines. The volume of all the samples, motility of 88.5% samples, pH of 80% of the samples was optimum. On culturing these samples on Brain Heart Infusion agar plates, it was found that 80% of the isolates so obtained were Gram positive cocci, 18% Gram negatives and 2% were yeast. Out of these isolates, 4% showed complete immobilization whereas 12% led to agglutination of spermatozoa. Scanning electron microscopy showed morphological alterations in sperm head, neck and mid piece etc. when incubated with the sperm immobilizing and sperm agglutinating strains. Further when the enzymatic activity was looked for, the results showed that these organisms were able to produce either protease, phospholipase, lipase or all of these. Hemolysis on sheep blood agar showed that only 2% of the isolates were capable of causing complete hemolysis and 6% showed partial hemolysis. The Gram positive bacteria were maximally sensitive to Oxacillin and Gram negative were sensitive to tobramycin and gentamycin. In conclusion, various Gram positive and Gram negative bacteria inhabiting male reproductive tract might produce certain factors which may impair sperm parameters. Eradication of these microbes by use of antibiotics can be a probable cure of microorganism induced infertility.

Introduction

Amongst the leading causes of male infertility, genitourinary tract infection accounts for about 15% of the cases (Pellati *et al*., 2008). These infections not only deteriorate the quality of spermatozoa and sperm cell function but also the process of spermatogenesis. Different microorganisms have been reported to alter reproductive functions in a number of ways and to varied degrees. These microorganisms found in the male urogenital tract are associated with sperm abnormalities, especially a reduced sperm count, poor morphology, aberrant motility, deficient mitochondrial function, and loss of DNA integrity (La Vignera *et al*., 2011). These microorganisms include *Escherichia coli*, *Enterococcus faecalis, Ureaplasma urealyticum*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma hominis*, *Candida albicans*, and *Trichomonas vaginalis*. The possible mechanisms by which infertility occurs include attachment of bacteria to sperm, immobilizing factor

produced by some bacteria, immune system recruitment, alteration of glandular function, production of enzymes such as proteases, elastases, metabolic end products, apoptosis and necrosis (Cottell *et al*., 1996).

The alleged pathogenic bacteria are found not only in the reproductive tracts of infertile patients, but also in those of healthy men (Kiessling *et al*., 2008). Reports are at variance regarding the effect of indigenous bacteria on semen quality. Some investigators have reported that there was not any significant difference in sperm parameters of infectious and non-infectious ejaculates (Golob *et al*., 2014) whereas others have observed significant differences in sperm characteristics between fertile and infertile men (Weidner *et al*., 2013).

Therefore, it remains a controversy if the microorganisms found in semen necessarily signify infection and significantly contribute to male infertility (Hou *et al*., 2013).This discrepancy might be due to number of factors such as use of different collection procedures, differing definitions of bacteriospermia, contamination of semen by non-pathogenic commensals of skin, glans penis or lower urethra (Domes *et al*., 2012). Although, isolation of microorganisms in seminal fluid especially of infertile men has been widely reported, their pathophysiologic role in male infertility has not been established (Maciejewska *et al*., 2005).

Due to shortcomings in diagnostic standards and asymptomatic nature of the microbial infections, their exact role in the aetiology of infertility is not very certain, however, their possible effect on the properties of seminal fluid has been suggested (Bukharin, 2002). Though many studies agree that bacterial infection in the genital system could be possible reason for a significant number of cases of male infertility, yet, there is no consensus on how these microbes specifically affect seminal parameters (Khalili *et al*., 2000). Given that, it is one of the potentially preventable forms of infertility; thus, culture-positive patients must undergo antibiotic profiling of the associated microorganisms, before the initiation of the therapy. In this regard, studies conducted by Mogra *et al*., (1981) have shown that out of the most frequently isolated microorganisms from the semen of infertile men, all the strains of *S. aureus* were resistant to Penicillin, thereby, indicating the pervasiveness of penicillin-resistant Staphylococci in semen. Recently, a study conducted by Pajovic *et al*., (2013) has shown a clear effect of antibiotic therapy on the volume and pH of the seminal fluid. Moreover, on completion of the therapy, a significant improvement in sperm concentration and motility was observed.

Therefore, it is necessary to scrutinize seminal fluid for the presence of bacteria so as to fill the gaps in knowledge about infections as cause of infertility.

Materials and Method

Semen Sample

Semen samples were obtained from the patients attending infertility clinic, PGIMER, Chandigarh, India. On liquefaction (37^0C, 30-45min), various macroscopic parameters viz. colour, volume and pH and microscopic parameters viz. sperm count, motility, viability were evaluated as per the WHO standards (WHO, 2010).

Isolation and Presumptive Identification of Microorganisms from Semen

Semen samples were streaked on Brain Heart Infusion agar plates and incubated at 37^0C for 48h and observed for bacterial growth. The isolates so obtained were subjected to various tests for identification according to the characteristics laid down in the Bergey's Manual of Determinative Bacteriology.

Effect of cell culture and cell free supernatant of isolates on motility/ agglutination/viability of human spermatozoa.

The effect of cell culture and cell free supernatants of isolates was studied on motility/agglutination/ viability of human spermatozoa. Briefly, isolates were grown for 72h at 37°C under shaking and stationary conditions. The cultures were then centrifuged, supernatant was separated and the cells were washed with PBS (50mM, pH 7.2) and resuspended in same buffer. Human semen ejaculates that satisfied WHO criteria of normal standards were selected and the sperm count adjusted to 39×10^6 per ml with sterile PBS (pH 7.2) used as diluent. Equal volumes of sperm suspensions and cell culture/ cell free supernatant (1:1) were mixed and incubated at 37^0C. For control PBS/ BHI was added instead of cell culture/cell supernatant. At different time intervals, a 10 µl aliquot of the mixture was placed on a clean glass slide, covered with a coverslip and observed under X400 magnification using a bright-field microscope (Olympus).

