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**Semen cultures analysis : retrospective study during a six-year period
and interest in the management of infertility**

Concise title : Management infertility and semen culture analysis

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Abstract

40

Purpose : In the **past** decade, the number of couples consulting **an** Assisted Reproductive
42 Techniques (ART) center for infertility **has increased in most European countries. In France, sperm**
bacterial examination must be performed every 6 months in couples undergoing In Vitro
44 **Fertilization (IVF) cycles, according to 2010 French ART Guidelines.** The aim of this study was to
analyze the results from such samples at Nantes University Hospital **and to assess** their potential
46 **value** for infertile couples undergoing ART cycles.

Methods : Between 2003-2008, semen cultures performed were analyzed according to bacterial
48 **enumeration**, type and number of bacterial species and their antibiotic resistance profile.

Results and discussion : 14,119 semen cultures were performed **showing an annual increase of 45**
50 **%** from the start to the end of the study. The proportion of positive semen cultures was stable
throughout the study period (40 to 45 %). **Many** bacterial species **were** considered as contaminants
52 (coagulase-negative staphylococci, alpha-hemolytic streptococci). For pathogen agents (in most
cases Enterobacteriaceae), the antibiotic resistance profile revealed mostly a **susceptible** phenotype.
54 **At last, every positive bacterial result had direct consequences on IVF cycle management, with**
subsequent reinforced advice on hygiene procedure before sample collection and/or antibiotics
56 **prescription.**

58 197 words

60

62

64 Introduction

66 In France, over the past decade, an increasing number of couples consulted an Assisted
Reproductive Techniques (ART) center for the management of infertility [1]. In this framework, a
68 clinical and biological assessment of infertility is realized for each partner. For men, this assessment
systematically requires a semen analysis including a microbiological semen culture [2-3]. This
70 bacteriological analysis is indicated for the safety of the partner and to optimize the preparation of
the semen before insemination [4]. The purpose of the semen culture is to identify a possible
72 bacteriospermia in order to eradicate it and to prevent contamination of embryonic culture media. A
positive bacterial culture could result in fertilization failure and altered embryonic development,
74 leading to lowered pregnancy rates, making it an essential consideration before *in vitro* fertilization
(IVF) cycle.

76 This analysis has to be performed in a systematic fashion, as most men are asymptomatic. Indeed,
chronic bacterial infection of semen is rare, but may be a potential cause of male infertility [5].
78 Bacteria involved in that case could act on semen quality either directly on spermatozoa
(spermatozoa motility and viability), or indirectly on the seminal plasma or by forming anti-semen
80 antibodies [5]. Semen bacterial analysis can recover various bacterial species, including potential
pathogens (i.e. Enterobacteriaceae or *Staphylococcus aureus*), but also bacterial species usually
82 considered as contaminants (coagulase-negative staphylococci, alpha-hemolytic streptococci...)
originating from the urethral or cutaneous flora [5,6].

84
The aim of this study was to evaluate the results of semen analysis in the bacteriology laboratory at
86 Nantes University hospital and to assess the relevance of the procedure in the global management of
infertile patients.

88

90 **Materials and methods**

92 This retrospective study has been conducted at Nantes University Hospital for a six-year period,
between 2003 and 2008.

94

Patient selection

96 During the study period, each bacterial semen analysis performed was included. Most of the men
were referred for semen analysis before an IVF cycle (approximately 2000 per year ; semen
98 analysis was performed after a first infertility consultation or 6 weeks before each IVF cycle ;
control semen analysis was performed after antibiotherapy). Others were semen donor or men
100 referred for semen cryopreservation before potentially gonadotoxic treatment.

102 *Bacteriological analysis of semen*

Sample collection procedure

104 Before semen analysis, men were previously instructed to drink abundantly (generally, up to 1.5
liter) the day before semen collection in order to increase diuresis and to assure a cleaning of the
106 urethra, as previously reported [6]. Semen samples were collected at the ART center after 2 to 3
days of sexual abstinence. Just before semen collection, both verbal and written advices were given
108 by the medical team to the patient to follow a drastic hygiene procedure : first, men were asked to
wash hands, then to urinate and finally to wash their penis with chlorhexidine (aqueous
110 chlorhexidine, 0.05 %, one dose recipient, Gilbert laboratory, France). Semen was collected in an
aseptic recipient (MT245, JCD, La Mulatiere, France) after masturbation and analyzed after 30
112 minutes of liquefaction at 37°C.

114

116 *Bacteriological culture procedure*

After collection of the ejaculate into a sterile plastic receptacle, samples were aliquoted. Semen
118 aliquots (150 µL) were transferred within three hours to the bacteriology laboratory to quantify
aerobic rods only [7]. Semen samples were plated pure (10 µL) and diluted with sterile saline
120 solution at 1:10 (100 µL) on horse blood agar plates (bioMérieux, Marcy-l'Etoile, France). Media
were incubated for 24 to 48 hours in atmosphere supplemented with 5 % of CO₂ at 37 °C.

