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Anaerobic seminal fluid micro-flora in chronic prostatitis/chronic pelvic pain syndrome patients ☆

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Abstract

We investigated the seminal micro-flora of 116 men. Eighty-four men had chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), and 34 of them were also leukocytospermic. Thirty-two asymptomatic men formed the control group. Micro-organisms were found in all of the 116 seminal fluid specimens. More than 20 different micro-organisms were found in both groups. *Neisseria gonorrhoeae* and *Chlamydia trachomatis* were not found. A high frequency of anaerobic bacteria was found in all groups (68–79%), and in most of the specimens, anaerobic micro-organisms were equal to or outnumbered the aerobic strains. We found 1–8 different micro-organisms in each semen sample, the total count of micro-organisms ranged from 10^2 to 10^7/mL of semen. Both parameters were significantly higher in leukocytospermic CP/CPPS (NIH IIIA category) patients (median = 5 different micro-organisms; total median count 5×10^4) than in the control group (median = 3 different micro-organisms; total median count 10^3). In the CP/CPPS patients, the prevalence and/or count of some opportunistic bacteria was higher than in the control group. To show that the micro-organisms do not originate from the urethra, first voided urine was also investigated in 17 prostatitis patients and 15 controls. One patient had significantly fewer micro-organisms (median 1 vs. 4) and a lower total count of micro-organisms (median 10^2 vs. 10^4/mL) in the first-catch urine than in the seminal fluid. We found only one third of the micro-organisms to be similar in urine and semen while anaerobic bacteria and some aerobic opportunists were infrequent in urine. Semen is a suitable specimen for the diagnosis of prostatitis.

Keywords: Chronic prostatitis; Semen; Anaerobic micro-flora; Seminal leukocytes; Quantitative testing

1. Introduction

Chronic prostatitis is a common but enigmatic condition with a symptom complex of pelvic area pain and lower urinary tract symptoms. It may affect up to 50% of men of all ages and demographics [1–3]. Although several theories have been proposed regarding its etiology, the nature of the disease remains elusive. At the NIH Chronic Prostatitis workshop in Bethesda, MD, 1995, a new prostatitis classification system was developed (Table 1) [4–6]. The conclusions from the workshop also stated that there are no uniformly agreed upon diagnostic or treatment protocols for chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), and in order to establishing them, extensive clinical and basic research studies are needed. Microbiological studies should be one of the priorities.

The diagnostic bacteriological studies for known urinary tract pathogens are mostly negative and usually the bacterial counts do not correlate with the severity of symptoms [7]. However, during the last several years, it has been suggested that micro-organisms can appear as causative agents in so-called non-bacterial prostatitis. Although dysfunctional and neuromuscular problems, immunologic disorders or chemical irritation have also been linked to the prostatitis syndrome, recent data strongly suggest that chronic prostatitis may represent an infectious disease in many, if not all cases [3,8–10]. Difficult-to-culture coryneforms, anaerobes, wall-deficient bacteria, bacteria that exist in aggregated

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Table 1
National Institutes of Health Classification of the Prostatitis Syndromes [4–6]

Category	Type	Description	Presentation
I	Acute bacterial prostatitis	Acute infection of the prostate gland	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	Non-inflammatory	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

"biofilms" adherent to the prostatic ductal walls or within obstructed ducts in the prostate might be important in the etiology of chronic prostatitis [8]. During routine studies, its infectious genesis is hard to prove due to insufficient laboratory methods. Usually, little attention is paid to anaerobic bacteria as they are sensitive to transportation, and their culture and differentiation is difficult, costly and time-consuming [11].

Another problem related to these microbiological studies is the presence of normal micro-flora in the urogenital tract. Since the prostate gland is a hidden site in the human body, indirect specimens such as fractionated urine, expressed prostatic secretions (EPS) or semen must be investigated. Therefore, interpretation of study results is complicated because of the indigenous micro-flora and a lack of evaluation criteria [3,10]. One way to overcome this problem is to use quantitative microbiological techiques.

