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The limit of leucocytospermia from the microbiological viewpoint

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Summary. The aim of the study was to find out the correlation between white blood cell (WBC) counts in semen and quantitative composition of seminal microflora, and to establish the minimum WBC count associated with significant bacteriospermia. The research included 159 men with different WBC counts in their semen, 84 of them with chronic prostatitis/chronic pelvic pain syndrome. Semen samples were cultivated quantitatively for detecting anaerobic, microaerophilic and aerobic bacteria. Bryan-Leishman stained slides were used for detecting WBC in semen. Seminal fluid was colonized by eight different microorganisms, and the total count of microorganisms in semen ranged from 10^2 to 10^7 CFU ml^{-1}. A high frequency of anaerobic microorganisms was found. A positive correlation was observed between the WBC count and the number of different microorganisms, and also between the WBC count and the total count of microorganisms in semen sample. The receiver operating characteristic curve analysis demonstrated that the WHO-defined WBC cut-off point (1×10^6 WBC ml^{-1}) has very low sensitivity for discriminating between patients with and without significant bacteriospermia, as a more optimal sensitivity/specificity ratio appears at 0.2×10^6 WBC ml^{-1} of semen. The quantitative microbiological finding of semen in the patients of National Institute of Health (NIH) categories IIIa and IV was very similar, i.e. a high number of different microorganisms and a high total count of microorganisms. In the control group (without leucocytospermia and prostatitis symptoms) both parameters were significantly lower.

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Introduction

Seminal fluid is one of the most problematic specimens for microbiological studies. Interpretation of study results is complicated because of the presence of indigenous genital tract microflora and the absence of some evaluation criteria (Dimitrakov *et al.*, 2001). Seminal fluid cultures are not only necessary in the case of prostatitis, but also in the case of asymptomatic occurrence of white blood cells (WBC) in semen.

The prevalence and clinical significance of leucocytes (WBC) in semen is currently a matter of controversy. It is generally accepted that, it is an indicator of inflammation in the genital tract. Leucocytospermia occurs frequently (10–44%) in infertile patients (Wolff, 1995; Omu *et al.*, 1999; Stanislavov, 1999; Arata de Bellabarba *et al.*, 2000; Sharma *et al.*, 2001) and is associated with poor semen quality parameters (Fedder, 1996; Arata de Bellabarba *et al.*, 2000; Ludwig *et al.*, 2001). However, some investigators have failed to prove it (Tomlinson *et al.*, 1993), and Kiessling *et al.* (1995) have even hypothesized that male reproductive tract leucocytes may function in the elimination of abnormal spermatozoa from ejaculated semen. Yet, leucocytes in seminal fluid generate reactive oxygen species (ROS), known to be toxic to spermatozoa and impair male fertility (Wolff, 1995; Conte *et al.*, 1999; Pasqualotto *et al.*, 2000; Sharma *et al.*, 2001), and sperm damage by WBC can be mediated by proteases and cytokines (Wolff, 1995) or by enhanced T-helper 1 modulation (Omu *et al.*, 1999). The diagnosis of leucocytospermia is usually based on the WHO definition of 1×10^6 WBC ml^{-1} of semen (Wolff, 1998; Stanislavov, 1999; Zorn *et al.*, 2000; Sharma *et al.*, 2001). However, this number is comparatively high and many sub-leucocytospermic patients in whom

other signs of inflammation are present could be disregarded. Some authors have used the lower (Jedrzejczak *et al.*, 1996) or higher (Kiessling *et al.*, 1995) WBC count in semen for determining leucocytospermia.

Though leucocytospermia is related to inflammation in genital tract, as much as 50–80% of leucocytospermic semen samples are microbiologically 'negative' (Wolff, 1995; Cottell *et al.*, 2000) if routine laboratory methods are used. Usually little attention is paid to anaerobic bacteria as they are sensitive to transportation and culturing, and their differentiation is difficult, costly and time-consuming (Szöke *et al.*, 1998, 2000). However, as routine bacteriological analysis cannot reveal the complexity of seminal fluid microbiocenosis, advanced methods should be introduced.

The aims of this study were to clarify the relationship between WBC count in semen and quantitative microbiological data obtained from total microflora analysis and to establish the minimum WBC count associated with significant bacteriospermia.