122

Diagnostic procedure

124 Each colony from the plate inoculated with diluted semen represented 10² CFU mL⁻¹. The bacterial
enumeration was categorized **as follows** : **enumeration** less than 10³ CFU mL⁻¹ **was** considered as
126 negative cultures **according to WHO criteria** [2] ; **enumeration** from 10³ CFU mL⁻¹ to 3 × 10³ CFU
mL⁻¹ or enumeration up to 3 × 10³ CFU mL⁻¹ were considered as positive **cultures**, in the case of
128 pure or predominant **flora** culture. According to the World Health Organization guidelines [2], a
bacterial concentration in the ejaculate up to 10³ bacteria mL⁻¹ **is a significant bacteriospermia**.
130 Therefore, no qualitative analysis was realized for samples with **enumeration less than** to 10³ CFU
mL⁻¹.

132 If necessary, bacteria were identified by fast and simple tests such as Lancefield's group
determination for streptococci (Streptex[®], DiaMondial, Sees, France) and agglutination test for
134 staphylococci (Statest[®], Biorad, Marnes-la-Coquette, France). For Enterobacteriaceae biochemical
identification was performed using **the** Vitek2[®] IDGN card (bioMérieux) and Api Coryne[®]
136 (bioMérieux) for *Corynebacterium seminale*.

Susceptibility testing was systematically performed **on bacterial strains potentially pathogenic or**
138 **resistant to antibiotics such as Enterobacteriaceae** (VITEK2[®] AST-N052, bioMérieux),
Staphylococcus aureus (VITEK2[®] AST-P551, bioMérieux) and *C. seminale* (disc diffusion
140 technique on horse blood agar plates at 37 °C in an atmosphere supplemented with 5 % of CO₂).

An antibiotic treatment was **indicated** in the following cases :

142 When an **Enterobacteriaceae** or a *S. aureus* was detected, an antibiotic treatment with good **uro-**
143 **genital** diffusion was recommended. Antibiotic regimen was in first intention, nitrofurantoin (150
144 mg per day, 8 days except for *Proteus-Providencia-Morganella*) or in second intention, ofloxacin
(400 mg per day, 10 days). Antibiotherapy was adapted according to susceptibility testing and a
146 control semen culture was systematically **performed** 10 days after the end of the treatment in order
to check the efficiency of the antibiotherapy.

148 **When *C. seminale*** was detected associated with leucospermia, an antibiotic treatment with good
uro-genital diffusion was also recommended.

150 **On the** opposite, no antibiotic treatment was established for the patient (i) when a monomicrobial
culture of *Enterococcus* (**enumeration** up to 3×10^3 CFU mL⁻¹) was detected, but a control semen
152 culture was systematically performed or (ii) when **a** plurimicrobial culture (i.e. up to 3×10^3 CFU
mL⁻¹) with cutaneous flora bacteria or *Enterococcus* was observed, suggesting a contamination
154 during semen collection.

156 **Results**

158 *Quantitative analysis*

From January 2003 1st to December 2008 31th, 14,119 samples from 7,723 men were included in
160 this study, representing an average of 1,287 patients per year.

The activity increased from 1,843 samples in 2003 to 2,664 in 2008, (+ 45 % - Figure 1). Although
162 the ratio of requests **per** patient was stable (ranging from 1.74 to 1.86 between 2003 and 2008),
there was a 35 % increase in the number of patients consulting and then included in the ART
164 program (1,060 in 2003 to 1,435 patients in 2008), reflecting the increasing activity of the ART
center during the study period.

166 The results have been interpreted according to bacterial **enumeration** and number of species
recovered (Table 1). **Very few samples were** classified as **non-interpretable** due to the invasion of

168 the culture by *Proteus* species. Furthermore, for a limited number of samples, the volume was
insufficient for bacterial enumeration. The percentage of positive semen cultures was relatively
170 stable during the 6 years with an average of 44.8 % (39.9 to 46.6 %), respectively 11 % with a
bacterial enumeration from 10^3 CFU mL⁻¹ to 3×10^3 CFU mL⁻¹ and 33.8 % with an enumeration up
172 to 3×10^3 CFU mL⁻¹. Whatever the enumeration for positive semen cultures, a majority of these
samples was polymicrobial (77 %).

174

Qualitative analysis : bacterial ecology of semen samples

176 The different types of bacteria recovered from semen cultures are summarized in Table 2. In most
cases, they corresponded to bacteria considered as contaminants or “belonging” in the male genital
178 tract (average : 50.3 %). These results were confirmed all over the study period. Enterococci were
found from 10.5 to 24.4% of the isolated species and Enterobacteriaceae from 11.7 to 27.5 %,
180 mostly *Escherichia coli* (57.1 % to 65.6 %).