Our aim was to prove the need for quantitative full-micro-flora semen analysis for determining the role of infection in CP/CPPS.

2. Material and methods

2.1. Study group

The research included 116 men and was carried out between September 1999 and May 2001 in the Andrology unit of Tartu University Clinicum.

In 84 out of 116 men, the pain or discomfort in the pelvic area for at least 3 months had been the main symptom; therefore, CP/CPPS was diagnosed in them according to the suggestion of the NIH workshop on chronic prostatitis in Bethesda, MD, 1995 [4–6]. Their mean age was 34.2 yr (median 33, range 22…50). These men were divided into subgroups according to the number of leukocytes (white blood cells, WBC) in their semen [12] and NIH Classification of the Prostatitis Syndromes (Table 1): NIH IIIA category ($\geqslant 0.2 \times 10^6$ WBC/mL, $n = 34$) and NIH IIIB category ($< 0.2 \times 10^6$ WBC/mL, $n = 50$).

Thirty-two healthy men (mean age 28.7 yr, median 28.5, range 21…36) consulted a physician due to infertility of the couple or for prophylactic purposes. They did not have complaints for chronic pelvic pain; they were normospermic, not responsible for the infertility of the couple, supposedly monogamous for at least 1 year, and with an insignificant number of leukocytes in their semen ($< 0.2 \times 10^6$ WBC/mL). Therefore, they served as a control group for this study.

All subjects were at least 18 yr old. Exclusion criteria were stated according to the suggestion of the NIH workshop on chronic prostatitis in Bethesda, MD, 1995 [5]. None of the patients had received antimicrobial therapy within 3 months. All men gave consent for the additional microbiological studies of their semen.

2.2. Samples

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

In 32 randomly selected patients (17 prostatitis patients and 15 control patients), the first-catch urine was also investigated.

All semen and urine samples were transported to the microbiology laboratory (located near the Andrology unit) immediately and processed within 1 h.

2.3. Cytological analysis

The white blood cell concentration in semen (WBC/mL) was determined using a microscopic method. The smears of semen were air-dried, stained with Bryan–Leishman stain and examined under oil immersion (magnification = 1000 ×) by an experienced microscopist.

2.4. Microbiological analyses

Semen samples were cultivated quantitatively for detecting anaerobic, micro-aerophilic and aerobic bacteria. The seedings were performed within 1–2 h of collection onto Wilkins–Chalgren agar (Oxoid, Unipath, Basingstoke, UK) supplemented with 5% horse blood, Schaedler medium (Oxoid) supplemented with 5% horse blood, vancomycin and nalidixic acid, *Gardnerella vaginalis* selective agar (Oxoid), MRS agar for lactobacilli (Oxoid), freshly prepared blood agar and chocolate agar. Wilkins–Chalgren and Schaedler media were incubated in an 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 *G. vaginalis* selective agar were incubated in a micro-aerophilic 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 examined microscopically. The micro-organisms were identified mostly on a genus level. Standard methods were used for identification of enteric and other Gram-negative bacteria [13]. A latex test (Oxoid) was employed for differentiation of *Staphylococcus aureus* and coagulase negative staphylococci (CONS). Streptococci and enterococci were identified by the absence of catalase production and differentiated by fermentation of esculine. Group B streptococci were identified using a latex test (Oxoid). *Corynebacterium seminale* was differentiated by testing its beta-glucuronidase activity using MUG medium (Oxoid). *G. vaginalis* was detected by its ability to grow on selective medium (Oxoid), characteristic morphology and negative catalase test. The anaerobes were identified by their growth on selective media, colony and cellular morphology, Gram stain reaction and some rapid tests and diagnostic disks (catalase, oxidase, spot indole, fluorescence, oxgall, brilliant green, bile esculin, colistin, vancomycin, kanamycin). All anaerobic micro-organisms were tested for absence of growth under aerobic and micro-aerophilic conditions.

