The presence of bacteria species in semen and sperm quality

Elena Moretti · Serena Capitani · Natale Figura ·
Andrea Pammolli · Maria Grazia Federico ·
Valentina Giannerini · Giulia Collodel

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Abstract

Purpose To verify the prevalence of semen bacterial contamination and whether the contamination could decrease sperm quality.

Methods Spermogram, semen culture, and sperm transmission electron microscopy (TEM) analysis were performed. TEM data were elaborated using a mathematical formula that calculates a fertility index (FI)—able to define patients as fertile or infertile—and the percentage of sperm apoptosis, immaturity and necrosis. We aligned the amino acid sequence of beta-tubulin with protein of the most frequent species isolated from semen.

Results Patients were divided according to the contaminating species; in each group, we observed fertile individuals, in whom the semen quality was similar to that of controls and infertile men whose sperm quality was significantly

decreased, in terms of motility, FI, apoptosis and necrosis. Partial homology between β -tubulin and bacterial proteins was observed.

Conclusion Sperm bacterial contamination is quite frequent and could contribute to the deterioration of the sperm quality of infertile men.

Keywords Bacteria · Semen quality · Spermatozoa · TEM

Introduction

Infections of the male genitourinary tract account for up to 15% of cases of male infertility [1]. Acute and chronic infections and consequent inflammation in the male reproductive system may compromise the sperm cell function and the whole spermatogenic process [2–4], causing qualitative and quantitative sperm alterations. Recent studies have shown that the simple presence of bacteria in semen samples may compromise the sperm quality. The bacteria responsible for semen contaminations generally originate from the urinary tract of patients or can be transmitted by the partner via sexual intercourse [5].

The most frequently isolated microorganism in male patients with genital tract infections or semen contamination is *Escherichia coli*. The negative influence of this species on sperm quality is partially due to its effect on motility [6] and to the impaired acrosomal function, as demonstrated at the ultrastructural level by Diemer et al. [7].

The influence of gram-positive uropathogenic bacteria on sperm morphology and function has been poorly investigated until now. Mehta et al. [8] reported that aerobic cocci are present in about 50% of semen samples of male partners in infertile couples. *Enterococcus faecalis* was isolated from 53% of patients, micrococci from 20% and

Capsule the presence of bacteria in semen may decrease sperm motility and increase apoptosis and necrosis particularly in infertile men.

E. Moretti · M. G. Federico · V. Giannerini · G. Collodel (✉)
Department of Biomedical Sciences, Biology Section,
Interdepartmental Centre for Research and Therapy
of Male Infertility, University of Siena,
Policlinico Le Scotte, Viale Bracci, 14,
53100 Siena, Italy
e-mail: collodel@unisi.it

S. Capitani · A. Pammolli
Department of Physiopathology,
Experimental Medicine and Public Health, University of Siena,
Siena, Italy

N. Figura
Department of Internal Medicine, Endocrine-Metabolic Sciences
and Biochemistry, University of Siena,
Siena, Italy

alpha-haemolytic streptococci from 16% of the infected samples. Increased prevalence of genital tract infections caused by *E. faecalis* is associated with compromised semen quality in terms of sperm concentration and morphology. The presence of micrococci and alpha-haemolytic streptococci does not appear to exert any detrimental effect on sperm quality.

Although no significant depressor effect of enterococci on sperm motility was observed, some researchers described, in an *in vitro* study, a negative influence on membrane integrity of human sperm head, neck and mid-piece [9], probably mediated by hemolysin, a well-known virulence factor of enterococci.

Genital ureaplasmas and mycoplasmas may colonize male urethra and contaminate the semen during ejaculation. However, these microorganisms and particularly *Ureaplasma urealyticum* are potentially pathogenic species playing an etiologic role in both genital infections and male infertility [10]. *U. urealyticum*, one of the most frequent causes of the male infertility [11], due to its ability to reduce semen quality and the fertilizing potential of sperm, negatively influences the sperm motility, density and morphology and reduces the oxidoreductive potential of the ejaculate, which makes sperm more vulnerable to peroxidative damage.

The cause-effect relationship between bacterial infections and semen contamination and male infertility is still being debated. To complicate the problem even more, the presence of bacteria in semen samples of infertile men has a similar prevalence to that observed in fertile males [12]. The clinical significance of bacteria in semen is still unclear.

The aim of this study was to investigate the semen quality in the presence of different bacterial species. Semen samples were processed for bacteriological analysis and examined to evaluate sperm concentration and motility. The morphological sperm characteristics were studied by transmission electron microscopy (TEM). TEM data were elaborated with a mathematical formula used in our laboratory and validated by a large number of papers that we have published over the past 10 years. This method enables the identification of fertile and infertile individuals and the percentages of sperm apoptosis, necrosis and immaturity. Finally we aligned the aminoacid sequence of β -tubulin, a major constituent of sperm flagellum, with those of proteins expressed by the most frequent agents of sperm contamination. Our purpose was to verify whether a phenomenon of antigenic mimicry may influence the sperm motility. Should a linear aminoacid homology among bacterial and sperm proteins really exist, the immune response against bacterial infection could also react with the human cells with possible detrimental consequences.

