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Research Article

SEMEN ANALYSIS: CORELATION WITH RELAVENT TO PATHOLGOY AND MICROBIOLOGY PARAMETERS IN MALE INFERTILITY

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A B S T R A C T

The study was aimed to assess microbiological quality of male patients with infertility at Vivek Laboratories, Nagercoil. Infertility is a matter of concern in India because of its social problems. Seventy semen specimens were collected from males with infertility. The seminal fluids were diluted with sterile saline, centrifuged and cultured on Nutrient agar, Blood agar, Chocolate agar and MacConkey agar then incubated aerobically and in 5% CO_2 at 37°C for 24 hours for the isolation of pathogenic microorganism. Isolates were identified based on Gram's staining and biochemical tests. *Escherichia coli* was the most common isolate followed by *Staphylococcus aureus, Pseudomonas aeruginosa, Proteus* sps and *Enterococcus* sps. Of the total samples, 63% were infected and significantly affected the semen parameters. The semen motility rate was reduced significantly in samples infected with pathogenic microorganisms.

INTRODUCTION

Semen is composed of secretions and cells from the testes, epididymides, prostate, seminal vesicles and urethral glands. Male infertility has been associated with a number of non-genetic and genetic factors. The non-genetic factors include hypogonadotrophic hypogonadism, previous inguinal and scrotal surgery, and environmental factors such as genital infections. In respect to male urogenital tract infection, it was found that asymptomatic bacteriospermia had an important role in male infertility through affecting different sites of male reproductive tract, such as the testis, the epididymus and male accessory gland (Ali *et al*., 2013).

Although only one sperm is needed for conception, millions are released during intercourse to increase the chance of pregnancy. A fertile male ejaculates at least 2 milliliters of semen with the right consistency to transport at least 40 million sperm toward the waiting egg(s). Of these, at least 50% should be moving vigorously through the liquid, 2% must be moving forward and at least 4% should have a normal shape - an elliptical head and tail to provide the thrust they need. Poor sperm count and quality includes too many white blood cells, bacteria, sexually transmitted diseases or infections are indicators of male-related infertility. These parameters are a major risk factor in infertility (Bhatt *et al*., 2015).

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The medical definition of infertility is the inability to conceive *via* unprotected sex over the course of one year. About one in six couples is unsuccessful when trying to get pregnant for the first time. Thus, infertility constitutes medical and socio-cultural problems in worldwide and bacterial infections contribute to about 15% of male infertility Both men and women can suffer reproductive issues either directly or indirectly caused by bacterial infections. Infections, whether bacterial or due to other issues such as sexually transmitted diseases, inflict negative changes upon the reproductive and genital tracts.

Acute and chronic infections and consequent inflammation in the male reproductive system may compromise the sperm cell function and the whole spermatogenetic process. Therefore, these infections are considered to be a major source of causing sperm damage, elevated leukocyte response (pyospermia), poor motility (asthenospermia) and immature forms (tetratospermia) in male (Elena *et al*., 2009). For successful fertilization, motility is the most obvious and most essential sperm function and has been repeatedly shown to be predictive of fertilization in vitro.

Microbiological examination in infertile man can be helpful in detecting male urogenital tract infection and its treatment. Presence of pathogenic microorganisms in semen may be related to a breach in the integrity of the blood-testes barrier and serve as early warning signals of impairment of male fertility. Along with the routine semen analysis parameters,

microbiology study of semen can be an asset in dealing with primary infertility (Khan *et al*., 2014)

It is found that the simple presence of bacteria in semen samples may compromise the sperm quality by altering the morphological structure of sperm (Kaur *et al.*, 2014). The bacteria responsible for semen contaminations generally drawn from the urinary tract of patients or can be transmitted by the partner via sexual intercourse. It is well documented that bacteria, yeasts and protozoa may interact directly with sperm. These interactions result in attachment between bacteria and sperm, which cause morphological alterations to sperm (Kaur *et al.*, 2014). Commonly, bacteriospermia includes *Neisseria gonorrheae*, *Treponema pallidum* and *Chlamydia trachomatis* and non-specific (facultative) organisms like g. *E. coli*, *Staphylococci, Streptococci*, *Klebsiella* spp., and yeast-like cells (a fungus) because of their ability to impair fertility both in man and women (Gerais *et al*., 1992).