Mycoplasma hominis and *Ureaplasma urealyticum* were detected using Mycoplasma IST test (BioMerieux, France). *Neisseria gonorrhoeae* and *Chlamydia trachomatis* were detected by a PCR method using Amplicor C. trachomatis/N. gonorrhoeae Test (Roche, Mannheim, Germany).

2.5. Statistical methods

Statistical analyses were performed using SigmaStat (Jandel Scientific) and Exel (Microsoft Corp.) software programs. The following tests were employed: Mann–Whitney rank sum test, Fisher exact test.

2.6. Ethical considerations

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

3. Results

3.1. Micro-flora of seminal fluid

None of the 116 seminal fluid specimens was sterile. More than 20 different micro-organisms were isolated (Table 2). Some opportunistic bacteria such as beta-haemolytic streptococci, GBS, enterococci, coliforms, actinomyces and *C. seminale* appeared more frequently and/or in higher counts in CP/CPPS patients, however, these differences were not significant. *N. gonorrhoeae* and *C. trachomatis* were not found.

In the leukocytospermic subgroup (NIH IIIA category) the prevalence of beta-haemolytic streptococci and peptostreptococci was significantly higher than in the control group. Also, the count of peptostreptococci was remarkably high in NIH IIIA patients. In the non-leukocytospermic subgroup (NIH IIIB) the prevalence of enterococci was significantly higher than in the control group.

A high frequency of anaerobic bacteria was found in all groups (Table 3). In most of the specimens, the counts of anaerobic micro-organisms were equal to or outnumbered the aerobic strains.

We found 1–8 different micro-organisms in each semen sample, and the total count of micro-organisms in the samples was $10^2 \ldots 10^7$ CFU/mL. Both parameters were significantly higher in leukocytospermic CP/CPPS patients (Table 4) than in the control group.

3.2. Micro-flora of first-catch urine

The first-catch urine was investigated in 32 consecutive men (17 prostatitis patients and 15 control patients). We found 6 sterile samples. In the remaining 26 samples,

Table 2
Comparison of seminal micro-organisms in prostatitis patients (leukocytospermic and non-leukocytospermic subgroups) and the control group