Materials and methods

Patient selection

Between January 1998 and December 2006, 1256 male individuals were referred to the Interdepartmental Centre for Research and Therapy of Male Infertility for semen analysis. The primary reason to seek counselling in our Centre was fertility disorder. The median duration of infertility was 3.75 years (range 3–7 years) of unprotected intercourse without conception, however we also analysed semen samples from men who wanted to check their fertility status.

Clinical and instrumental examinations of genital tract, lymphocyte karyotype analysis, hormonal levels evaluation, spermogram, TEM sperm analysis and culture for identification of the most common bacteria that colonize the male reproductive tract were routinely performed for each patient.

We applied the following exclusion criteria: altered hormonal levels, anatomical problems, such as varicocele and cryptorchidism, altered lymphocyte karyotype, a previous or ongoing treatment for fertility disorders and the presence of sperm defects of supposed genetic origin, *i.e.* characterised by an identical and specific alteration affecting the vast majority of the sperm population. To avoid misinterpretation of results, we excluded cases in which the spermoculture yielded two or more bacterial species and those with a non significant bacterial colony count, except for *U. urealyticum*, whose simple presence indicated remarkable contamination.

Semen samples from twenty men of proven fertility (aged 22 to 35 years) with a normal karyotype, without anatomical problems or infections, were used as controls. These fertile men had fathered one or more children during the previous 3 years.

Patients and controls provided a written informed consent before inclusion in this study.

Semen analysis

Semen collection

Semen samples were collected by masturbation after 3–5 days of sexual abstinence. Patients were asked to urinate and wash the hands, penis and scrotum before ejaculation to avoid possible contamination from the urine or external genitalia.

Light and electron microscopy

Samples were examined after liquefaction for 30 min at 37°C. Volume, pH, concentration and motility were evaluated

according to World Health Organization guidelines (WHO, [13]).

Leukocytes were identified by peroxidase stain; WHO [13] considered normal a leukocytes concentration $\leq 10^6$ cells/ml.

For the electron microscopy, sperm samples were fixed in cold Karnovsky fixative and maintained at 4°C for 2 h. Fixed semen samples were washed in 0.1 mol/L cacodylate buffer (pH 7.2) for 12 h, postfixed in 1% buffered osmium tetroxide at 4°C for 1 h, then dehydrated and embedded in Epon Araldite. Ultrathin sections were cut with a Superno-va ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate and lead citrate and then observed and photographed with a Philips CM10 transmission electron microscope (TEM; Philips Scientifics, Eindhoven, The Netherlands).

For each sample, three hundred ultra-thin sperm sections were analysed. Major submicroscopic characteristics were recorded by trained examiners who were blind to the experiment, applying the same evaluation criteria. TEM data were elaborated using the mathematical formula by Baccetti et al. [14], based on the Bayesian technique. This formula considers 16 selected submicroscopic characteristics of sperm organelles on the basis of which it is possible to define the sperm function and calculate the number of spermatozoa free from structural defects (fertility index, FI). The lowest number of spermatozoa free from defects, conferring normal fertility, was two million *ca.* per ejaculate, by the application of the formula for TEM data obtained with sperm from men of proven fertility [14, 15].

This formula was able to quantify the percentages of the three main sperm pathologies: immaturity, necrosis and apoptosis [15]. The characteristic features of immaturity were: altered acrosomes; misshaped, round or elliptical nuclei with uncondensed chromatin and the presence of cytoplasmic droplets. Marginated chromatin, cytoplasmatic translucent vacuoles and badly assembled mitochondria were the typical ultrastructural markers of apoptosis, whereas spermatozoa with broken plasma membrane, reacted or absent acrosome, misshaped nuclei with disrupted chromatin, poor axonemal and periaxonemal cytoskeletal structures were affected by necrosis.

Bacteriological analysis

Samples were seeded using a calibrated loop on agar plates, which were incubated overnight at 37°C in normal air with 5% CO₂. The microorganisms were identified by gram stain, oxidase, catalase and other biochemical tests using Bio-Mérieux products (Bio-Mérieux, Florence, Italy). For the isolation of *U. urealyticum*, the urea-arginine broth (Bio-Mérieux) was used. Spermicocultures were considered positive when the number of colonies was $\geq 10^4$ CFU ml⁻¹ in case of gram positive cocci and $\geq 10^5$ CFU ml⁻¹ in case

of gram negative rods. As far as *U. urealyticum* is concerned, its simple presence indicated positivity. Culture for strict anaerobes was not carried out.

