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[Konstantinos Stamatiou](#) <sup>\*</sup>, [Hippocrates Moschouris](#), Konstantinos Tzelepis, [Gianpaolo Perletti](#), Vittorio Magri

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Article

# Three-Glass Test to Culture Prostate Secretion and Semen of Chronic Prostatitis Patients

Konstantinos Stamatiou <sup>1</sup>, Hippocrates Moschouris <sup>2</sup>, Konstantinos Tzelepis <sup>3</sup>, Gianpaolo Perletti <sup>4</sup> and Vittorio Magri <sup>5</sup>

<sup>1</sup> Urology Department, Tzaneio General Prefecture Hospital, 18536 Piraeus, Greece

<sup>2</sup> Radiology Department, Tzaneio General Prefecture Hospital, 18536 Piraeus, Greece

<sup>3</sup> Urology Department, Agios Panteleimon General Hospital, 18450 Nikaia, Greece

<sup>4</sup> Department of Biotechnology and Life Sciences, Section of Medical and Surgical Sciences, University of Insubria, 21100 Varese, Italy

<sup>5</sup> Urology clinic, ASST Fatebenefratelli Sacco Hospitals, 20026 Milan, Italy

\* Correspondence: stamatiouk@gmail.com; Tel.: 00306934917578

**Abstract: Background/Objectives:** Currently, the Meares-Stamey 4-glass and the 2-glass tests are used for diagnosing chronic prostatitis subtypes. Both tests include prostatic massage. Failure to extract prostatic secretions -for any reason- results in an undiagnostic test. Evidence from everyday practice and studies shows that expressed prostatic secretion is successfully recovered in less than 50% of the examined patients and an important number of post-massage urine samples are missing prostatic secretions. This study evaluated a simpler test, the 3-glass (pre-ejaculation, ejaculation, and post-ejaculation) test. We compared it with the 4-glass and the 2-glass tests to detect inflammation and bacteria in men with chronic prostatitis symptoms. **Methods:** The study population included patients with chronic prostatitis symptoms. Subjects were assigned in each visit to undergo either the 4-glass or the 2-glass test or the 3-glass test. The comparison among the three tests was based on the percentage of bacterial detection, the percentage of false negative diagnoses, and the percentage of shifts among chronic prostatitis subtypes in the follow-up visits of recurrent patients. **Results:** A total of 157 patients were finally evaluated. Fifty-nine (59) patients underwent the 4-glass test (group A), sixty-seven (67) underwent the 3-glass test (group B) and thirty-one (31) underwent the 2-glass test (group C). No statistically significant differences in the aforementioned comparisons were found. **Conclusions:** A comparison of the three diagnostic tests showed no superiority of the total ejaculate culture-based 3-glass test to the conventional prostatic secretions culture-based tests

**Keywords:** Chronic bacterial prostatitis; Meares-Stamey 4-glass test; 3-glass test; 2-glass test

## 1. Introduction

Despite the recent progress in chronic –either bacterial or non-bacterial- prostatitis management, many cases relapse. The reasons remain unknown however they were mainly attributed to host, bacterial, and treatment-related factors. An ignored obvious reason could be misdiagnosis and misclassification among chronic prostatitis subtypes. Accumulating evidence suggests pitfalls and caveats in the diagnostic approach of chronic prostatitis to contribute to treatment failure [1]. Currently, the Meares-Stamey 4-glass test is the standard method of assessing inflammation and the presence of bacteria in the lower urinary tract in men. Since it has strong concordance with the 4-glass test, the 2-glass test (pre-massage and post-massage) was introduced as a reasonable alternative when expressed prostatic secretions are not obtained [2]. Both tests have value in diagnosis and treatment since they are also used for distinguishing between chronic prostatitis subtypes. Both tests include prostatic massage. This technique is performed by stroking the prostate several times to extract expressed prostatic secretions (EPS). EPS alone or mixed with urine (VB3/post-massage urine sample) is examined under a microscope. Failure to extract prostatic secretions results in an undiagnostic test. Evidence from everyday practice and studies shows that expressed prostatic

secretion is successfully recovered in less than 50% of the examined patients and an important number of post-massage urine samples are missing prostatic secretions [3,4]. In addition to the abovementioned pitfalls, most urologists do not use prostatic secretions culture-based tests in daily practice because of the time and difficulty in performing them and many patients refuse follow-up since both tests are somehow awkward [5].