Micro-organisms	Prostatitis patients ($n = 84$)			Prostatitis patients, with leucokytospermia (NIH IIIA, $n = 34$)			Prostatitis patients, without leukocytospermia (NIH IIIB, $n = 50$)			Control group ($n = 32$)		
	Mean (log)[a]	Median/range (log)[a]	%	Mean (log)	median/range (log)	%	Mean (log)	Median/range (log)	%	Mean (log)	Median/range (log)	%
S. aureus	3.6	<2/<2–5.0	37	2.7	<2/<2–4.0	32	3.8	<2/<2–5.0	40	2.7	<2/<2–4.0	37
CONS	4.2	2.3/<2–6.0	68	4.5	2.3/<2–6.0	79	3.7	2/<2–5.0	60	2.9	2.2/<2–4.0	62
β-haemolytic streptococci	4.5	<2/<2–6.0	26	4.8	<2/<2–6.0	32*	3.7	<2/<2–5.0	22	2.6	<2/<2–4.0	9*
Group B streptococci	3.5	<2/<2–5.0	14	3.7	<2/<2–5.0	12	3.4	<2/<2–5.0	16	1.8	<2/<2–3.3	3
Streptococci	4.4	<2/<2–6.0	36	4.6	<2/<2–6.0	32	4.2	<2/<2–5.7	38	2.5	<2/<2–4.0	37
Enterococci	4.2	<2/<2–6.0	24	4.5	<2/<2–6.0	9	3.6	<2/<2–5.0	34*	2.0	<2/<2–3.3	9*
C. seminale	5.1	<2/<2–7.0	24	3.6	<2/<2–5.0	29	5.3	<2/<2–7.0	20	2.0	<2/<2–3.3	13
Corynebacteria	5.4	<2/<2–7.3	44	4.0	<2/<2–5.0	47	5.6	<2/<2–7.3	42	2.9	<2/<2–4.0	31
Bacillus sp.	1.7	<2/<2–3.3	2	2.1	<2/<2–3.3	6		<2	0		<2	0
G. vaginalis	4.1	<2/<2–6.0	10	4.5	<2/<2–6.0	6	3.0	<2/<2–4.0	12	5.5	<2/<2–7.0	16
Coliforms	3.4	<2/<2–5.0	12	3.8	<2/<2–5.0	15	2.4	<2/<2–4.0	10	2.8	<2/<2–4.3	3
Haemophilus sp.	5.1	<2/<2–7.0	4	5.5	<2/<2–7.0	3	4.3	<2/<2–6.0	4		<2	0
Yeasts	0.3	<2/<2–2.3	1		<2	0	0.6	<2/<2–2.3	2		<2	0
Actinomyces	4.2	<2/<2–6.0	14	4.6	<2/<2–6.0	18	3.4	<2/<2–5.0	12	1.5	<2/<2–3.0	3
Peptostreptococci	5.5	<2/<2–7.0	49	5.6	3/<2–7.0#	62*	5.4	<2/<2–7.0#	40	4.6	<2/<2–6.0	38*
Gram–neg. anaerobic rods[b]	5.4	<2/<2–7.0	40	5.5	<2/<2–7.0	47	5.4	<2/<2–7.0	36	5.5	<2/<2–7.0	31
Veillonella	4.2	<2/<2–6.0	8	3.5	<2/<2–5.0	9	4.4	<2/<2–6.0	8	2.5	<2/<2–4.0	6
Bifidobacteria	4.2	<2/<2–6.0	11	3.8	<2/<2–5.0	15	4.3	<2/<2–6.0	8	3.5	<2/<2–5.0	6
Propionibacteria	3.2	<2/<2–5.0	4	3.5	<2/<2–5.0	6	2.3	<2/<2–4.0	2	4.5	<2/<2–6.0	16
Megasphaera	3.1	<2/<2–5.0	1		<2	0	3.3	<2/<2–5.0	2		<2	0
Sarcina	5.1	<2/<2–7.0	2	5.5	<2/<2–7.0	6		<2	0	2.5	<2/<2–4.0	6
Eubacteria	3.1	<2/<2–5.0	4	1.8	<2/<2–3.0	6	3.3	<2/<2–5.0	2	1.8	<2/<2–3.3	3
Mobiluncus sp.	3.1	<2/<2–5.0	2	3.5	<2/<2–5.0	3	2.3	<2/<2–4.0	2		<2	0
Anaerobic lactobacilli	1.1	<2/<2–3.0	1	1.5	<2/<2–3.0	3		<2	0		<2	0
M. hominis			0			0			0			0
U. urealyticum			16			24			12			13

*$P < 0.05$ (Fisher test).
#$P < 0.05$ (Mann–Whitney rank sum test).
[a] Counts in $\log_{10}$ CFU/mL (detection level was 2.0).
[b] In 13 patients Gram negative anaerobic rods were identified as follows: in NIH IIIA group 3 *Bacteroides ureolyticus*, 2 *Prevotella* sp., in NIH IIIB group 2 *B. ureolyticus*, 1 *B. fragilis*, 1 *B. ureolyticus*+*Fusobacterium* sp., in control group 2 *Fusobacterium* sp., 1 *B. ureolyticus*, 1 *B. ureolyticus*+*Prevotella* sp.