Material and methods

Study group

The study included 159 men whose mean age was 32.3 years (median = 31 years, range 20–50 years), with 148 of them being married. The study was carried out between September 1999 and May 2001 in the Andrology Unit of Tartu University Clinicum. In 84 men (group I) the pain or discomfort in the pelvic area for at least 3 months had been the main symptom, as they had chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS). Their mean age was 34.2 years (median = 33 years, range 22–50 years). Fifty-six men consulted physicians because of infertility or for prophylactic purposes. They did not have complaints of chronic pelvic pain and were supposedly monogamous for at least 1 year (group II, mean age 29.4 years, median = 29 years, range 21–38 years). Nineteen men consulted physicians because of their partners' chronic gynaecological infections (group III, mean age 32.5 years, median = 30 years, range 20–47 years). All subjects were at least 18 years of age. Exclusion criteria were as suggested in the National Institute of Health (NIH) workshop on chronic prostatitis in Bethesda, MD, in 1995 (National Institutes of Health Summary Statement, 1995; Krieger *et al.*, 1999; Nickel *et al.*, 2001). None of the patients had received antimicrobial therapy in the last 3 months. All men consented to have additional microbiological studies of semen.

Semen samples were collected by patients following washing of the glans penis with soap and water and urinating. The samples were obtained by masturbation were ejaculated into a sterile collection tube. After ejaculation, semen was incubated at 37 °C for 25–45 min for liquefaction.

Cytological analysis

Semen smears were made for detecting WBC. The smears were air-dried, Bryan–Leishman stained and examined using oil immersion microscope by an experienced microscopist. The WBC concentration in semen was calculated using the known sperm concentration (as 10^6 ml^{-1}) according to the following formula:

$$[\text{WBC}] = \frac{\text{number of WBC counted}}{\text{number of sperm counted}} \times \text{semen sperm concentration}$$

One hundred round cells were counted twice and their mean value was registered.

Microbiological analyses

Semen samples were cultivated quantitatively for detecting anaerobic, microaerophilic and aerobic bacteria. The seedings were performed within 2 h of collection onto Wilkins–Chalgren medium (Oxoid, Unipath, Basingstoke, UK), Schaedler medium (Oxoid), *Gardnerella vaginalis* selective agar (Oxoid), MRS agar (Oxoid), freshly prepared blood agar and chocolate agar. Wilkins–Chalgren and Schaedler media were incubated in anaerobic glove box (Sheldon Manufacturing Inc. with a gas mixture: 5% H_2, 5% CO_2, 90% N_2) for 5–6 days. MRS medium, chocolate agar and *Gardnerella vaginalis* selective agar were incubated in a microaerophilic atmosphere (10% CO_2) for 72 h. Blood agar was incubated aerobically at 37 °C and inspected after 24 and 48 h.

Colonies with different morphology were Gram-stained and subjected to microscopy. The microorganisms were identified mostly up to the genus level. Standard methods were used for identification of enterobacteria and other Gram-negative bacteria (Balows *et al.*, 1991). A latex test (Oxoid) was employed for differentiation of *Staphylococcus aureus* and coagulase-negative staphylococci. Streptococci and enterococci were identified by the absence of catalase production and differentiated by the fermentation of esculine. Group B streptococci were identified using latex test (Oxoid). *Corynebacterium seminale* was differentiated by testing its beta-glycuronidase activity using MUG supplement (Oxoid) containing medium. *Gardnerella vaginalis*

was detected by its ability to grow on selective medium (Oxoid), its characteristic morphology and a negative catalase test. The anaerobes were identified up to the family and genus level by their growth on selective media, the colony and cellular morphology and Gram stain reaction. All anaerobic microorganisms were tested for absence of growth under aerobic and micro-aerophilic conditions.

The absence of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in these patients was confirmed by polymerase chain reaction using Amplicor *C. trachomatis/N. gonorrhoeae* test (Roche, Mannheim, Germany).

Statistical methods

Statistical analyses were performed using SIGMASTAT (Jandel Scientific) and R (The R Development Core Team) software. The following tests were employed: receiver operating characteristic (ROC) curve analysis (evaluation of different WBC levels), Mann–Whitney rank sum test (comparison of different study groups); Spearman rank order correlation (the counts of WBC and microorganisms).

Ethical considerations

Informed consent was obtained from the patients. The study was approved by the Institutional Review Board of University of Tartu.