Scanning Electron Microscopy

Scanning electron microscopy was used to investigate the morphology of the spermatozoa. The sample processing was done according to the

standard method (Hafez and Kanagawa, 1973) with slight modifications. For this, semen sample was washed twice with PBS at 700rpm for 10min and finally resuspended in same buffer so as to get a final count of 40 x 10^6/ml. 200µl of this sample was mixed with 200µl of bacterial culture and incubated for 2h for 37^{0}C. As control, 200µl of sample was mixed with 200µl of PBS. After incubation, 2.5% of gluteraldehyde was added and incubated at 37^{0}C for 30min. The samples were then washed thrice with PBS. One drop of fixed and washed spermatozoa was placed on a silver painted adhesive tape mounted on brass stubs and air dried. 100 A^0 gold coating was done using fine coat; Jeol ions sputter (JFC-1100). This gold coated stub was finally examined at different magnifications under the scanning electron microscope (model JSM-6100, SM-Jeol 20kV). SEM was carried out at sophisticated analytical instrumentation facility (SAIF), Panjab University, Chandigarh.

Screening of Isolates for Various Enzymatic Activities

Protease: Proteolytic activity was determined by the method described by Iida *et al*., 1982. For this, 1% milk casein agar plates were prepared and isolates were inoculated on by spotting and plates were incubated for 24-72h at 37^{0}C. The activity was revealed by the clear zone formation around the inoculum spot.

Phospholipase: Phospholipase activity was analysed by the procedure described by Collee and Miles (1989). Briefly, egg yolk was collected under sterile condition and was mixed with normal saline in equal volumes (1:1). 50% egg yolk agar plates were made by and the isolates were spotted on the plate and incubated at 37°C for 72h. Clear zone formation around the colonies were taken as phospholipase positive.

Lipase: Lipase activity was checked by using tributyrin agar plates (Collee and Miles, 1989). Tributyrin agar plates were prepared by adding 1% tributyrin (v/v) to nutrient agar medium. Isolates were spotted on the plates and incubated at 37^{0}C for 72h. Clear zone formation around the colonies indicated lipase positive.

Quantitative Assay for Extracellular Enzymatic Activity of Organisms

All the positive isolates were further used for quantitation of extracellular enzymes like protease, phospholipase, and lipase. 5mm steel wells were placed on 1% milk casein agar plates, 5% egg yolk agar plates, 1% tributyrin agar plates. 100µl of 72h old cell free supernatant was added to each well. Plates were kept at 4^{0}C for 2h and incubated at 37^{0}C for 24h.

Production of Haemolysin by the Isolates

Certain bacteria produce extracellular enzymes that lyse red blood cells in the blood agar (haemolysis). These haemolysins (exotoxin) radially diffuse outwards from the colony causing complete or partial destruction of the red cells (RBC) in the medium and complete denaturation of haemoglobin within the cells to colourless products. β-haemolysis (complete or partial haemolysis) was seen on sheep blood agar. For this 5% blood agar plates were prepared. The isolated colonies were streaked on plates and the plates were incubated at 37^{0}C for 24h. After incubation, the plates were observed for zone of haemolysis surrounding the colony.

Antibiotic Susceptibility Test

To check the antibiotic susceptibility, bacterial isolates were inoculated in 10ml BHI broth under shaking conditions at 37^{0}C for 24h.100µl of culture was spread plated on sterile Muller Hinton Agar plates. Antibiotic disk was removed from container under sterile conditions with the help of sterile forceps and carefully placed on the surface of the medium. The disk was slightly pressed with the help of forceps to make complete contact with surface of medium. The plates were incubated at 37^{0}C for 24h. After incubation, diameter of zone of inhibition was recorded in mm.

Results

Semen Analysis

A total of 35 semen samples were obtained from patients attending infertility clinic PGIMER, Chandigarh, India. The samples were macroscopically and microscopically examined according to WHO standards. Macroscopic parameters such as colour, pH and volume of the semen sample were looked for. All the samples were homogeneous and had grey-opalescent appearance. The volume of the sample was in the normal range of 1.5 to 6 ml. The pH of all the samples was also in optimal range i.e. 7.2 to 7.6 except for 20% samples where pH range (pH 6- 6.8) was below optimum.

The microscopic parameters such as % motility, agglutination, % viability, morphology and presence of other cells were also assessed. The results revealed

that percentage motility of 88.5% of the samples was normal (lower reference limit 40) whereas 25.7% of the samples showed agglutination. The percentage viability of 68.6% of the samples was above 58% which was the reference limit of WHO. All the samples had normal sperm morphology however, 34.3% of the samples showed presence of round cells.

Isolation of Microorganisms from Semen Samples

For isolation of microorganisms, semen samples were cultured on Brain Heart Infusion (BHI) agar plates and the plates were incubated aerobically at 37^0C for 24 to 48 h. In total 50 isolates were obtained and Gram staining of these isolates showed the presence of Gram positive (80%), Gram negative (18%) microorganisms and also yeast 2% (Figure 1).