Alignment

The amino acid sequence of human β -tubulin was “blasted” in the protein databases at the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA; www.ncbi.nlm.nih.gov) [16], with proteins of *E. coli* (strain UTI89) and *M. morganii*, *U. urealyticum* and *E. faecalis*. Sequences longer than five amino acids were also included, even if the alignment was interrupted by one or two non-matching amino acids.

Statistical analysis

Analysis was performed using the StatGraphics Plus (Ver. 5.0) statistical package. Patients with positive spermicoculture were grouped according to the species isolated in semen sample. Semen parameters and sperm pathology scores in each group were expressed as percentages, medians, means and standard deviations (SD). Standardized skewedness (a measure of symmetry or more precisely, the lack of symmetry) and standardized kurtosis (a measure of whether the data are peaked or flat, related to a normal distribution) values were used to determine whether data were normally distributed; in this case the *t* test was carried out. If data were not normally distributed, the non parametric Mann Whitney W test was used. P values <0.05 were considered significant.

The difference in the prevalence of infertility in the various groups was examined by the chi-square test with the Yates correction or the Fisher's exact test.

Results

Out of the 1,256 patients examined, 417 men (33.2%) showed the presence of bacterial species in semen samples. A total of 171 patients were excluded from the study for one or more of the reasons listed in the exclusion criteria; thus, the final number of patients included in the study was 246.

Spermicoculture in the 246 patients yielded *E. faecalis* in 79 samples (32.1%, 9 men were azoospermic), *E. coli* in 50 samples (20.3%, 4 men were azoospermic), *Streptococcus agalactiae* in 33 samples (13.4%, none was azoospermic), *U. urealyticum* in 29 samples (11.8%, one man was azoospermic), *Staphylococcus epidermidis* in 24 samples (9.7%, none was azoospermic), *Streptococcus anginosus* in 23 specimens (9.3%, two men were azoospermic) and *Morganella morganii* in eight samples (3.2%, none was azoospermic).

In each group semen volume, sperm concentration, progressive motility and leukocyte concentration were evaluated according to WHO guidelines [13] and analysed by light microscopy (Table 1). The mean sperm concentration in each group was significantly lower than that determined in controls, even though it was always higher than the lowest concentration considered normal by WHO [13], i.e. 2×10^7 sperm/ml (Table 1). The progressive motility ratios were lower than that established by WHO guidelines ($a + b > 50\%$, rapid- $a > 25\% +$ slow- b) and significantly lower than that observed in controls, except for the samples positive for *S. agalactiae* and *S. anginosus* (Table 1). In the same groups sperm morphology was evaluated by TEM. Results were elaborated using the mathematical formula by Baccetti et al. [14], which calculates, in each sample, the FI that in a fertile sample is around two million, and the percentage of sperm pathologies, such as immaturity, apoptosis and necrosis [15].

TEM values related to the FI and the percentage of sperm pathologies observed in the different groups and in controls are reported in Table 1. The mean scores concerning sperm apoptosis and necrosis were generally significantly higher than those detected in the control group, whereas the immaturity score was not significantly different from that of controls. The means of FI score were significantly lower than those of fertile controls, however they were all $> 2 \times 10^6$ with the exception of the score related to the samples contaminated by *M. morganii* (Table 1).

Since our mathematical method enables the identification of fertile and infertile patients, we divided the men of each group with a FI $> 2 \times 10^6$ (fertile) from those with FI $< 2 \times 10^6$ (infertile) and compared the variables considered for each bacterial species. The results are reported in Table 2.

Enterococcus faecalis

Mathematically elaborated TEM analysis revealed that 54 out of the 70 patients (77.1%) were infertile, whereas 16 patients (22.9%) were fertile. The differences of the various parameters between infertile and fertile individuals and controls were significant, except for sperm volume (Table 2), which suggests a poor semen quality in the former group. The comparison between data from fertile men and from controls did not reach statistical significance (Table 2).

Escherichia coli

TEM analysis highlighted that 31 out of 46 individuals (67.4%) were infertile and 15 men were fertile (32.6%). Sperm quality observed in infertile patients was significantly poorer than that detected in fertile individuals, except for the apoptotic score, and than that of control except for immaturity and volume. Necrosis was the only parameter

Table 1 Means $\pm$ standard deviations (SD), medians of considered variables in each group of patients with contaminated sperm samples