Results

No sterile sample was found. The seminal fluid was colonized by one to eight different microorganisms, the total count of microorganisms in semen ranged from 10^2 to 10^7 CFU ml^{-1}. More than 20 groups of microorganisms were isolated (Fig. 1). A high frequency of anaerobic microorganisms was found: 73% in group I, 77% in group II, and 79% in group III.

Determining the limit of leucocytospermia

When all 159 men were analysed together, a positive correlation could be observed between the WBC count and the number of different microorganisms ($r = 0.182$, $P = 0.022$), and between the WBC count and the total count of microorganisms in the semen sample ($r = 0.146$, $P = 0.056$); however the latter was somewhat over the significance level.

For further analysis, a concentration of 10^4 microorganisms per millilitre of semen was used as the significant bacteriospermia level (Lewis *et al.*, 1981; Maier, 1983; Corradi *et al.*, 1992; Arakawa *et al.*, 1999). The ROC curve analysis (Fig. 2a) demonstrates that the WHO-defined WBC cut-off point (1×10^6 WBC ml^{-1}) has very low sensitivity (23%) for discriminating between patients with low ($<10^4$ CFU ml^{-1}) and high ($\geq 10^4$ CFU ml^{-1}) total count of microorganisms.

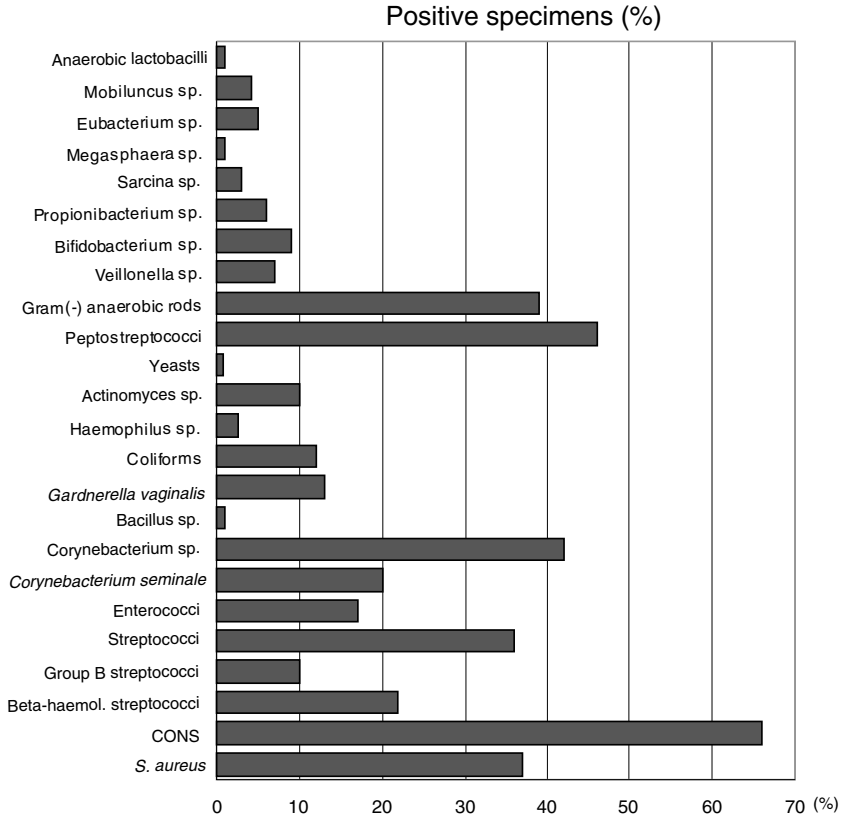

Figure 1. Occurrence of microorganisms in seminal fluid.