Presumptive Identification of Microorganisms

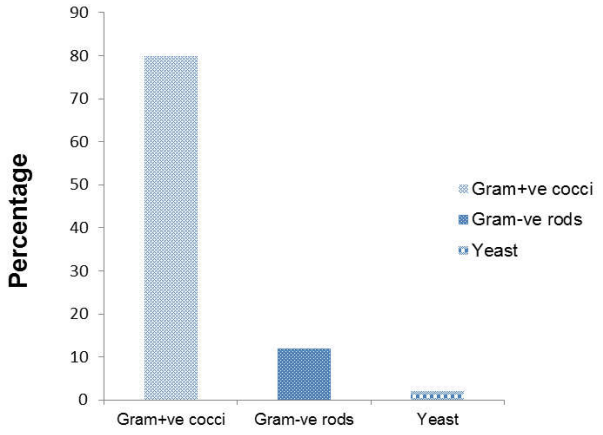

Fig. 1: Distribution of Gram positive, Gram negative and yeast in semen samples

Gram Positive

For the presumptive identification, Gram positive microorganisms were subjected to catalase test and the results showed that all the isolates were catalase positive indicating that they belonged to either *Micrococcus* or *Staphylococcus*. These isolates were further tested for their ability to ferment mannitol under aerobic and anaerobic conditions. It was observed that 80% of the isolates could ferment mannitol under both aerobic and anaerobic conditions revealing these isolates to be staphylococci whereas 20% of the isolates which fermented mannitol aerobically only were identified as micrococci.The different species of *Staphylococcus* were further identified by coagulase test. 45% of the total gram positive isolates were positive for coagulase leading to their presumptive identification as *Staphylococcus aureus* and rest 35% of the total isolates were coagulase negative staphylococci. *Staphylococcus aureus* was confirmed by presence of yellow zones around the colonies on mannitol salt agar (MSA) (Figure 2).

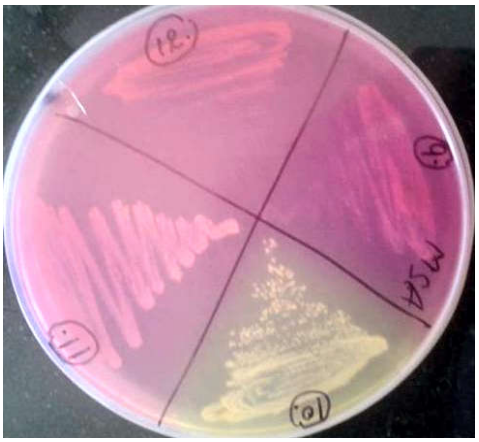

Fig 2: Representative photograph of *Staphylococcus aureus* showing yellow colonies on MSA and pink coloured colonies indicating coagulase negative staphylococci.

Gram Negative Microorganisms

Gram negative organisms so obtained were subjected to oxidase test. The result showed that 55.6% of the total gram negative isolates were oxidase negative indicating these isolates to be belonging to *Enterobacteriaceae*. These isolates were further biochemically identified. Based on biochemical characterization, it was observed that out of total isolates, 44.4% were *Escherichia coli* whereas 11.1% were *Serratia*. *E. coli* was further confirmed by the presence of green metallic sheen on eosin-methylene blue agar *Serratia* was also found to give red pigmented colonies on BHI (Figure 3).

The oxidase positive isolates (44.4%) were identified to be *Pseudomonas* by their ability to utilize various biochemicals. They were also found to produce green pigmented colonies on BHI.

Effect of cell culture and cell free supernatant of the isolates under stationary and shaking conditions on motility, agglutination and viability of Human spermatozoa.

All the 50 isolates obtained from semen samples were grown in BHI for 72h (shaking and stationary conditions) and the interaction of culture and cell free supernatant with human spermatozoa was studied with respect to change in motility, agglutination and viability of spermatozoa.

Cell Culture

Motility and Agglutination

When semen samples were mixed with 72h old cell culture, it was observed that 4% of the isolates under stationary as well as shaking conditions could cause 100% immobilization of spermatozoa. 4% and 12% of the isolates grown under stationary and shaking conditions resulted in ≥ 50% sperm immobilization, respectively. However, immobilization in 80% of isolates grown under stationary conditions and 72% of the isolates in shaking conditions was not significant. The 12% of the isolates in both the conditions could result in agglutination of spermatozoa however, the size of agglutination clumps were larger under shaking conditions (Figure 4)

Cell free Supernatant

The effect of cell free supernatant from 72h old cultures of semen sample isolates on motility of spermatozoa was studied. The results showed that 4% of supernatants from the isolates under stationary as well as shaking conditions could cause 100% immobilization of spermatozoa. ≥ 50% of sperm immobilization was observed in 8% and 24% of the isolates grown under stationary and shaking conditions respectively. However immobilization in 88% of isolates grown under stationary conditions and 72% of the isolates in shaking conditions was insignificant. No agglutination was observed in any of the supernatants.

Percentage Decrease in Viability

The decrease in motility was more pronounced with cell culture and supernatant under shaking conditions, therefore viability of the spermatozoa was studied with cell culture and supernatant under shaking conditions only. Results showed that cell culture as well as supernatant of only 18% of the isolates could lead to ≥ 50% death of spermatozoa (Figure 5).

Fig. 3: Representative photographs showing (A) Green metallic sheen of *E. coli* on EMB agar plate(B) Red pigmented colonies of *Serratia* on BHI agar plate (C) Green pigmented colonies of *Pseudomonas* on BHI agar plate.