Characteristics	E. faecalis (70)	E. coli (46)	S. agalactiae (33)	U. urealyticum (28)	S. epidermidis (24)	S. anginosus (21)	M. morganii (8)	Controls (20)
Volume (ml)	3.7 $\pm$ 1.9 3.2	3.7 $\pm$ 1.6 3.3	3.0 $\pm$ 1.3 3.0	3.8 $\pm$ 1.7 3.6	4.2 $\pm$ 1.9 4.1	3.3 $\pm$ 1.2 3.0	3.3 $\pm$ 2.4 3.2	3.7 $\pm$ 1.2 3.7
Number sperm /ml $\times 10^6$	81.7 $\pm$ 97.6 52.6***	75.0 $\pm$ 85.9 41***	120.7 $\pm$ 106.6 110*	112.3 $\pm$ 155.8 38*	96.7 $\pm$ 126.8 79.2**	84.9 $\pm$ 136.3 36***	82.3 $\pm$ 99.3 31.5*	190.9 $\pm$ 102.6 162
Motility %	30.6 $\pm$ 16.8 29**	31.9 $\pm$ 15.6 30.5**	37.9 $\pm$ 17.7 39	24.2 $\pm$ 16.2 24***	27.7 $\pm$ 19.1 28**	35.2 $\pm$ 15.1 39	18.4 $\pm$ 14.3 22*	46.4 $\pm$ 12.5 51
Fertility Index	3,431,119 $\pm$ 8,690,042	2,710,722 $\pm$ 4,779,520	15,245,498 $\pm$ 34,864,969	3,748,949 $\pm$ 12,373,016	3,079,603 $\pm$ 7,697,472	2,961,707 $\pm$ 4,398,247	151,606 $\pm$ 225,012	17,743,046 $\pm$ 19,178,712
Apoptosis %	227,413*** 8.8 $\pm$ 7.2	387,707*** 10.5 $\pm$ 9.1	2,026,757* 6.8 $\pm$ 5.6	156,378*** 9.2 $\pm$ 6.5	432,263*** 2.3 $\pm$ 5.7	713,581** 7.9 $\pm$ 4.6	38,303*** 12.1 $\pm$ 8.4	4,882,415 4.1 $\pm$ 3.5
Necrosis%	7.4* 44.9 $\pm$ 19.5	8.2* 39.5 $\pm$ 16.1	4.7 40.4 $\pm$ 17.6	7.3* 38.9 $\pm$ 12.4	7.4* 37.4 $\pm$ 11.6	7.1** 41.6 $\pm$ 14.5	10.5* 52.6 $\pm$ 21.1	3.3 21.6 $\pm$ 14.9
Immaturity%	45.1*** 66.2 $\pm$ 14.0	37.2** 67.0 $\pm$ 11.5	38.2** 57.8 $\pm$ 11.1	35.4** 72.3 $\pm$ 17.2	36.0** 66.1 $\pm$ 15.2	43.7* 59.8 $\pm$ 15.3	58.1* 61.2 $\pm$ 16.0	21.8 59.6 $\pm$ 17.7
	68.0	68.9	58.9	76.1	65.3	63.8	68.9	58.9

We compared the different variables in each group with those of the control group (20 fertile individuals). The number of patients considered for each group is reported in parentheses.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 2 Means±Standard Deviation (SD), *medians* of variables in each group of patients with positive spermiculture, divided in fertile and infertile according to fertility index