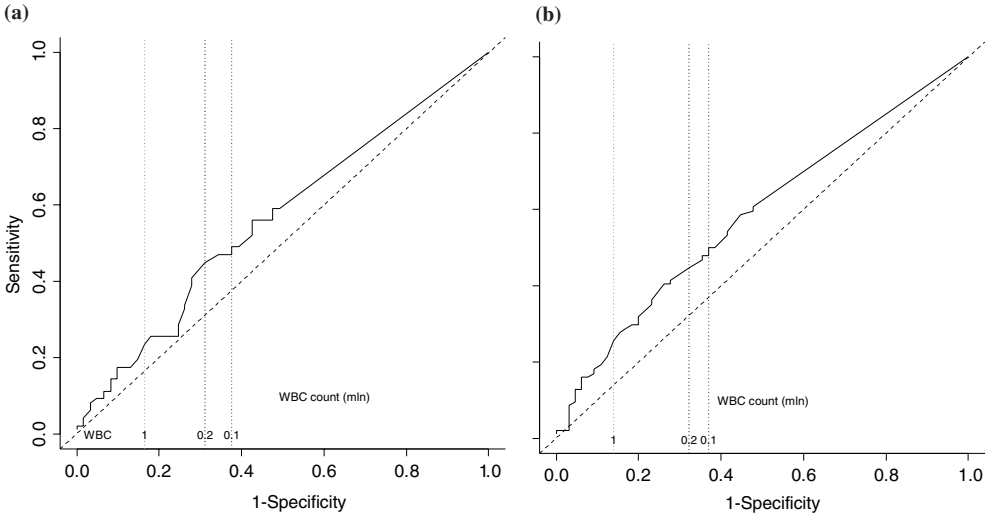

Figure 2. (a) The receiver operating characteristic (ROC) curve of white blood cell (WBC) levels in case of 104 microorganisms per millilitre of semen: the WHO-defined WBC cut-off point (1×10^6 WBC ml^{-1}) has very low sensitivity (23%) for discriminating between patients with low ($<10^4$ CFU ml^{-1}) and high ($\geq 10^4$ CFU ml^{-1}) total count of microorganisms. (b) The curve of WBC levels in the case of four different microorganisms in semen. The WHO-defined WBC cut-off point has very low sensitivity (25%) again. ROC curve analysis of WBC levels.

A good correlation was also found between the total count of microorganisms per millilitre of semen sample and the number of different microorganisms in the semen sample ($r = 0.601$, $P < 0.001$). In the patients with low total count of microorganisms ($n = 61$), the mean number of different microorganisms per semen sample was 3.05 (median = 3; SD = 1.371) while in the patients with high total count of microorganisms ($n = 98$), the mean number of different microorganisms per semen sample was 4.78 (median = 5; SD = 1.583). Therefore it is proposed that the number of different microorganisms in semen sample can be used as an additional parameter of bacteriospermia. Figure 2b shows the receiver operating characteristic curve of WBC levels in the case of four different microorganisms in semen. The WHO-defined WBC cut-off point has very low sensitivity (25%). The more optimal sensitivity/specificity ratio appears at 0.2×10^6WBC ml^{-1} of semen, both in respect of number of different microorganisms per semen sample and total count of microorganisms per ml of semen sample. The sensitivity and specificity rates for different WBC counts are given in Table 1.

Evaluation of the patients using the new cut-off point (0.2×10^6 WBC ml^{-1})

Patients were re-grouped according to this new cut-off point and NIH Classification of the prostatitis syndromes (Table 2). Eighty-four men who had clinically CP/CPPS (group I) were divided into group IA ($\geq 0.2 \times 10^6$ WBC ml^{-1}, NIH IIIA

Table 1. Comparison of sensitivity and specificity rates for different WBC counts in semen

WBC count per millilitre of semen ($\times 10^6$)	Total count of microorganisms per millilitre of semen (10^4 CFU ml^{-1} is considered significant)		Number of different microorganisms per sample (four different microorganisms is considered significant)	
	Sensitivity	Specificity	Sensitivity	Specificity
0	1.0	0.00	1.0	0.00
0.05	0.53	0.57	0.54	0.59
0.1	0.49	0.62	0.50	0.63
0.2	0.45	0.69	0.45	0.68
0.5	0.33	0.74	0.35	0.77
0.8	0.26	0.79	0.29	0.83
1.0	0.23	0.84	0.25	0.86
2.0	0.11	0.93	0.13	0.95
3.0	0.09	0.95	0.09	0.95
5.0	0.03	0.98	0.02	0.97

Table 2. National Institutes of Health Classification of the Prostatitis Syndromes (by National Institutes of Health Summary Statement, 1995; Krieger *et al.*, 1999; Nickel *et al.*, 2001)