In this study, we evaluated a simpler test, the 3-glass (pre-ejaculation, ejaculation, and post-ejaculation) test, and we compared it with the Meares-Stamey 4-glass and the 2-glass tests to detect inflammation and bacteria in men with chronic prostatitis symptoms.

## 2. Methods

The study population included 157 patients with chronic prostatitis symptoms presented to the prostatitis clinic of the "Tzaneio" General Hospital between January 2023 and January 2025. Subjects were assigned in individual visits to undergo either the 4-glass, the 2-glass, or the 3-glass tests.

**Exclusion criteria:** Patients suffering from conditions affecting either bacterial virulence or host response (eg. immunodeficiencies, abnormalities of the urogenital system) and individuals who received antibiotics or immunosuppressive treatment within 4 weeks from the visit were excluded from the study.

**Microbiological evaluation:** All 3 tests were considered positive when: 1) bacteria grew in the culture of the expressed prostatic secretion (EPS) or total ejaculate (TE) and VB3/PoPM/PoE (post-prostate massage/post ejaculate) urine sample and did not in VB1 and VB2/PrPM/PrE (pre-prostate massage/pre-ejaculate) sample; 2) bacterial colonies in VB3/PoPM/PoE were higher than that of VB1 and VB2/PrPM/PrE urine samples. Specimens were cultured undiluted in blood, and MacConkey agar plates (Kallestad Lab., TX, USA) and subjected to centrifugation for microscopic examination of the sediment. Evaluation of culture results was performed by two specialist microbiologists, who were not informed of the patient record. Identification was performed by conventional methods and the Vitek-2 Compact (bioMerieux, France) system. Susceptibility testing was performed by disc diffusion and/or the Vitek-2 system. Interpretation of susceptibility results was based on Clinical and Laboratory Standards Institute (CLSI) guidelines.

To avoid misinterpretation due to contamination of the various biological materials a threshold of 200 cfu/ml was defined by consensus for diagnosis of chronic bacterial prostatitis in prostatic secretions culture-based tests.

**Clinical evaluation:** Differentiation between chronic non-bacterial prostatitis subtypes was based on the presence/absence of leukocytes in EPS/VB3/PoPM/PoE samples.

The EPS/VB3/PoPM/PoE cultures that considered negative (bacteria unable to grow) despite bacterial presence in the relative specimens were indicated as cases showing "possible chronic bacterial prostatitis" and represented false negative cases.

A higher number of bacterial colonies in the VB1, VB2, PrPM, PrE compared to that of VB3, PoPM, PoE samples were considered mixed prostatitis/cystitis cases.

Cases diagnosed with different types of prostatitis on follow-up were reported as shifts between subtypes of chronic prostatitis and were attributed to the bias associated with each test.

The comparison among the three tests was based on the percentage of bacterial detection, the percentage of false negative diagnoses, and the percentage of shifts among chronic prostatitis subtypes in the follow-up visits of recurrent patients.

**Statistical analysis:** Differences between proportions were analyzed by Pearson's Chi-square test and Fisher's exact test of significance. Analyses were performed in the "R" environment for statistical computation. The accepted significance level was 0.05 (P value <0.05 is significant).

The locally appointed Ethics Committee approved the research protocol 7312/12-12-22

### 3. Results

A total of 157 patients were finally evaluated. Fifty-nine (27 at initial evaluation and 32 at follow-up) patients underwent the 4-glass test (group A), Sixty-seven (35 at initial assessment and 32 at follow-up) underwent the new 3-glass test (group B) and thirty-one (12 at initial evaluation and 19 at follow-up) underwent the 2-glass test (group C). No difference in median symptom severity and age among the three groups was found (table 1).

**Table 1.** Differences between proportions analyzed by Pearson's Chi-square test.

	Mean Age	Mean NIH-CPSS
4 glass Meares-Stamey test	51.4	17.5
3 glass test	48.7	20.4
2 glass test	49.2	17.4
	p>0.05	p>0.05

Upon initial evaluation bacterial detection (chronic bacterial prostatitis and mixed prostatitis/cystitis) was achieved in 51.8% of patients of Group A, 68.5% of patients of Group B, and 66.6% of patients of Group C (table 2).