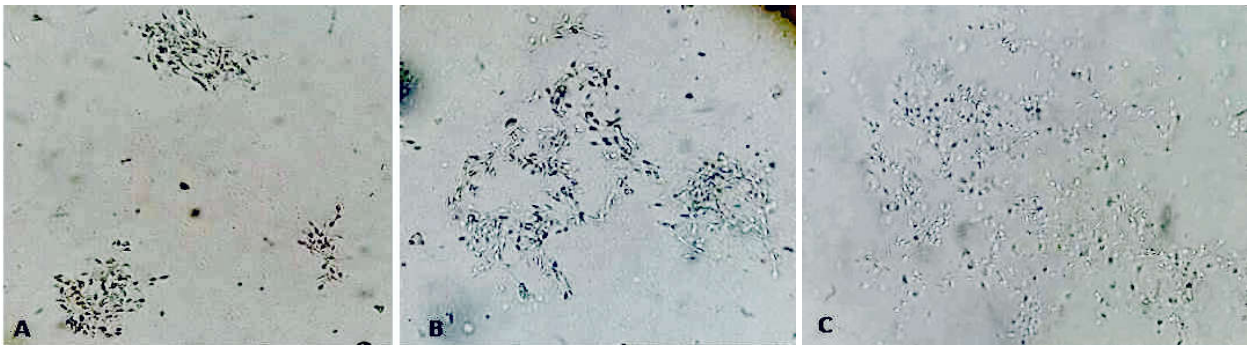

Fig. 4: Photomicrograph showing grades of agglutination of human spermatozoa (A) Grade 2; (B) Grade 3; (C) Grade 4

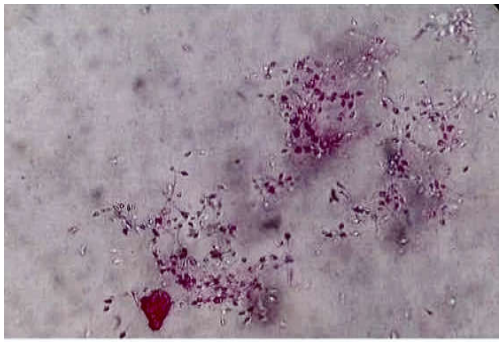

Fig. 5: Representative photograph of eosin staining of human spermatozoa after incubation with isolates showing live spermatozoa (unstained) and dead spermatozoa (pink stained)

Scanning Electron Microscopy

The effect of cell cultures of sperm agglutinating *E. coli, Serratia* and sperm immobilizing *Pseudomonas* sp. on human spermatozoa was studied by scanning electron microscopy. Cell cultures were incubated with 200µl of sperm suspension (40×10^6 sperms ml^{-1}). BHI was added to the sperm suspension in case of control.

Normal human spermatozoa were observed in control which was characterized by flattened ovoid heads covered anteriorly by rough rigid surface and posteriorly by smooth surface (Figure 6A).

From the results, it was observed that sperm agglutinating *E. coli* and *Serratia* could adhere to spermatozoa. Further, *E. coli* resulted in morphological alterations in head, neck and midpiece region due to loosening and disruption of membrane (Figure 6B). *Serratia* also led to loosening and disruption of membrane causing damage in head and tail. Breakage in the neck region and coiling of end piece of tail was also observed with *Serratia* (Figure 6C). However, decrease in thickness around neck region was observed with sperm immobilizing *Pseudomonas* sp. (Figure 6D).

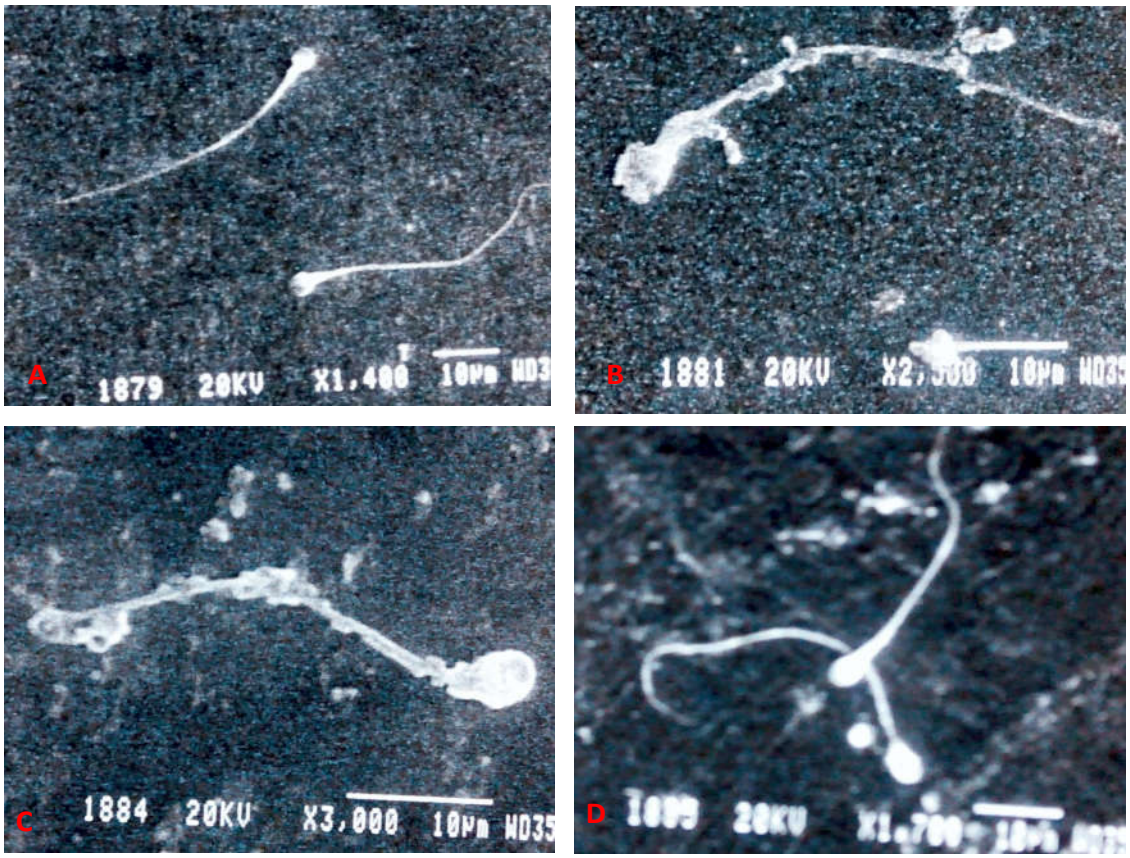

Fig. 6: Scanning electron micrograph of (A)control showing normal spermatozoa, (B) spermatozoa showing morphological alteration on treatment with sperm agglutinating *E. coli*, (C)spermatozoa showing loosening and disruption of membrane on treatment with spermag glutinating *Serratia* (D)spermatozoa showing decrease in thickness around neck region on treatment with sperm immobilizing *Pseudomonas* sp.