Bacterial species	Patients	Volume (ml)	Number sperm / ml × 10 ⁶	Motility %	Fertility Index	Apoptosis %	Necrosis%	Immaturity %
<i>E. faecalis</i>	Fertile (16)	3.8±2.2	173.78±37.6	49.6±7.7	13,946,133±13,916,501	4.5±3.9	35.1±17.0	52.06±16.38
	Infertile (54)	3.2	127 ³	52.5 ³	9,920,058 ³	4.1 ²	32.0 ¹	56.3 ³
		3.6±1.8	54.5±7.0	24.9±14.8	315,560±484,020	10.1±7.4	47.8±19.4	70.3±10.0
<i>E. coli</i>	Fertile (15)	3.1	39.7 ^c	24 ^c	96,822 ^c	9.0 ^b	49.1 ^c	70.6 ^a
	Infertile (31)	4.7±1.6	128.3±113.9	42.9±14.2	7,662,719±5,833,433	6.9±7.8	30.6±8.2	60.05±9.93
		4.5 ²	110 ²	44 ³	7,071,831 ³	4.0	33.1 ^{1,*}	58.51 ²
<i>S. agalactiae</i>	Fertile (17)	3.3±1.4	49.1±53.8	26.5±13.4	314,594±457,712	12.2±9.3	43.8±17.3	70.38±10.78
	Infertile (16)	3	34.5 ^c	25 ^c	170,955 ^c	10.7 ^b	39.3 ^c	69.01
		3.0±1.0	181.853±103.4	42.2±1	29,125,353±44,818,916	4.3±3.2	31.8±11.6	53.8±12.14
<i>U. urealyticum</i>	Fertile (4)	2.97±1.55	55.7±63.8	29.1±16.4	11,478,242 ³	4.2 ²	30.6 ²	53.86 ¹
	Infertile (24)	2.6	38.2 ^c	24.5 ^b	498,154±486,395	9.3±6.5	48.9±18.7	61.7±8.5
		4.9±2.1	268.7±325.2	45.5±10	416,626 ^c	9.2 ^a	48.0 ^c	63.5
<i>S. epidermidis</i>	Fertile (6)	4	166.375	47 ²	23,457,697±27,747,190	8.9	30.1±2.7	54.7±20.3
	Infertile (18)	3.6±1.7	86.2±98.7	20.6±14.2	11,813,052 ³	9.2±6.5	30.4	45.2
		3.1	37.5 ^b	17.0 ^c	464,158±693,336	7.3 ^a	38.0±13.0	75.2±15.2
<i>S. anginosus</i>	Fertile (7)	4.4±2.0	207.9±208.3	44.5±7.8	100,224 ^c	7.3 ^a	38.5 ^b	77.6 ^a
	Infertile (14)	4.4	142.5 ¹	43 ¹	10,866,667±13,179,562	4.0±3.6	30±11.9	55.0±10.8
		4.1±2.0	59.7±56.4	22.0±18.0	5,503,227 ³	3.5 ¹	28.3	55.3
<i>M. morganii</i>	Fertile (1)	4.1	41 ^c	17 ^c	477,249±626,316	9.7±5.7	39.8±10.8	68.9±14.8
	Infertile (7)	3.2±1.0	173.2±210.4	35.3±17.2	224,008 ^c	8.5 ^b	41.7 ^c	70.4
		3.5	75 ²	38	8,026,671±4,338,764	6.7±4.5	32.3±11.0	54.63±13.17
Controls	Fertile (20)	3.3±1.2	40.7±42.8	31.2±16.1	6,025,031 ²	4.8	32.7 ¹	48.73
	Infertile (1)	3	27.9 ^c	42	429,225±593,348	8.5±4.7	46.2±14.1	62.4±16.1
		/	/	/	81,609 ^c	7.3	44.9 ^c	64.0
	Fertile (1)	3.3±2.4	82.3±99.3	18.4±14.3	/	/	/	/
	Infertile (7)	3.2	31.5 ¹	22 ³	151,607±225,012	12.7±8.4	52.6±21.1	61.24±15.95
		3.7±1.2	190.9±102.6	46.4±12.5	38,303 ³	10.5 ¹	58.0 ²	68.88
	Fertile (20)	3.7	162	51	17,743,046±19,178,712	4.1±3.5	21.7±14.9	59.62±17.74
					4,882,415	3.3	21.8	58.9

The following values were compared: infertile patients vs fertile patients

¹ $P<0.05$, ² $P<0.01$, ³ $P<0.001$; fertile patients vs controls * $P<0.05$, ** $P<0.01$, *** $P<0.001$; infertile patients vs controls ^a $P<0.05$, ^b $P<0.01$, ^c $P<0.001$. The number of patients considered for each group is reported in parentheses

whose score was significantly increased in contaminated fertile subjects compared to controls.

Streptococcus agalactiae

In this group, 16 out of 33 individuals (48.5%) were infertile and 17 men were fertile (51.5%). Infertile patients showed significantly lower sperm quality than that observed in fertile men, except for the volume, and than that observed in controls, except for the volume and the immaturity score. Fertile patients and controls showed a similar semen quality (Table 2).

Ureaplasma urealyticum

In this group, 24 out of 28 patients (85.7%) were infertile and four fertile (14.3%). The motility score and FI detected in infertile patients were significantly lower than those observed in fertile men. Infertile patients showed a significantly lower sperm quality than that of controls, except for the sperm volume (Table 2). Fertile patients and controls showed similar sperm parameters.

Staphylococcus epidermidis

TEM elaboration enables to individuate 18 infertile men out of 24 individuals (75%) and six fertile men (25%). We observed that the sperm concentration, the percentage of progressive motility and FI scores were significantly decreased in infertile patients, compared to fertile individuals, whereas the apoptotic score value was increased. All values of the different parameters, with the exception of volume and immaturity, were significantly poorer in infertile patients than those observed in control subjects. No statistical difference was found between the scores of sperm parameters of fertile men and controls.

Streptococcus anginosus

In this group, 14 out of 21 individuals (66.6%) were infertile and seven were fertile (33.4%). The sperm quality in infertile patients was significantly lower than that observed in fertile men particularly regarding sperm concentration, FI and necrosis. Fertile men and controls had similar scores of sperm parameters, whereas the infertile patients showed a significant decrement in sperm concentration and FI scores, concomitant with an increase of necrosis compared with control values (Table 2).