Table 3
Relation of aerobic and anaerobic bacteria in semen

Group (number)	Number of specimens harbouring anaerobic bacteria (%)	No of specimens with		
		Aerobes > anaerobes	Aerobes = anaerobes	Aerobes < anaerobes
NIH IIIA (34)	27 (79%)	14	6	14
NIH IIIB (50)	34 (68%)	19	23	8
Control (32)	24 (75%)	11	9	12

up to 4 different micro-organisms were identified. One patient had significantly fewer micro-organisms (median of 1 vs. a median of 4, $P < 0.001$) and a lower total count of micro-organisms (median 10^2 CFU/mL vs. 10^4 CFU/mL, $P < 0.001$) in the first-catch urine than in seminal fluid. The prevalence and counts of some aerobic and anaerobic opportunists were significantly lower in the first-catch urine than in seminal fluid (Table 5).

We found only 37% of the micro-organisms to be coinciding in urine and semen, though in 22 men the same micro-organisms were present. The most frequent concurrent micro-organisms were staphylococci in 19 men, present in 10 as a single coinciding micro-oganism and in 9 other men in combination with one (3 streptococci, 1 coliform, 1 *Bacteroides ureolyticus*, 1 peptostreptococcus, 1 enterococcus) or two (2 streptococci + peptostreptococci) other micro-organisms. In the remaining 3 men, the coinciding micro-organisms were streptococci, group B streptococci, and a combination of streptococci and *S. aureus*.

There were slightly fewer different micro-organisms (median of 1 vs. a median of 2, $P = 0.079$) and a lower total count of micro-organisms (median 3.3 CFU/mL vs. median 4.1 CFU/mL, $P = 0.141$) in the first-catch urine of the control patients than in the prostatitis patients, but no difference in species composition was noted.

4. Discussion

We found anaerobic bacteria in three-quarters of the semen samples, and in a majority of them, anaerobic bacteria were equal to or outnumbered aerobic species. Our results correspond to the data of Szöke et al. [11]. Several other investigators have detected only aerobic bacteria and sometimes also causative agents of sexually transmitted diseases [7,14,15]. Anaerobic bacteria may have been overlooked in these studies.

Our study revealed that leukocytospermic CP/CPPS patients (NIH IIIA category) had a high number of different micro-organisms and a high total count of micro-organisms in their semen. The control group without leukocytospermia and prostatitis symptoms was significantly different having a low number of different micro-organisms and a low total count. Berger et al. [16] have shown that men with EPS confirmed inflammation were more likely to have bacterial growth, positive cultures for anaerobic bacteria, higher total bacteria counts, and more bacterial species isolated in prostate biopsy cultures than men without EPS confirmed inflammation. We have shown that similar results are obtained by culturing seminal fluid. This finding is important since non-invasive specimens are preferred in clinical practice [17].

We detected opportunistic organisms, including anaerobic bacteria, somewhat more frequently and/or in higher counts in CP/CPPS patients. This tendency was clearer in the case of leukocytospermic (NIH IIIA) patients. An interesting finding is the higher number of corynebacteria, especially *C. seminale*, in CP/CPPS patients. Similar data have also been described in other papers [18,19].

Table 4
Comparison of the total count of micro-organisms and number of different micro-organisms in semen in different study groups

Group	Total count of micro-organisms/mL of semen (CFU/mL)	Number of different micro-organisms in semen
	Median (range)	Median (range)
NIH IIIA	5×10^4 (10^2…10^7)*	5 (2…8)#
NIH IIIB	10^4 (10^2…10^7)	4 (2…8)
Control	10^3 (10^2…10^7)*	3 (1…7) #

*$P = 0.01$; #$P = 0.005$ (both Mann–Whitney rank sum test).

Table 5
Comparison of micro-flora of seminal fluid and first-catch urine specimens