Enzymes Produced by Microorganisms

All the 50 isolates from semen samples were screened for their abilities to produce various enzymes like protease, phospholipase, and lipase on milk agar, egg yolk agar and tributyrin agar plates, respectively (Figure 6). From the results, it could be observed that out of total isolates, 40% hydrolysed casein indicating protease activity, 4% showed phospholipase activity and 56% isolates possessed lipase activity (Figure 7).

Quantitation of Extracellular Enzymatic Activity of Microorganisms

All positive isolates were further used for quantitation of extracellular enzymatic activity like protease, phospholipase and lipase by means of quantitative test following the well diffusion technique. From the results, it was observed that 29% of the protease positive isolates, all the phospholipase positive isolates and 22% of the lipase positive isolates produced enzymes extracellularly(Fig:8).

Protease, Phospholipase and Lipase activity in terms of clear zone (mm) produced by 100µl of cell free supernatant on milk agar, egg yolk agar and tributyrin agar plates after 48h of incubation at 37°C.

On the basis of preliminary screening of seminal isolates with respect to their enzyme production, organisms were grouped under $P^+P_L^+L^+$, $P^+P_L^+LÉ$, $P^+P_L^ÉL^+$, $P^ÉP_L^+L^+$, $P^+P_L^ÉLÉ$, $P^ÉP_L^+LÉ$, $P^ÉP_L^ÉL^+$, $P^ÉP_L^ÉLÉ$ (P-protease, P_L-phospholipase, L- lipase). From the results, it was observed that 4% were $P^+P_L^+L^+$, 26% were $P^+P_L^ÉL^+$, 10%were $P^+P_L^ÉLÉ$, 26% were $P^ÉP_L^ÉL^+$ and 19%were $P^ÉP_L^ÉLÉ$ whereas none of the isolates fell under the groups $P^+P_L^+LÉ$, $P^ÉP_L^+L^+$, and $P^ÉP_L^+LÉ$.

When the organisms were grouped on the basis of enzyme activity and their ability to impair sperm motility, it was observed that all the isolates of group $P^+P_L^+L^+$(2/2) could cause 100% immobilization of spermatozoa.

7.6% of the isolates from group $P^+P_L^ÉL^+$, 20% of the isolates belonging to $P^+P_L^ÉLÉ$ group, 7.6 % of the isolates of group $P^ÉP_L^ÉL^+$ and 17.6% isolates of the group $P^ÉP_L^ÉLÉ$resulted in agglutination of spermatozoa.

Thus protease and lipase seemed to be important enzymes affecting sperm motility. However, immobilization of spermatozoa was also observed in the 17.6% of isolates belonging to group $P^ÉP_L^ÉLÉ$, producing none of the above enzymes, indicating that some other factors might be causing sperm impairment.

Production of Haemolysin by Isolates

The isolates were further checked for haemolysin activity on sheep blood agar plates. It was observed that only 2% of the isolates showed complete haemolysis (transparent zone was observed due to lysis of red blood cells), 6% of the isolates showed partial haemolysis whereas all the remaining isolates failed to lyse sheep RBCs (Figure 9).

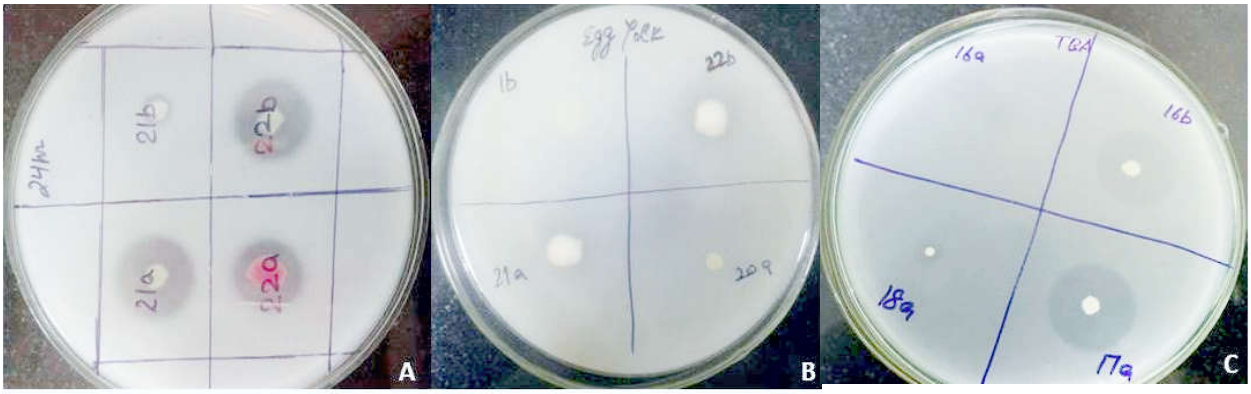

Fig. 7: Representative photographs showing (A) protease, (B) phospholipase and (C) lipase activity in terms of clear zone produced by isolates on milk agar, egg yolk agar and tributyrin agar plates respectively

Fig. 8: Quantitative assay of protease, phospholipase and lipase activity in terms of clear zone size (mm) produced by isolates on milk agar, egg yolk agar and tributyrin agar plates by plate method

Antibiotic Susceptibility

In vitro, antimicrobial susceptibility was determined for clinical isolates by disk diffusion method, recommended and interpreted according to CLSI (Clinical and Laboratory Standard Institute) guidelines.