Among many isolates*, E.coli* is the most frequent one from male patients with genital tract infections or semen contamination as reported by Diemer *et al*. (2003). They also reported that the negative influence (qualitative and quantitative alterations) of this species on sperm quality was associated partially to its effect on motility and to the impaired acrosomal function In this background, the present attempt was made to examine the quality of semen which infected with different bacterial pathogens from infertile men and their antibiotic susceptibility pattern *in vitro* for the purpose of formulating and monitoring the antibiotic policy and proper empiric therapy.

MATERIALS AND METHODS

Patient Selection

The aim of this research and methods were explained to the patients and a written informed consent was taken from each subject before collection of specimen. The methodology of this current study was approved by our institutional ethical committee. The subjects were instructed to urinate and wash their hands, penis and scrotum before ejaculation to avoid contamination. A total number of 70 samples of primarily male infertility from the age group of 25 to 45 years who had 3 to 7 days of sexual abstinence from intercourse were collected in the sterile containers during 1st January 2017 to 30thJune 2017. Patients who administrated any medication in the last one week were excluded.

Qualitative analysis of semen samples

The semen samples were checked after liquefaction within one hour of ejaculation. A normal sample was appeared like homogenous gray opalescent. It may appear less opaque if the sperm concentration is very less or brown when red blood cells are present. The volume of the seminal fluid was measured by decanting the whole sample aseptically into a graduated centrifuge tube and the level was recorded in ml ± 0.1. The pH of the sperm sample was examined by spreading a drop of the sample evenly onto the pH paper and the colour of the impregnated zone was compared with the calibrated strip. The viscosity of the sample was observed by the length of thread formation when a drop of semen was fall back to the sample with the help of Pasteur pipette. A normal sample left the pipette as small discrete drops while in abnormal cases, the drop formed a thread greater than 2 cm long. Total motility of the samples was done by applying a drop of the sample onto a slide, covered with cover slip. Each sample was then focused under the microscope using x40 objective lens. The microscopic field was scanned systemically and the motility of each spermatozoon encountered was graded a, b, c and d that is, (a) rapid progressive motility, (b) slow or sluggish motility, (c) non-progressive motility and (d) immobility. The total number of spermatozoa in each category was enumerated with the use of a laboratory counter. In this procedure, four to six fields were observed to classify 100 successive spermatozoa and their movements were recorded. The sperm count was done by using improved Neubauer Counting Chamber and one twenty (1/20) dilution of semen was done with formol saline as diluents. Count = $N \times 10^6$/ml (Ekhaise and Richard, 2011). The reference values used, with the lower reference limit (95% confidence intervals) of the above-mentioned semen parameters, were taken from the criteria provided by the EAU guidelines (5).

Calculation

Depth = 0.1 mm
Area = $1/5 \times 1/5 = 1/25 \times 5 = 1/5$ mm^2
Volume= 0.1 mm × 1/5 mm^2 =1/50 mm^3
If N cell is in 1/50, N = n × 50 × 20 = 1000
Converting to ml,
$1000 \times 1000 = 10^6$ N. Dilution factor is 20

Bacteriological examination of semen samples

A total of 44 samples of subjects who had sperm count less than 20 million were taken for bacteriological analysis and cultured onto Nutrient Agar, Blood Agar, and Mac-Conkey Agar at 37°C for 24 h. After gram staining, differential tests were used for detection of bacterial species in accordance with Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1978). The positive seminoculture culture was confirmed against the number of bacterial colonies was $\geq 10^4$CFU ml^{-1}for Gram positive cocci and $\geq 10^5$CFU ml^{-1}for Gram negative rods (Enwuru *et al*., 2016). Culture for strict anaerobes was not done.