Category	Type	Description	Presentation
I	Acute bacterial prostatitis	Acute infection of the prostate glad	Acute febrile illness associated with perineal and suprapubic pain, dysuria, and obstructive voiding symptoms
II	Chronic bacterial prostatitis	Chronic infection of the prostate gland	Recurrent urinary tract infections with pain and voiding disturbances
III	Chronic prostatitis/chronic pelvic pain syndrome	Chronic genitourinary pain in the absence of uropathogenic bacteria localized to the prostate gland employing standard methodology	Chronic perineal, suprapubic, testicular, penile or ejaculatory pain associated with variable dysuria and obstructive and irritative voiding symptoms
IIIA	Inflammatory	Significant number of white blood cells in expressed prostatic secretions, post-prostatic-massage urine sediment, or semen	See category III
IIIB	Noninflammatory	Insignificant number of white blood cells in expressed prostatic secretions, post-prostatic-massage urine sediment, or semen	See category III
IV	Asymptomatic inflammatory prostatitis	White blood cells (and/or bacteria) in expressed prostatic secretions, post-prostatic-massage urine sediment, semen, or histological specimens of prostate gland	Asymptomatic

Table 3. Quantitative bacteriological data of semen in different andrology unit patients

Study group	Total count of microorganisms per millilitre of semen (median/range)	Number of different microorganisms in semen (median/range)
Group IA (NIH IIIA)	$5 \times 10^4/10^2$– 10^{7a}	5/2–8[b]
Group IIA (NIH IV)	$5 \times 10^4/10^2$–10^{7c}	5/2–8[d]
Group IIB (control)	$10^3/10^2$–10^7	3/1–7

Difference from control group (Mann–Whitney rank sum test): [a]$P = 0.010$; [b]$P = 0.005$; [c]$P = 0.013$; [d]$P = 0.015$.

category, $n = 34$) and group IB ($<0.2 \times 10^6$ WBC ml^{-1}, NIH IIIB category, $n = 50$). Fifty-six men who consulted physicians because of infertility or for prophylactic purposes and those who did not have complaints for chronic pelvic pain (group II) were divided into group IIA ($\geq 0.2 \times 10^6$ WBC ml^{-1}, NIH IV category, $n = 24$) and group IIB ($<0.2 \times 10^6$ WBC ml^{-1}, $n = 32$). Group III was quite small, and only four patients had $\geq 0.2 \times 10^6$ WBC ml^{-1}.

The patients belonging to categories NIH IIIA and NIH IV were compared with group IIB patients who were nonleucocytospermic and without CP/CPPS, and therefore can serve as control group here. The results are given in Table 3. The NIH IIIA category patients had significantly more different microorganisms and higher total microbial counts than the control group patients. The same could be noted for NIH IV category and control group patients. Hence, the NIH IIIA and NIH IV category patients were microbiologically quite similar, both with respect to the number of different microorganisms per sample ($P = 0.45$) and the total count of microorganisms per millilitre of sample ($P = 0.75$).

Discussion

A positive correlation was found between WBC count and number of different microorganisms, and between WBC count and total count of microorganisms per millilitre of semen sample. It was also found that the WHO-defined WBC cut-off point (1×10^6 WBC ml^{-1}) has very low sensitivity for discriminating between patients with and without significant bacteriospermia.

For analysing the data, the bacterial concentration 10^4 CFU ml^{-1} was used as the limit of significant bacteriospermia. This value has also

been used in previous studies (Lewis *et al.*, 1981; Maier, 1983; Corradi *et al.*, 1992; Arakawa *et al.*, 1999), although lower or higher limits have also been proposed (Weidner *et al.*, 1985; Gregoriou *et al.*, 1989; Jedrzejczak *et al.*, 1996; Szöke *et al.*, 1998). However, the concentration 10^4 CFU ml^{-1} seems to be clinically significant, as Monga & Roberts (1994) have shown that this bacterial concentration can cause sperm agglutination. At the same time, a good correlation was found between total count of microorganisms per millilitre and the number of different microorganisms in the semen sample. In the patients with low total count of microorganisms, the mean number of different microorganisms per semen sample was only 3.05 while in the patients with high total count of microorganisms it was 4.78. Therefore it is thought that the number of different microorganisms in semen sample can be used as an additional parameter of bacteriospermia in the case of a complete microflora analysis (including anaerobes). Also Szöke *et al.* (1998) have found that a group of chronic prostatitis patients had, on average, 3.9 anaerobic bacteria per patient.