**Table 2.** Initial evaluation: diagnoses per group.

Group A: 4 glass test	Diagnosis	N°	%
N = 27	CBP	13	48.1
	CNBNI	6	22.2
	CNBI	5	18.5
	C&CBP	1	3.7
	PCB	3	11.1
	Other	1	3.7
Group B: 3 glass test	Diagnosis	N°	%
N = 35	CBP	20	57.1
	CNBNI	5	14.2
	CNBI	4	11.4
	C&CBP	4	11.4
	PCB	1	2.8
	Other	1	2.8
Group C: 2 glass test	Diagnosis	N°	%
N = 12	CBP	7	58.3
	CNBNI	3	25
	CNBI	0	0
	C&CBP	1	8.3
	PCB	1	8.3
	Other	0	0

More precisely, 13 (48.1%) patients in Group A, 20 (57.1%) patients in Group B, and 7(58.3%) patients in Group C were diagnosed with chronic bacterial prostatitis (CBP). Similarly, 1(11.1%), 4(11.4%), and 1(8.3%) patients in Group A, B and C respectively were diagnosed with cystitis & chronic prostatitis (C&CBP). Chronic non-bacterial, non-inflammatory subtype (CNBNI) was diagnosed in 22.2%, 14.2% and 25% of the patients in groups A, B, and C respectively. Chronic non-bacterial, inflammatory subtype (CNBI) was diagnosed in 18.5 and 11.4% of the patients in groups A and B respectively. The percentage of false negative diagnoses (possible chronic bacterial prostatitis/PCB) was 11.1 in Group A, 2.8% in Group B and 8.3% in Group C (table 2).

Regarding follow-up visits, CBP remissions and reinfections were detected in 9.3%, 28.5%, and 5.2% of groups A, B, and C respectively. Finally, the percentage of shifts among chronic prostatitis subtypes was 18.7%, 18.7%, and 26.3% respectively (table 3).

**Table 3.** Follow up evaluation: diagnoses per group.

Group A: 4 glass test	T	U	CBP	CNBI	CNBNI	C&CBP	PCB
CBP	12	3			1		1
CNBI	1	2	1	2			
CNBNI	6		1				
C&CBP	2						
PCB	1						
Group B: 3 glass test	T	U	CBP	C-NBI	CNBNI	C&CBP	PCB
CBP	9	5				1	
CNBI	4						1
CNBNI	2	1				1	1
C&CBP	4		1				
PCB	1		1				
Group C: 2 glass test	T	U	CBP	CNBI	CNBNI	C&CBP	PCB
CBP	6	1			2		
CNBI	2	1	1				
CNBNI							
C&CBP	2		1				
PCB	2		1				

T treated. U untreated. CBP Chronic Bacterial Prostatitis. CNBNI Chronic Non-Bacterial, Non-Inflammatory. CNBI Chronic Non Bacterial, Inflammatory. C&CBP Cystitis & Chronic Prostatitis. PCB Possible Chronic Bacterial

Differences between proportions analyzed by both Pearson's Chi-square test and Fisher's exact test revealed no statistically significant differences in bacterial detection (tables 4 and 5) .

**Table 4.** Differences between proportions analyzed by Pearson's Chi-square test.

	4GT	3GT	2GT	4GT vs 3GT (p)	4GT vs 2GT (p)	3GT vs 2GT (p)
Patients with CBP (N)	16	29	14	0.19	0.23	0.91
Total patients tested (N)	59	67	31			

**Table 5.** Differences between proportions analyzed by Fisher's exact test.

	4GT	3GT	2GT	4GT vs 3GT (p)	4GT vs 2GT (p)	3GT vs 2GT (p)
Patients with CBP (N)	16	29	14	0.22	0.27	0.99
Total patients tested (N)	59	67	31			

From a microbiological viewpoint, the detected microorganisms in the prostatic fluid and the ejaculate practically do not differ from each other (table 6).

**Table 6. and 7.** Pathogens found (alone or in combination) in each group and susceptibility rates.

4 glass test		3 glass test		2 glass test	
<i>Enterococcus faecalis</i>	13	<i>Escherichia coli</i>	25	<i>Enterococcus faecalis</i>	4
<i>Escherichia coli</i>	12	<i>Enterococcus faecalis</i>	17	<i>Escherichia coli</i>	4
<i>Staphylococcus epidermidis</i>	4	<i>Proteus mirabilis</i>	10	<i>Staphylococcus epidermidis</i>	3
<i>Staphylococcus hominis</i>	4	<i>Staphylococcus epidermidis</i>	9	<i>Staphylococcus hominis</i>	2