Antibiotic Sensitivity Test

The positive cultures were tested for their antibiotic sensitivity pattern using Kirby-Bauer disk diffusion method according to CLSI recommendations. The commercial antibiotics applied and their concentrations per disc were as follows: Gentamycin (10mcg), Tobramycin (10mcg), Cefazolin (30mcg), Ampicillin (10mcg), Meropenem (10mcg), Amikacin (30mcg), Imipenem (10mcg), Ampicillin + Sulbactem (10mcg), Co-trimozole (25mcg), Cefuroxime (30mcg), Piperacillin +Tozobactum (30mcg), Erythromycin (15mcg), Amoxycillin + Culvulanic acid (30mcg), Azithromycin (15mcg), Levofloxacin (5mcg), Tetracycline (30mcg), Chloramphenicol (30mcg), Cefepime (30mcg), Clarithromycin (15mcg), Aztreonam (30mcg), Ceftriaxone (30mcg), Linezolid (30mcg), Penicillin G (10mcg), Cefotaxime (5mcg), Cefoxitin (30mcg), Ciprofloxacin (5mcg), Clindamycin (5mcg), Oxacillin (1mcg), Minocycline (30mcg), Vancomycin (30mcg) and Moxifloxacin (5mcg).

RESULTS

Among 70 semen samples tested, 44 men samples indicated the incidence of bacterial speices. Of these 70 samples, a total of 26 samples were excluded from this analytical study for one or more of the reasons enlisted in the exclusion criteria. Therefore, the final number of samples included in the study

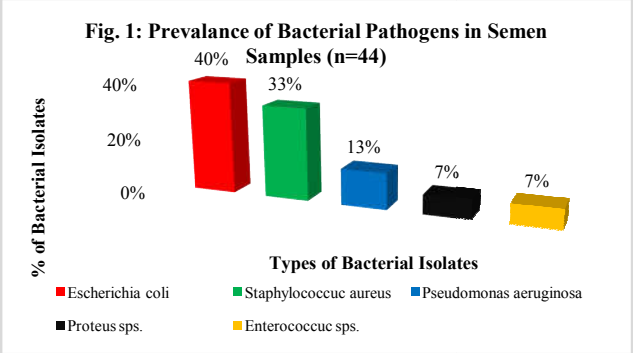
was 44. Spermioculture in the 44 patients yielded *Escherichia coli* (40 %), *Staphylococcus aureus* (33%), *Pseudomonas aeruginosa* (13%), *Proteus* sp. (7%), and *Enterococcus* sps. (7%) (Table 2 and Figure 1).

Table 1 Frequency of culture positive respect to age groups

S.No.	Age Groups (Years)	Number of Samples (n)	No. of Positive Cultures (%)	Pattern of Positive Cultures (n)	
				Monomicrobial	Polymicrobial
1.	25-35	25	19	19	-
2.	36-45	35	26	26	-
Mean Age: 40.5		70	44 (63%)	44	-

Table 2 Prevalence of Bacterial Pathogens in Semen samples (n =44)

S. No.	Organisms Isolated	Total Pathogens Isolated
1.	*Escherichia coli*	40%
2.	*Staphylococcus aureus*	33%
3.	*Pseudomonas aeruginosa*	13%
4.	*Proteus* sps.	7%
5.	*Enterococcus* sps.	7%



Each bacterial isolate was characterized as either obligate aerobic or facultative anaerobic. No strictly anaerobic isolate was detected. In this present study, 5 bacterial species (*E.coli, S. aureus, P.aeruginosa, Proteus* sp., *Enterococcus* sp.) while other authors have counted an additional 7 bacterial species in several countries (Elena et al., 2009; Chika *et al*., 2013; Bhatt *et al*., 2015). The majority of the contaminants identified in semen were Gram-negative bacteria, almost all belonging to the *Enterobacteriaceae* family. *E. coli* was the most frequently isolated contaminant of this study.

All bacteriological positive samples were evaluated for observing their volume, concentration, progressive motility by light microscopy according to CLSI guidelines. The results depicted in table 3 demonstrated that rate of fertility was significantly decreased in the men whose semen was contaminated with the examined pathogenic species. The mean sperm concentration in all bacterial contaminated samples were significantly lower than normal value as suggested by WHO i.e. 2×10^7 sperm/ml (Table 3).