Morganella morganii

Quantitative TEM analysis indicated that 7 out of 8 individuals (87.5%) were infertile, with only one case (12.5%)

being fertile. The semen quality of the infertile patients was significantly poorer than that observed in controls; in particular, the sperm concentration, motility percentage and FI scores were significantly decreased, whereas apoptosis and necrosis scores were significantly increased (Table 2).

Finally, in order to verify whether the sperm contamination by determined organisms could possibly be associated with an increased prevalence of infertile patients, we compared the number of infertile men, present in each group, with those found in the other groups. The statistical calculation showed that the number of infertile patients in the groups with *U. urealyticum* and *E. faecalis* contamination was significantly higher than that with *S. agalactiae* ($P=0.005$ and $P=0.007$, respectively). The total of infertile patients in *M. morganii* group was significantly higher than those in *S. epidermidis* ($P=0.003$) and *S. anginosus* ($P=0.012$).

Leukocytospermia

Fifteen percent of patients showed the presence of leukocytospermia ($>10^6$ leukocytes/ml of semen). Patients with leukocytospermia were present in all groups of infertile and fertile contaminated patients. The control group, encompassing men of proven fertility, did not show leukocytospermia.

Alignment

Bacterial proteins that show the most significant homologies with the human β -tubulin are reported in Table 3. In Fig. 1, we show the aminoacid sequences of such proteins that align with tubulin.

Discussion

Many studies have examined the impact of genital tract infections and bacterial semen contamination in male fertility; however, the putative detrimental effect of bacteria on the sperm quality is still controversial [17]. Microorganisms can affect the male reproductive function directly, causing the agglutination of motile sperm, reducing the ability of acrosome reaction and causing alterations in cell morphology—and indirectly, through the production of reactive oxygen species generated by the inflammatory response to the infection [18]. However, there is not complete agreement on the detrimental role of the presence of bacteria in the semen. Sanocka-Maciejewska et al. [4] have reported that the bacteria most frequently isolated from the genitourinary tracts of men have no effect on semen quality in normozoospermic males; however, in infertile patients with pathological semen parameters

Fig. 1 The alignments between human β -tubulin and amino acid sequences of proteins expressed by different bacterial species are shown. When two proteins are made up by at least five identical (or with the same chemical properties) aminoacids in sequence they are considered homologue. *Query*: tubulin amino acid sequence; *Subject*: amino acid sequence of the bacterial protein; the *numbers* in the sequence represent the initial and final positions of amino acid; + indicates that the aligned aminoacids are different, although, from an antigenic point of view, they are similar; *identities* represent the percentage of identical aminoacids in the two compared sequences; *positives* indicate the percentage of aminoacids that share the same behaviour from an antigenic point of view; *gaps* mean the number of intervals lacking linear homology between two homologue sequences