11 commonly used antibiotics belonging to different classes were chosen for Gram positive

organisms and zone of inhibition in mm was measured (Figure 10). Most of isolates (96%) were found to be sensitive to Oxacillin, only 3.4% isolates were resistant, 79% of isolates were resistant and 21% were sensitive to Penicillin G and 48% isolates were found to be intermediate, 3.4% were resistant and 48% were sensitiveto cefotaxime (Figure 11).

For Gram Negative organisms, 6 antibiotics belonging to different classes were chosen and zone of inhibition in mm was measured (Figure 12). All isolates were sensitive to Tobramycin and Gentamycin and all isolates were seen to be resistant to Ampicillin and Amoxyclav. 22.2% of isolates were seen to be intermediate, 11.1% were resistant and 67% were sensitive to cefotaxime whereas 55.5% were resistant and 44.4 % were sensitive to co-trimaxazole (Figure 13).

Fig. 9: Representative photograph showing clear zone indicating haemolysis on sheep blood agar

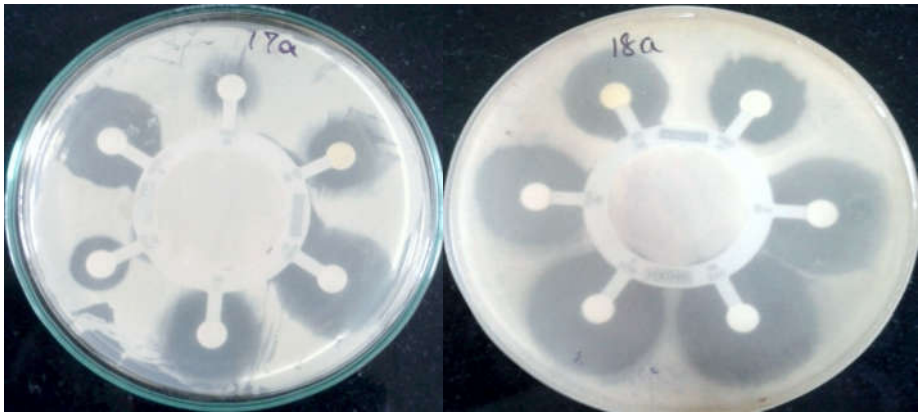

Fig. 10: Susceptibility of Gram Positive Organisms to different antibiotics. A disk diffusion test with an isolate of semen sample. The diameters of all zones of inhibition are measured and those values translated to categrories of susceptible, intermediate or resistant using the latest tables published by the CLSI.

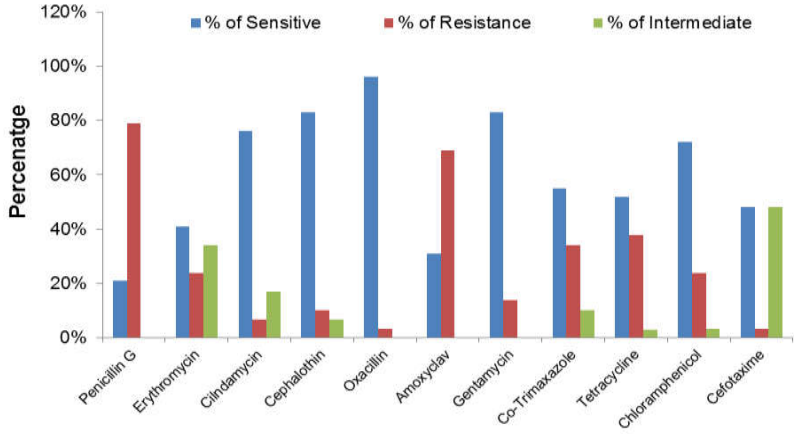

Fig. 11: Percentage susceptibility of Gram positive organisms

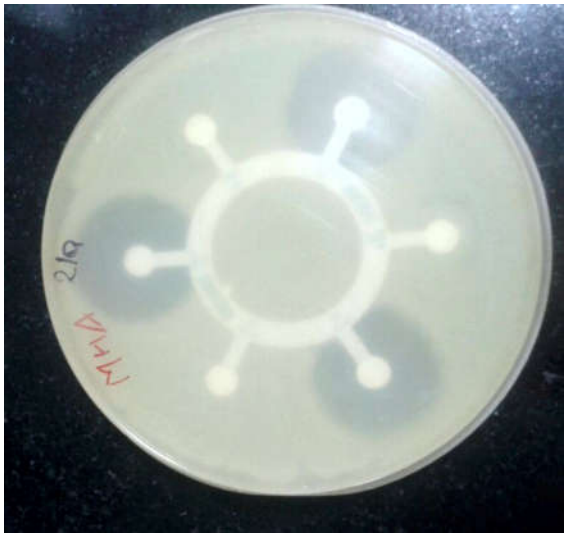

Fig. 12: Representative photograph shows antimicrobial susceptibilty of Gram negative organisms. A disk diffusion test with an isolate of semen sample. The diameters of all zones of inhibition are measured and those values translated to categories of susceptible, intermediate or resistant using the latest tables published by the CLSI.