Table 3 Qualitative analysis of bacterial contaminated semen samples

S.No	Particulars of samples	Bacterial Isolates (n=44)				
		E.coli	*S. aureus*	*P.aeruginosa*	*Proteus sp.*	*Enterococcus* sp.
1.	Volume (ml)	3	3.5	3.2	3.5	3.5
2.	P^H	9	8	8	8	8
2.	No. of sperm/mlx10^6	62.60	78.16	97.20	68.75	76.5
3.	Motility %	25	46.7	84	54	62.1
5.	Fertility Index	47.175	128.4717	261.2736	129.9375	166.27278

(Note: *Fertility Index = Volume (ml) x No. of Sperm (mlx10^6) x x motility % / 100)*

Among the gram negative bacterial isolates, *E.coli* was found 99.95 % sensitive to Meropenem followed by Gentamycin (99.95 ±0.05), Ceftriaxone (99.95 ±0.05), Piperacillin +Tazobactam (99.95 ±0.05), Meropenem 99.90± 0.10, Tobramycin (99.83 ±0.15), and Cefoxitin (99.97 ±0.02). *P.aeruginosa* was equally sensitive to Gentamycin, Tobramycin, Meropenem Amikacin, Imipenem, Piperacillin +Tozobactum, Cefepime, Ceftazidime, Aztreonam and Ciprofloxacin where as *Proteus* sp**.** showed sensitive to Gentamycin, Tobramycin, Cefazolin, Amikacin, Ampicillin + Sulbactem, Cefuroxime, Piperacillin +Tazobactam, Ceftazidime, and Ciprofloxacin. *Enterococcus* sp. exhibited sensitive to Tobramycin, Ampicillin, Tetracycline, and Linezolid, Penicillin G and Vancomycin.

In over all gram negative bacterial isolates, Gentamycin was found to be susceptible followed by Tobramycin, Amikacin, and Piperacillin + Tazobactam.

DISCUSSION

The present study revealed that 44 out of the 70 samples were found to be positive for the incidence of bacterial pathogens. The results of this study were shown that bacterial infection in semen might be related to this phenomenon in infertile couples. It was observed that sperm progressive motility, viability and normal morphology were significantly less than normal value in the semen samples infected with bacterial isolates. The microorganisms in semen can impair sperm function *via* agglutination of motile spermatozoa, impairment in acrosome reaction, morphology, and induction of apoptosis (Tremellen, 2008). Some bacteria may affect sperm function via their pili. Gram positive bacteria, like *Enterococcus*, can bind to mannose receptor in sperm surface and induce sperm damage (Villegas *et al*., 2005). *In vitro* studies using light microscopy illustrated that bacteria (*i.e. E. coli* and mycoplasmas) may lead to alteration in sperm morphology (Ali *et al*., 2013). They also demonstrated an immobilizing factor of *E. coli* can impair sperm motility and morphology. The inflammation in male genital tract impairs the secretory function of the male accessory glands and makes decline in semen volume (Marconi *et al*., 2009).

E. coli was identified as predominant bacteria comparatively others in 55% of samples which is similar to previous report of Ali *et al*. (2013). The reduction of sperm motility by *E. coli* could be due to depletion of adenosine triphosphate as documented by De Lamirande and Gagnon (1991). Seminal infection may act as the reservoir of infection and could cause urinary tract infection.

E. coli induces an agglutinating effect on 40-75% of motile sperm as reported in humans by Monga and Roberts (1994). Bacterial contamination overgrowth yields worse semen quality and longevity (Aurich and Spergser, 2007). The clinical importance of anaerobic microorganisms in semen is still remains a controversial issue. However, the possible role of anaerobic bacteria on semen quality and fertility potential needs to be further elucidated.

It was noted that more of the samples with abnormal morphology have yielded bacterial growth compared to those with normal forms. This agrees with the conclusion by Berger *et al* (1984) that bacterial infection causes deterioration of spermatogenesis. This also agrees with the findings of Chika *et al.* (2013).

Table 4 Antibiotic Susceptibility Pattern of Total Microbial Isolates of Semen Samples