<i>Staphylococcus haemolyticus</i>	3	<i>Staphylococcus hominis</i>	4	<i>Staphylococcus haemolyticus</i>	1
<i>Klebsiella aerogenes</i>	2	<i>Streptococcus agalactiae</i>	3	<i>Klebsiella aerogenes</i>	1
<i>Staphylococcus lugdunensis</i>	1	<i>Haemophilus parainfluenza</i>	2		
Pantoea sp	1	<i>Enterococcus Faecium</i>	1		
<i>Staphylococcus capitis</i>	1	<i>Klebsiella aerogenes</i>	1		
<i>Gonococcus</i>			1		
		<b>4GT</b>	<b>3GT</b>	<b>2GT</b>	
<b>Full sensitive</b>	32	29	11		
<b>Any resistance</b>	9	44	4		
<b>Total</b>	41	73	15		

#### 4. Discussion

According to our findings, the bacterial detection rate (diagnoses of chronic bacterial prostatitis and mixed prostatitis/cystitis cases) was higher in group B than in group A. However, the bacterial detection rate in groups B and C was similar. The percentage of false negative diagnoses (possible chronic bacterial prostatitis) was higher in Groups A and C than in Group B. Finally, the percentage of shifts among chronic prostatitis (CP) subtypes upon follow-up was relatively high in all three tests. The reasons explaining the abovementioned findings are probably the strict definitions used to classify CP syndromes following prostatic secretions culture-based tests. Traditionally, chronic bacterial prostatitis (CBP) is diagnosed by a 10-fold increase in bacteria in the EPS or VB3 specimens compared with VB1 and VB2 [6]. However, in a significant number of CBP cases in the present study, the increase in bacterial loads in VB3 specimens was between 2- and 3-fold compared to VB1 and VB2. In a similar number of cases, leucocyte counts in VB3 specimens were slightly higher than those in VB1 and/or VB2. These findings are identical with that of our previous study [4]. This criterion is somehow confounding given that, white blood cell (leucocyte) counts have not been indicated to correlate with symptoms or the presence or absence of infection [7,8]. On the other hand, certain drawbacks e.g. technical difficulties in performing prostatic massage (under the circumstances of obesity, rectal discomfort or recent ejaculation) for which prostatic secretion cannot be obtained and a variety of factors (such as hyper-hydration, pollakiuria, and medications) that hide differences in leucocyte counts between pre (VB1/VB2/prePM) and post prostatic massage samples (VB3/PoPM) may shift the diagnosis to one or another direction.

Similar to our previous study, the most possible diagnosis following failure to extract prostatic secretions is that of chronic non-bacterial, non-inflammatory subtype [4]. Of note, in the present study, almost 60% of the 4-glass tests yielded sufficient quantities of EPS; therefore, in the remaining cases, the diagnosis was based only on VB3 cultures. In confirmation to the above the detection rate of chronic non-bacterial, non-inflammatory subtype was significantly higher in Groups A and C than in Group B.

The difference in the rate of false-negative cases is probably due to the cut-off level of the number of bacterial colonies in both urine and prostate secretion samples. According to the literature, negative culture results may also occur for various reasons, including initiating empirical antibiotic therapy before obtaining the diagnostic test, high bacterial count cut-offs established by some laboratories (e.g., a threshold of 50,000 colony-forming units to report a test culture as 'positive') or insufficient sample volumes. On the other hand, the presence of fastidious organisms, anaerobic pathogens or bacteria not detectable with the usual tests may explain cases characterized by false-negative cultures despite the actual presence of bacteria and no recent exposure to antibiotic intake reported [4].

As semen contains diluted prostatic material (around 20%–30% of the total volume), together with material from the testes and other genital tract tissues, some authors have suggested the usefulness of semen analysis for detecting prostatic pathogens [9]. Although the culture of semen/total ejaculate for the diagnosis of bacterial prostatitis was introduced about 30 years ago [10], its usefulness and efficacy remain debated. The key factors that contribute to the continued discussion and assessment of semen culture for diagnosing bacterial prostatitis are the following: 1) Contamination: Semen samples can be easily contaminated by bacteria from the skin or surrounding environment, which can lead to misleading results and false positives. However, contamination of prostatic secretion can also occur during the collection process, which may similarly affect the accuracy of diagnostic tests.

2) Inconsistent Results: Studies and clinical experiences have shown varying levels of accuracy. Some studies report that total ejaculate cultures are reliable, while others find them less consistent in detecting the bacteria that actually cause prostatitis. In this study, the detection rate of the 3-glass test was higher than that of the 4-glass and 2-glass tests.