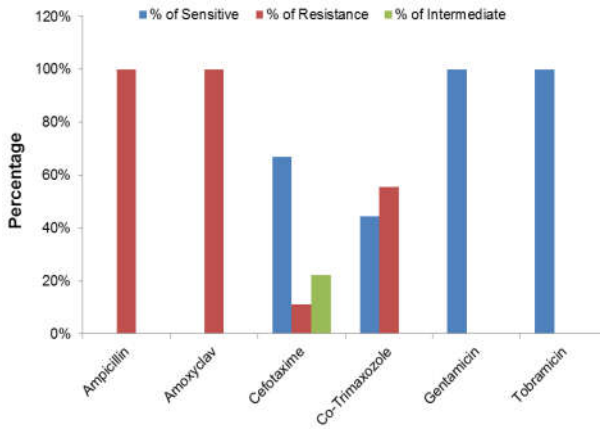

Fig. 13: Percentage susceptibility of Gram negative organisms

Discussion

Semen analysis is considered as an important step to examine various disorders distressing the genital tract of males (Andrade-Rocha, 2003). As the ejaculate is a blend of secretions from the urogenital tract and the male accessory glands, therefore, analysis of semen can offer a clear understanding in terms of sperm parameters viz. count, motility (ability to move), vitality and morphology (size and shape) and presence of non-sperm cells, if any (Samplaski *et al*., 2010). Investigation of these parameters helps in gaining important clinical information regarding spermatogenesis, the functional competence and the secretory pattern of glands (Moazzam *et al*., 2015). Also, evaluation of these factors may help us uncover the reasons for infertility.

Recently, role of microorganisms in male infertility has been constantly gaining consideration. Various microorganisms commonly found in the male genital tract include *Escherichia coli*, *Mycoplasma hominis*, *Enterococcus faecalis, Neisseria gonorrhoeae*, *Ureaplasma urealyticum*, *Chlamydia trachomatis*, *Candida albicans*, and *Trichomonas vaginalis* (Nelson *et al*., 2010). They may lead to altered semen parameters in different ways. Therefore, the present study was carried out with an aim to assess the frequency of occurrence of different microorganisms in semen and to study their effect on semen quality.

A total of 35 semen samples were obtained and were primarily checked for the macroscopic parameters viz. colour, pH and volume. All the parameters were found to be in normal ranges except in 20% of the samples where pH was lower than the reference limit. Further, when the microscopic seminal parameters like motility, viability and agglutination were evaluated, the results showed that morphology was normal in all the samples; % motility in 88.5% of the samples was normal (i.e. above 40%) and rest 11.5% of the samples showed motility below 40%. The % viability of 68.6% of the samples was normal (i.e. above 58%) whereas the remaining 31.4% samples showed viability less than 58%, which was lesser than the WHO standards. Furthermore, only 34.3% samples also showed the presence of other cells like pus cells and macrophages.

As the incidence of microorganisms in semen can be linked to infertility, therefore, semen samples were cultured on growth media to check the presence of any microbial isolates. It was found that out of all the isolates obtained, Gram positive bacteria were the predominant flora (80%) as compared to Gram negative bacteria (18%) and yeast (2%). These isolates were further identified based on their ability to utilize various biochemicals. The results showed that coagulase positive Staphylococci, coagulase negative staphylococci and micrococci constituted the Gram positive organisms. The major Gram negative bacteria identified were *E. coli, Serratia* sp. and *Pseudomonas* sp.

Similar results have been obtained by Momoh *et al*., (2011) wherein *S. aureus* was found to be the major organism with 75% prevalence in the semen samples studied whereas *E. coli* and *Pseudomonas* were lesser in number. These results are also in concordance with the work of Ekhaise and Richard (2014), who also reported the occurrence of *S. aureus* (77%) and *E. coli* (11.1%) in semen samples of men complaining of infertility. Similar findings have been documented by Ikechukwu *et al*., (2007) showing *S. aureus* (37.1%) as the highest bacterial isolate and *E. coli* (8.9%) as

the lowest.

A number of microorganisms are capable of interacting directly or indirectly with spermatozoa (Golshani *et al*., 2006) resulting in immobilization, agglutination phenomena and morphological alterations of the spermatozoa. In the present study, when the effect of these isolates was checked on sperm parameters *in vitro* under stationary conditions, it was found that12% of the isolates (comprising of *E. coli* and *Serratia* sp.) were capable of impeding sperm motility via agglutination of spermatozoa. On the other hand, only 4% of the isolates (belonging to *Pseudomonas* sp. and *S. aureus*) could cause 100% immobilization of spermatozoa while 50-100% of sperm immobilization was observed in another 4% of the isolates (belonging to *Pseudomonas* sp. and *S. aureus*). However, yeast isolates failed to agglutinate or immobilize spermatozoa. Moreover, the size of clumps of agglutinated spermatozoa was bigger and inhibition of motility was better when the cultures were grown under shaking conditions rather than stationary conditions.

Seminal parameters such as count, percent motility and percent viability play a vital role in fertility potential of men (Dohle *et al*., 2005). Hence, with an aim to find out the effect of these isolates on the viability of spermatozoa, the supra-vital staining was done. From the results, it was observed that cell cultures of only 18% of the isolates could lead to death of more than 50% of spermatozoa. Similar findings have been reported by Liu *et al*., (2002) wherein they have reported spermicidal effect of *S. aureus* when co-incubated with human spermatozoa. Similarly, Teague *et al*., (1971) has reported that upon incubation of *E. coli* with human spermatozoa, there was a significant inhibition of motility and viability.

Similar findings have been made by Diemer *et al*., (2003) wherein *E. coli* was seen to adhere to human spermatozoa *in vitro*, resulting in agglutination of spermatozoa. In another study, Vander and Prabha (2015) have also shown that *Serratia marcescens* causes agglutination of human spermatozoa. Also, while studying the effect of *Pseudomonas* on sperm motility parameters, Rennemeier *et al*., (2009) has reported that it could lead to reduction in motility of spermatozoa in a dose dependent manner. The results obtained by Huwe *et al*., (1998) are in concordance with our study, who also checked the influence of various uropathogens on human sperm parameters by means of CASA and reported that *S. aureus* retards the sperm motility. However, our results are in contrast to the work done by Tian *et al*., (2007) who have reported that yeast, has an inhibitory effect on human sperm motility.