Commercial Antibiotics used	Antimicrobial Susceptibility Pattern (%) (Mean ±SD value)									
	Escherichia coli (n=6)		*S.aureus* (n=5)		*P.aeruginosa* (n=2)		*Proteus sp.* (n=1)		*Enterococcus* sp. (n=1)	
	S	R	S	R	S	R	S	R	S	R
Gentamycin	99.95 ±0.05	0	99.03±0.05	0	99.90± 0.12	0	99.94 ±0.05	0	99.96 ±0.05	0
Tobramycin	99.83 ±0.15	0	**	**	99.98 ±0.04	0	99.95± 0.04	0	**	**
Cefazolin	67.10± 0.10	32.9± 0.11	**	**	**	**	99.90± 0.12	0	**	**
Ampicillin	20.80 ±0.10	79.15± 0.05	**	**	**	**	99.98 ±0.04	0	99.97 ±0.02	0
Meropenum	99.90± 0.10	0	**	**	99.90 ±0.16	0	0	99.65 ±0.56	**	**
Amikacin	99.96 ±0.04	0	**	**	99.98 ±0.04	0	99.69 ±0.27	0	**	**
Imipenum	99.93± 0.08	0	**	**	93.96± 0.22	0	0	99.93± 0.06	**	**
Ampicillin + Sulbactum	83.10± 0.10	16.09 ±0.14	**	**	**	**	99.69 ±0.27	0	**	**
Co-trimozole	60.70 ±0.10	39.30± 0.13	99.83 ±0.15	0	**	**	**	**	**	**
Cefuroxime	33.17 ±0.06	66.70 ±0.16	**	**	**	**	99.03±0.05	0	**	**
Piperacillin +Tozobactum	99.95 ±0.05	0	**	**	99.73± 0.26	0	99.83 ±0.15	0	**	**
Erythromycin	**	**	99.87 ±0.13	0	**	**	**	**	**	**
Amoxycillin + Culvulanic acid	0	99.83 ±0.15	**	**	**	**	0	99.69 ±0.27	**	**
Azithromycin	**	**	80.03±0.25	20.03±0.15	**	**	**	**	**	**
Levofloxin	50.40± 0.04	49.60 ±0.11	99.83 ±0.15	0	0	99.03±0.05	**	**	**	**
Tetracycline	74.38± 0.08	25.60± 0.09	50.30± 0.04	49.70 ±0.11	**	**	0	99.79 ±0.17	99.98± 0.01	0
Chloramphenicol	74.28± 0.08	25.70± 0.09	99.93± 0.06	0	**	**	**	**	**	**
Cefepime	79.15± 0.05	20.80 ±0.10	**	**	99.89± 0.16	0	0	99.93± 0.06	**	**
Clarithromycin	**	**	80.03±0.27	20.03±0.15	**	**	**	**	**	**
Ceftazidime	99.95 ±0.05	0	**	**	99.95± 0.04	0	99.94± 0.05	0	**	**
Aztreonam	50.40± 0.04	49.60 ±0.11	**	**	99.89± 0.16	0	0	99.68 ±0.23	**	**
Ceftriaxone	99.95 ±0.05	0	**	**	**	**	99.97 ±0.02	0	**	**
Linezolid	**	**	99.89± 0.16	0	**	**	**	**	99.98± 0.02	0
Penicillin G	**	**	50.27± 0.04	49.73 ±0.11	**	**	**	**	99.97 ±0.02	0
Cefotaxime	66.70 ±0.16	33.17 ±0.06	**	**	**	**	0	99.58 ±0.23	**	**
Cefoxitin	99.97 ±0.02	0	66.70 ±0.16	33.17 ±0.06	**	**	0	99.88 ±0.13	**	**
Ciprofloxacin	66.70 ±0.16	33.17 ±0.06	99.89± 0.12	0	50.27± 0.04	49.73 ±0.11	0	99.34 ±0.73	**	**
Clindamycin	**	**	99.99± 0.26	0	**	**	**	**	**	**
Oxacillin	**	**	99.94± 0.06	0	**	**	**	**	**	**
Minocycline	**	**	80.03±0.27	20.03±0.15	**	**	**	**	**	**
Vancomycin	**	**	99.93± 0.06	0	**	**	**	**	99.89± 0.12	0
Moxifloxacin	**	**	99.95 ±0.05	0	**	**	**	**	**	**

**: Not Applicable; S: Sensitive; R: Resistance

It is essential to understand that bacteria may get access to the seminal tract through the urinary tract. This infected seminal tract (the prostate) which may be a reservoir of infection to the bladder due to its inaccessible by many antibiotics. This implies that chronic urinary tract infection is to be expected in some of the patients with significant seminal tract infection.

CONCLUSIONS

The results obtained in the present study attributed that male semen has a consistent microbiological content. The current study suggested that comprehensive bacteriological investigations with subsequent antibiotic sensitivity assay and antibiotic therapy would be highly essential before admission of infertile men for infertility treatment, particularly in recurrent pregnancy loss cases.

Conflict of interest statement

We declare that we have no conflict of interest.

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