Micro-organism	Micro-organisms in semen ($n = 116$)		Micro-organisms in first-catch urine ($n = 32$)	
	Median/range (log)[a]	%	Median/range (log)	%
S. aureus	<2/<2–5.0#	37*	<2/<2–3.6#	9*
CONS	2.3/<2–6.0	65	<2/<2–3.5	66
β–haemolytic streptococci	<2/<2–6.0	22*	<2	0*
Group B streptococci	<2/<2–5.0	11	<2/<2–3.6	13
Streptococci	<2/<2–5.7	36	<2/<2–3.4	22
Enterococci	<2/<2–6.0	19	<2/<2–3.6	16
C. seminale	<2/<2–6.7	21*	<2	0*
Corynebacteria	<2/<2–7.3#	41*	<2#	0*
Bacillus sp.	<2/<2–4.0	2	<2/<2–3.6	3
G. vaginalis	<2/<2–4.0	11*	<2	0*
Coliforms	<2/<2–5.0	9	<2/<2–3.2	3
Haemophilus sp.	<2/<2–6.0	3	<2	0
Yeasts	<2/<2–3.0	1	<2	0
Actinomyces	<2/<2–5.0	11*	<2	0*
Peptostreptococci	<2/<2–7.0#	45*	<2/<2–4.0#	25*
Gram-neg. anaerobic rods	<2/<2–7.0#	37*	<2/<2–2.6#	3*
Veillonella	<2/<2–7.0	8	<2	0
Bifidobacteria	<2/<2–4.0	9	<2	0
Propionibacteria	<2/<2–5.0	7	<2	0
Megasphaera	<2/<2–6.0	1	<2	0
Sarcina	<2/<2–7.0	3	<2	0
Eubacteria	<2/<2–5.0	3	<2	0
Mobiluncus sp.	<2/<2–4.0	2	<2	0
Anaerobic lactobacilli	<2/<2–3.0	2	<2	0

*$P < 0.05$ (Fisher test); #$P < 0.05$ (Mann–Whitney rank sum test).
[a] Counts in $\log_{10}$ CFU/mL (detection level was 2.0).

In prostatitis studies, the question regarding a suitable specimen always arises. Prostate biopsy seems ideal but is technically difficult, inconvenient and potentially dangerous for the patient. Fractionated urine (Stamey or Nickel test) is classical and informative, but its collection and processing is cumbersome. Semen is a very suitable specimen since 30% of semen originates from the prostate, and therefore it should reflect infection of the prostate.

At the same time the question regarding the source of micro-organisms arises when semen is used as a specimen. Indigenous normal micro-flora exists in the urethra [20,21] as well as on genital skin [22], which complicates the interpretation of study results [3]. On the other hand, the upper genital tract should be normally sterile and the presence of normal micro-flora in the prostate is unlikely [23]. In our 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 micro-flora of first-catch urine (that represents micro-flora of the urethra) and seminal fluid. Only 37% of micro-organisms were similar in urine and semen, and the total bacterial count and number of different micro-organisms were significantly lower in urine. Hence, most of the micro-organisms in semen do not originate from the urethra, but can indicate an infection in the upper genital tract, and therefore semen can be considered a suitable specimen in the case of prostatitis. In our study, micro-organisms were always present in semen including in low counts in healthy men, probably reflecting normal urethral micro-flora.

When dividing the chronic prostatitis patients into two subgroups, leukocytospermic and non-leukocytospermic, we have used the limit 0.2×10^6 WBC/mL of semen. This limit was set according to our previous study [12], where we have shown that the WHO-defined limit of leukocytospermia 1×10^6 WBC/mL [24] is too high. A similar opinion has also come from some other studies [24,25].

We were somewhat surprised that in the case of high total bacterial count, not monoculture but rather mixed culture with a high number of different bacteria appeared. This may indicate polymicrobial infection, possibly similar to bacterial vaginosis in a female patient. To clarify this issue, carefully planned biopsy studies are necessary.

5. Conclusions

Leukocytospermic CP/CPPS patients (NIH IIIA category) have significantly higher numbers of different micro-organisms and a higher total count of micro-organisms in their semen than men without leukocytospermia and prostatitis symptoms.

It is also emphasized that anaerobic bacteria are frequently present and predominant in seminal samples. Several opportunistic organisms, including anaerobic bacteria, appear more frequently and/or in higher counts in CP/CPPS patients than in the control group.

We can also conclude that semen is a suitable specimen for diagnosis of prostatitis.

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