Further, in order to detect the ultrastructural anomalies of spermatozoa caused by these isolates, scanning electron microscopy (SEM) was carried out. The results showed the adherence of sperm-agglutinating *E. coli* and *Serratia* sp. to spermatozoa, thereby, causing prominent morphological defects. However no adherence was observed in case of spermatozoa upon incubation with sperm immobilizing *Pseudomona s*sp. or PBS.

This observation is supported by the study made by Wolff *et al*., (1993) wherein they have shown the attachment of *E. coli* to both heads and tails of spermatozoa. In another study, Diemer *et al*., (2000) have revealed multiple and intense alterations in the ultrastructure of spermatozoa, such as membrane defects as well as cytoplasmic vacuoles, when incubated with *E. coli*. Morphological alterations involved all of the superficial structures of sperm, in particular the plasma membrane of the mid-piece and neck and the acrosomal membrane, showing that these morphological defects might be responsible for the immobilization of spermatozoa. Similar results were obtained by Ohri and Prabha (2005) wherein SEM of sperm samples incubated with *Staphylococcus aureus* showed spermatozoal structural abnormalities.

Moreover, to check whether the immobilizing activity was the property of supernatant or cells, the supernatants of the isolates were checked for their effect on spermatozoa *in vitro*. From the results, it could be seen that supernatants from 4% of the isolates were capable of causing 100% immobilization of spermatozoa whereas 8% of the isolates rendered 50% of the sperms immotile. However, no agglutination was observed in any of the supernatants. Our results are in agreement with findings of Rennemeier *et al*., (2009), who have reported deleterious effects on sperm motility by quorum sensing signalling molecule, a secretory factor from *P. aeruginosa*. The decrease in sperm motility and viability was also observed earlier by Schluz *et al*., (2013), when *E. coli* supernatant was incubated with spermatozoa. Also, Paulson and Polakoski (1977) have reported a soluble, heat-stable factor of low molecular weight isolated from *E. coli* filtrates as being the causative agent for the immobilization of human spermatozoa. These results further corroborate earlier findings of our laboratory wherein sperm immobilization factor isolated and purified from supernatant of *E. coli* (Prabha *et al*., 2010) and *S. aureus* (Prabha *et al*., 2009) were shown to cause sperm immobilization *in vitro*.

Since the effect of various microorganisms on sperm parameters under *in vitro* conditions is multifactorial, therefore microbial products such as

enzymes were looked for their negative influence on spermatozoa. Some researchers have reported different pathogenetic mechanisms exerted by microorganisms upon spermatozoa, such as production of proteases, phospholipases A and C, and lipases, may injure sperm parameters (Fraczek and Kurpisz, 2007). On the similar grounds, screening of all the isolates for production of various enzymes viz. protease, phospholipase and lipase was done. From the results, it was observed that out of 50 isolates, 56% were found to be positive for lipase production, 40% for proteases, and rest 4% for phospholipases indicating that production of these enzymes may, in part, be responsible for the various detrimental effects on human spermatozoa. Further, the isolates positive for enzymatic activity associated with cell culture were screened for extracellular enzymatic activity. From the results, it was observed that 29% of the protease positive isolates, all the phospholipase positive isolates and 22% of the lipase positive isolates produced enzymes extracellularly. Based on the above grouping, it was observed that 100% of the isolates possessing all the three enzymatic activities could cause 100% immobilization of spermatozoa. However, 7.6% of the isolates which were positive for both protease and lipase activity; 17.6% negative for both; 20% and 7.6% positive for protease and lipase activity, respectively, resulted in agglutination of spermatozoa. Our results are in consistence with those of Villegas *et al.,* (2005) who have demonstrated that *E. coli* could cause sperm deterioration by activating several proteases, responsible for alterations in membrane symmetry. However, the isolates which lacked all the three enzymatic activities were also found to impair spermatozoa via agglutination which paved the way for the involvement of some other factor which might be responsible for the same.

A few researchers have described, in an *in vitro* study, a negative influence of hemolysin, a virulence factor of Enterococci, on membrane integrity of sperm head, neck and mid piece (Qiang *et al*., 2007). Similar observations were made by Boguen *et al*., (2007) who have reported that damaging effect of *E. coli* on sperm could be attributed to alpha hemolysin production. In view of these findings, in the present study, all the isolates were tested for haemolysin production, and the results revealed that 2% of the isolates showed complete hemolysis, 6% showed incomplete hemolysis whereas 92% of the isolates showed no hemolysis. The results in the present study indicate that haemolytic condition might not be important in pathogenicity affecting human sperm parameters; rather some other factor might be more relevant.

Antibiogram typing is a traditional epidemiological typing method used to distinguish between individual strains. The organisms exhibit remarkable versatility in their behaviour towards antibiotics (Uwaezuoke *et al*., 2004), with some strains have overcome most commonly used drugs. Globally there is an increasing concern about the spread of antibiotic resistance in various strains of microbes. Hence, for specific therapy for infertility resulting from bacterial infection, sensitivity of microorganisms to an array of antibiotics must be determined.

With this aim, in the present study, antibiogram typing of all the isolates was done by disc diffusion method and zone of inhibition was measured. From the results, it was observed that out of all the gram positive isolates tested,96%of the isolates were sensitive to most of the antibiotics tested except Penicillin G (to which 79% were resistant), whereas intermediate sensitivity was seen in case of Cefotaximine. In line with our findings, Mogra *et al*., (1981) have also shown that all the strains of *S. aureus* isolated from infertile patients were resistant to Penicillin, thus indicating the high prevalence of penicillin resistant Staphylococci in seminal fluid. This development of bacterial resistance to Penicillin G could be attributed to their frequent and indiscriminate use. In case of gram negative isolates, all the isolates were sensitive to Tobramycin and Gentamycin but were resistant to Ampicillin and Amoxyclav. Intermediate sensitivity was seen in 22.2% of the isolates.

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