3) Clinical Relevance: The presence of bacteria in semen doesn't always correlate with the symptoms of prostatitis. This can make it challenging to determine whether the detected bacteria are indeed responsible for the patient's condition, potentially leading to unnecessary treatments. In fact, the ejaculate is a mixture of sperm from the testicles and fluids from the seminal vesicles, prostate, and bulbourethral glands. Therefore, the microbial flora in the ejaculate can include bacteria from the urethra, seminal vesicles, and prostate. In addition, seminal fluid is rich in fructose and other nutrients that support sperm -and microbial- viability. On the other hand, the prostatic fluid is secreted by the prostate gland and contains various enzymes, lipids, and citric acid. Although citric acid is a weak organic acid has natural antimicrobial properties, making it effective in inhibiting the growth of bacteria, mold, and yeast. Moreover, it can lower the pH of its environment, creating conditions that are unfavorable for many microbes. Most pathogenic bacteria prefer neutral or slightly alkaline conditions, so the acidity from citric acid can hinder their growth. For the above reasons it is believed that some bacteria may be more prevalent in one type of fluid than the other. According to our findings, the microorganisms found in the ejaculate were similar to those found in prostatic fluid (*Escherichia coli*, *Enterococcus faecalis*, various species of *Staphylococcus*, etc). Although these bacteria can form biofilms and adhere to surfaces becoming resistant to antibiotics, in this study, most bacterial colonies were sensitive to most of the tested antibiotics. Of note, the resistance rate was found to be significantly higher in Group B (3 glass test) than in Groups A and C.

4) Alternative Methods: There are other diagnostic methods available, such as segmented urine cultures (Stamey Meares 4 glass test and 2-glass test), which some healthcare providers prefer, due to their perceived higher reliability. However, some authors have suggested that segmented tests do not display sufficient sensitivity and may underestimate the prevalence of bacterial prostatitis cases, thus misdirecting the therapeutic approach to the disease [11,12]. Moreover, the coexistent urethral infection found in patients with CBP indicates the continuity of the infection within the genitourinary tract system making thus the segmentation of urine cultures less important [13]. The total ejaculate culture test is much simpler to perform than the 4-glass or 2-glass tests and avoids the need for an unpleasant prostatic massage. A positive test diagnoses CBP even if the colony counts are low. However, a negative test does not necessarily rule it out [14,15]. Moreover, there is a misleading evaluation of chronic non-bacterial, inflammatory prostatitis due to the difficulty distinguishing immature sperm from leukocytes [6]. Given that the distinction between CP syndromes (bacterial/non-bacterial and inflammatory/non-inflammatory types) is based on the presence or absence of bacteria and/or inflammatory cells in the TE and PoE samples, the abovementioned fact may shift the diagnosis to another direction.

As shown in this study, both the total ejaculate-based culture test and the prostatic secretions culture-based tests can play significant roles in CBP diagnosis, but they suffer from different drawbacks and limitations. The fact that the findings from sperm cultures were comparable to those of EPS and post-PM cultures supports a supplementary role. Interestingly, Magri and associates

found that the addition of microscopic examination and sperm culture to the standard Meares-Stamey test (five-glass test) increased the sensitivity 3.6 and 6.5 times more than the 4-glass test and the 2-glass test respectively [14]. In the light of a novel perspective of inflammation of the prostate gland, as part of an infection affecting the entire male genitourinary tract system, CBP patients should indeed undergo a comprehensive investigation to identify the underlying cause and appropriate treatment. This investigation typically includes the standard Meares-Stamey test (four-glass test) as well as Urethral Smear and Total Ejaculate Culture. These tests are crucial for diagnosing and managing infections in the male genitourinary tract system, ensuring that patients receive the appropriate care and treatment.

## 5. Conclusions

A comparison of the three diagnostic tests showed no superiority of the total ejaculate-based culture test to the prostatic secretions culture-based tests. However, the addition of total ejaculate culture to either the 4-glass or the 2-glass tests may improve their diagnostic accuracy. Criteria for differentiating types of prostatitis that render the interpretation of the culture results difficult could be revised

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper

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## Abbreviations

The following abbreviations are used in this manuscript:

EPS	Expressed prostatic secretion
TE	Total ejaculate
VB1	first-void urine
VB2	second-void urine
VB3	third-void urine
PoPM	post-prostate massage
PoE	post ejaculate
PrPM	pre-prostate massage
PrE	pre-ejaculate

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