Using the above-described bacteriospermia limits, an attempt was made to set up the microbiologically significant limit of leucocytospermia. This is important as leucocytospermia is significantly related to male reproductive function (Branigan *et al.*, 1995; Wolff, 1995; Omu *et al.*, 1999; Stanislavov, 1999; Arata de Bellabarba *et al.*, 2000; Ludwig *et al.*, 2001). Until now, the limit of leucocytospermia has been usually set up at 1×10^6 WBC ml^{-1} of semen according to WHO definition (Wolff, 1998; Stanislavov, 1999; Zorn *et al.*, 2000). However, some investigators have considered it too high (Jedrzejczak *et al.*, 1996; Sharma *et al.*, 2001). Sharma *et al.* (2001) were even unable to determine the safe WBC count, as oxidative stress evaluated by ROS levels also occurred in patients having very low seminal WBC counts. In this study, the WHO-defined leucocytospermia limit correlated very poorly with the quantitative bacteriological data. The possible new limit was proposed to be 0.2×10^6 WBC ml^{-1}. However, even this value leads to the misdiagnosis of many bacteriospermic patients and may require to be lowered more.

Leucocytospermia can occur in men having symptomatic genital tract infection but it can be found occasionally in non-symptomatic men too. One of the most frequent clinical diagnosis for the leucocytospermic patients is CP/CPPS that is grouped to NIH IIIA category as suggested in the National Institutes of Health/National Institute of Diabetes and Digestive and Kidney Diseases Chronic Prostatitis Workshop (Table 2). Although the aetiology and pathogenesis of CP syndrome can be related to dysfunctional or neuromuscular problems, immunologic disorders or chemical irritation also, the data available strongly suggest that, CP may, in most cases, represent an infectious disease (Nickel, 2000). Another poorly understood condition is asymptomatic leucocytospermia that was grouped in the NIH IV category at the NIH workshop (Table 2). Its role in male infertility has been shown repeatedly (Branigan *et al.*, 1995; Wolff, 1995; Omu *et al.*, 1999; Stanislavov, 1999; Arata de Bellabarba *et al.*, 2000; Ludwig *et al.*, 2001; Sharma *et al.*, 2001), but its deeper nature is still unclear.

The study revealed that quantitative microbiological finding of semen in these patients (NIH IIIa and NIH IV categories) was very similar: high number of different microorganisms and high total count of microorganisms. It was significantly different in the case of control group without leucocytospermia and prostatitis symptoms: low number of different microorganisms and low total count of microorganisms. Berger *et al.* (1997) have shown that men with expressed prostatic secretion (EPS) indicating inflammation were more likely to have bacterial isolation, positive cultures for anaerobic bacteria, higher total bacteria counts, and more bacterial species isolated in prostate biopsy cultures than men without EPS indicating inflammation. Now it is shown that it is true also if seminal fluid is used as specimen. This finding is important as non-invasive specimens are preferred to in clinical practice (Aus *et al.*, 1996).

The question about the source of microorganisms always arises when semen is used as specimen. Normal microflora exists in urethra (Mazuecos *et al.*, 1998; Spaine *et al.*, 2000) and on genital skin (Diemer *et al.*, 2000). The upper genital tract is normally sterile and the presence of normal microflora in the prostate is extremely unlikely (Hochreiter *et al.*, 2000). In this study, contamination of the specimen from the skin and urethra was minimized by washing the hands and genitals before sampling with soap and water, and urinating. We were unable to disinfect the urethra but we have compared the microflora of first catch urine (that represents the microflora of urethra) and the seminal fluid in 32 men and found only 37% of microorganisms to be similar in the urine and semen, and that the number of microorganisms was much lower in urine (unpublished data). Hence, most of the microorganisms in semen do not originate from urethra but can indicate the infection in upper genital tract.

It has been concluded that the WHO-defined WBC cut-off point (1×10^6 WBC ml^{-1}) has very low sensitivity for discriminating between patients with and without significant bacteriospermia, and it

has been proposed that 0.2×10^6 WBC ml^{-1} is the possible new cut-off point. The men whose semen WBC count exceeds this limit (with or without a clinical picture of prostatitis) have significantly more different microorganisms and higher microbial counts in their seminal fluid than those having semen WBC count beneath this limit. If confirmed, these findings may contribute to a better knowledge of clinically significant leucocytospermia.

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