[GENE ID: 3992625 ftsZ](#) | cell division protein FtsZ [Escherichia coli UTI89]
Identities = 18/81 (22%), Positives = 35/81 (43%), Gaps = 10/81 (12%)

```

Query  171  TVVEPYN-----ATLSVHQLVENTDETYCIDNEALYDICFRTLRLTPTYGDLNHL  221
          V +P+N          A  + +L ++ D    I N+ L  +  R + L  +G N +
Sbjct  130  VVTKPFNFEGKKRMAFAEQGITELSKHVDSLITIPNDKLLKVLGRGISLLD-AFGAANDV  188

Query  222  VSGTMECVTTCLRFPGQLNAD  242
          + G ++ +    + PG +N D
Sbjct  189  LKGAVQGI AELITRPLMNVD  209



pir|A82895 S-adenosylmethionine synthetase UU412 [imported] - Ureaplasma urealyticum. Length=376


Identities = 22/74 (29%), Positives = 37/74 (50%), Gaps = 14/74 (18%)

Query  328  NKNSSYFVEWIPNNVKTAV----CDI-----PPRGLKMAV-TFIGNSTAIQELFKRIS  375
          +++SYF WI NV A    C+I          P+ + M V TF N    +++F+ I+
Sbjct  264  DRSASYFARWIAKNVVAAKLAKQCEIQLAFAIGEPQPVAMFVNTFKTNLVEKKIFEAIN  323

Query  376  EQFTAMFRRKAFLH  389
          + F    F K F++
Sbjct  324  KSFN--FDIKTFIN  335



GENE ID: 1200064 EF1162 | helicase, putative [Enterococcus faecalis V583] (10 or fewer PubMed links)


Identities = 25/83 (30%), Positives = 35/83 (42%), Gaps = 11/83 (13%)

Query  135  GTGSGMGTLL--ISKIREEYPDRIMNTFSVVPSPKVSdTVEPYNATLSVHQLVENTDET  192
          GTG M TL  + I EE +RI  +  + +V++ V+          L E  +T
Sbjct  220  GTGKTMSTLFPAMKAIGEEGERIFYLTAKTITRQVAEDAVKA-----LSEKQAQT  270

Query  193  YCIDNEALYDICFRTLRLTPTY  215
          I A ICF T R TP +
Sbjct  271  KSITLTAKDKICFLTERNCTPEH  293



emb|CAF18337.1 | hypothetical protein [Morganella morganii subsp. morganii] Length=513


Identities = 12/37 (32%), Positives = 16/37 (43%), Gaps = 0/37 (0%)

Query  206  RTLRLTPTYGDLNHLVSGTMECVTTCLRFPGQLNAD  242
          R R+ PTY +LN L    + C+ G L D
Sbjct  208  RFHRVKAPTYDELNTLAHTLSHRIARCMKRGILERD  244
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bacteria usually determined a diminished antioxidant capacity of sperm. These findings have in part been confirmed in the present study, *i.e.*, the bacterial contamination of semen samples of fertile individuals did not compromise the sperm quality, while in infertile men, it is possible that bacteria further deteriorated the whole quality of the seminal plasma.

The presence of leukocytes in the semen often complicates the interpretation of results of sperm analyses and alterations of sperm parameters. In this study, in order to

avoid the additional factors that could increase sperm damage, such as the cellular inflammatory response [19], we enrolled only asymptomatic individuals who were referred to this centre for sperm analysis. Nevertheless, 15% of our patients had leukocytospermia. The prevalent opinion is that leukocytospermia is always concomitant with bacteriospermia; however the lack of leukocytospermia does not preclude the development of genitourinary symptoms of disease [20]. The fact that in the present study the prevalence of leukocytospermia was equally distributed

Table 3 Proteins of some bacterial species producing the most significant homology with human beta-tubulin are shown

Gene	Protein and species	(Bits)	E Value
ref YP_539145.1	Cell division protein FtsZ [<i>Escherichia coli</i> ...	30.8	0.21
pir A82895	S-adenosylmethionine synthetase UU412 [<i>U. urealyticum</i>]	27.3	2.0
ref NP_814892.1	Helicase, putative [<i>Enterococcus faecalis</i> V5...	26.6	3.6
emb CAF18337.1	Hypothetical protein [<i>Morganella morganii</i> subspecies...	23.9	0.48

in all groups analysed and was independent of bacterial species isolated from the samples reinforces our interpretation that the presence of white blood cells in the semen only had a negligible pathogenic importance.

Our findings show that the simple presence of bacteria might alter the sperm quality. The mean sperm concentration in the group of individuals positive for bacteria was significantly lower than that observed in controls; however, it was always $> 20 \times 10^6$ sperm/ml, value considered normal for WHO [13].

The negative influence of bacteria on sperm motility is well known [4, 19]. In our study, motility was significantly reduced in all groups except for in those with *S. agalactiae* and *S. anginosus*. This suggests that the bacterial flagella and pili, i.e. constant accessory structures of *E. coli* and *M. morganii*, could be an important determinant of pathogenicity. Recent studies have hypothesized that the mechanisms of sperm damage caused by bacteria passes through the expression of the adhesive properties of the flagella and pili to mannose receptors [21]. The fact that receptors to mannose have been demonstrated also at the surface of human spermatozoa [22] suggests that flagella and pili could play a considerable causative role in sperm damage. As far as gram positive organisms are concerned, the production of pili is not a characteristic present in all clones of the same species. Some studies [23, 24] have underlined the pathogenetic importance of such structures also in gram positive organisms, as they have shown that bacteria isolated from the site of infection are more likely to be piliated, while when they are simple bystanders they are not; this is the case of bacteria colonizing the urethral tract of human beings. These observations suggest that pili could possibly constitute a putative determinant of pathogenicity in gram positive cocci, too.

Sperm morphology is generally evaluated by light microscopy, although a deep analysis of subcellular sperm anomalies can only be performed by TEM. Applying the statistical mathematical formula to TEM data [14], FI, an indicator of sperm quality, was determined together with the percentage of sperm pathologies such as immaturity, apoptosis and necrosis. FI score was significantly reduced in the studied groups compared to controls; however, as observed for sperm concentration, the values of standard deviations indicated a wide variability in each group. To better clarify this issue we divided the patients into fertile and infertile following the FI score. Despite fertile individuals distributed in each group (20.2% in *E. faecalis* group, 30% in *E. coli*, 51.5% in *S. agalactiae*, 13.8% in *U. urealyticum*, 33.3% in *S. epidermidis*, 30.4% in *S. anginosus*, and 12.5% in *M. morganii* group), the statistical calculation showed that the number of infertile patients in the groups with *U. urealyticum* and *E. faecalis* contamination was significantly higher than that with *S. agalactiae*.

Similarly, *M. morganii* seems to damage the spermatozoa more than *S. epidermidis* and *S. anginosus*, even though the number of the cases was very small.

The characteristics of the contaminated semen samples from fertile patients were similar to those from men of proven fertility (controls), except for sperm necrosis in *E. coli* group. The observation that the presence of bacteria in semen samples did not compromise the sperm quality of fertile men could be explained by the fact that the bacterial colonization was possibly recent and the contact of bacteria with spermatozoa had not been long enough to result in damage. Alternatively, we can suppose that the semen of fertile individuals is capable of hampering the potential mechanisms by which bacteria may damage spermatozoa.

Regarding sperm pathologies, only necrosis and apoptosis were significantly increased in the groups with semen bacterial colonization compared to controls, whereas the frequency of immaturity, a pathology generally related to the presence of varicocele [25], was equally dispersed in the various groups. As previously reported, apoptosis and necrosis seem to have an important role in spermatogenic damage associated with semen bacterial colonization [26]. In an *in vitro* study performed by Villegas et al. [21], a single incubation with *E. faecalis*, *E. coli* and *S. aureus* induced apoptosis in human sperm with two possible, putative mechanisms: a direct cytotoxic activity of bacterial toxins and the contact with pili and flagella. It has also been demonstrated that *E. coli* can start the apoptotic process by activating several caspases, proteases responsible for mitochondrial changes, alterations in membrane symmetry, and DNA fragmentation [21, 27].

In the present study, *E. faecalis*, *E. coli*, *U. urealyticum* and *M. morganii* have been shown to exert a negative influence on the sperm quality of infertile males as a whole; similar findings have also been reported by other researchers [2, 6]. The possible mechanisms of sperm damage, in addition to adhesion by flagella and pili, include the production of toxins and metabolic products originating from bacterial proliferation. Some researches reported different pathogenetic mechanisms exerted by *U. urealyticum* upon sperm, such as the production of H_2O_2 , a source of hydroxide anion, and phospholipases A and C which may injure sperm membranes. In addition, the presence of *U. urealyticum* in semen samples may decrease the amount of microelements such as zinc and selenium, which are important to maintain the antioxidative defensive properties of such biological fluids [11, 19].

In conclusion, our data suggest that the presence of bacteria in semen samples may influence the sperm quality, mainly by the induction of apoptosis and necrosis, which may in part be responsible for the observed reduction of sperm motility. However, another possible explanation may reside in the putative existence of an antigenic mimicry

between some constituents of sperm flagella such as tubulin, one of the major component of axoneme, and bacterial proteins. Molecular mimicry of host structures with proteins encoded by microorganisms can have pathogenetic consequences; infection may induce antibodies and T cells to react against bacterial cell constituents, which can also recognize self components and immunomediated damage may follow. To verify such a possibility, we blasted the aminoacidic sequence of tubulin with proteins of *E. coli* (strain UTI89), *M. organii*, *U. urealyticum* and *E. faecalis* and we found significant linear homologies with proteins expressed by all these species (Fig.1). Human spermatozoa may therefore share epitopes with antigens of the most frequent species colonizing the genitourinary tract of man. In case of infection or contamination, these antigens may induce an antibody response, which could cross-react with the flagella of spermatozoa, thus reducing their motility.

In conclusion, we have shown that contamination of sperm samples by some species is more closely associated with infertility. However, this is only part of the problem. In this study, we only dealt with aerobic and facultative anaerobic bacteria. The clinical significance of strict anaerobes in sperm samples is a subject of dispute. Anaerobic bacteria are not routinely sought in sperm samples, because they are fastidious to cultivate and may be damaged by the contact with oxygen for the duration of transportation. In fact, the studies concerning the presence of anaerobes in semen samples are neither numerous nor conclusive; however, already in 1995, Eggert–Kruse et al. [28] reported that almost all the ejaculates they examined were positive for anaerobic microorganisms and potentially pathogenic species were found in 71% of men. It is noteworthy that, in a recent study, Kiessling et al. [29] detected and identified bacteria, including anaerobes, in the semen of men undergoing fertility evaluation, by sequencing polymerase chain reaction amplified rRNA gene. By this method they found a significant contamination ($\geq 2 \times 10^4$ bacteria/mL) with strict anaerobic microorganisms in a discrete proportion of cases. It is not clear, yet, whether the presence of anaerobes in the semen may influence fertility.

Future studies, also based on this molecular approach, should be addressed to this topic in order to provide new insights into the microorganisms coming into contact with sperm, helping to monitor the health of male genitourinary organs. In any case, a re-evaluation of sperm characteristics after patients have been treated, could confirm the role, if any, of bacterial presence in the determination of the sperm abnormalities observed.

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