Resistance pattern of the bacteria recovered

Between 2003 and 2008, out of the 14,119 semen cultures performed, 1,376 antibiotic susceptibility
184 tests were realized (9.75 %). In 2003, according to our criteria, 16.7 % of the requests required an
antibiotic susceptibility test compared with 7.2 % in 2008 (Table 3). In most cases, regardless of the
186 identified species, a susceptible phenotype was generally recovered. Isolation of multi-drug-
resistant bacteria (MDR) remained an exceptional phenomenon as shown in table 3. Six of these 10
188 cases concerned Enterobacteriaceae resistant to third-generation cephalosporins. Most of the
corresponding patients had spinal cord injury resulting in paraplegia, with a permanent urinary
190 catheter.

192

194 Discussion

196 This retrospective study during a six-year period revealed a 45% increase in the annual number of
198 semen cultures requests. Over a 10 year period (1998-2008) this increase was 137 % with a 72 %
200 increase of the number of patients consulting the ART center and a doubling of the number of
202 medical consultations from 2003 to 2008 (data not shown). These observations are part of the
204 national trend of increasing number of couples consulting for infertility [1].

It could be partly due to the increasing maternal age at the time of the first pregnancy in most
202 European countries (30 years in France) [8]. Moreover, improved and extensive medical and media
204 information about infertility allows faster and more efficient referral of couples experiencing
difficulties to conceive to ART centers.

206 During this study, the proportion of positive semen cultures remained stable during the study period
with an average of 44.8 %. Previously reported data appeared to be highly variable from one study
208 to another. Indeed, values ranging from 20 to a surprising 100 % of positive semen cultures were
reported [5-6, 9-12]. For example, Virecoulon *et al* worked with a threshold of 10^3 CFU mL⁻¹ [6].
210 Stovall *et al* reported 69 % of positive cultures with a threshold of 10^2 CFU mL⁻¹ [13]. Moretti *et al*
worked with a threshold of 10^4 CFU mL⁻¹ in case of Gram positive cocci but a 10^5 CFU mL⁻¹ in case
212 of Gram negative rods [12]. Finally, comparison between different studies is difficult since some
authors do not mention a specific positivity threshold [5].

214 An interesting parameter to consider is the influence of hand washing on the number of positive
semen cultures. Indeed, a study among couples followed for IVF has shown that the number of
216 positive semen cultures could fall from 92.9 to 39 % with instructions for washing hands before
sampling [14]. Furthermore, Boucher *et al.* have shown that direct verbal counseling of patients by
218 a member of the medical staff improved the bacterial quality of the sample compared with short
written instructions [15]. In our study, there was no change in the procedure of the ART center for

220 semen sampling (verbal instructions) and the bacterial ecology of semen sample was relatively
stable during the 6 years.

222

According to the results, a large proportion of species can be considered as contaminants, resulting
224 from bad asepsis during sampling in agreement with the literature [5-6,12]. High-prevalences for
coagulase-negative staphylococci (CNS) and for alpha-haemolytic streptococci were previously
226 reported [5,13]. CNS usually **predominate** in the distal urethra and reflect the aerobic and facultative
bacterial ecosystem of the **male genital tract**.

228 In our cohort, there was also a large proportion of enterococci which is controversial in the
literature. For example, Rodin *et al.* found a low proportion of 7.4 % [16]. Different teams have
230 worked on the role of enterococci in infertility and as far as we know, only one study suggested an
high incidence of oligozoospermia and teratozoospermia in human semen infected with *E. faecalis*
232 [17]. **Although the French guidelines in Microbiology recommended systematic search for
anaerobes [18], due to contradictory data in the literature, these bacteria were not searched. Indeed,
234 anaerobes normally colonize the male urethral orifice and therefore are not considered to be a major
cause of male infertility [7]. Urogenital *Mycoplasma* spp. were not systematically searched in
236 agreement with the clinicians and biologists of the ART center except for specific request [19].**

238 For Enterobacteriaceae, different data from the literature found a proportion of around 15 to 20 %
[5,20], which is in agreement with our results. A **recent** study has shown their potential **role** in
240 semen motility alteration [21]. Presence of Enterobacteriaceae in semen involves the prescription of
antibiotic treatment, with a significant potential impact on **the** digestive flora [22]. Here, the
242 presence of Enterobacteriaceae in semen involved a delay in the IVF procedure but had no
influence on the assisted reproductive technique outcome. The management of patients was shifted :
244 the IVF procedure was not started before negative semen culture was obtained, confirming that the
semen was free of infection.

246 The impact of bacteriospermia on semen parameters remains controversial [5, 13, 14, 20, 23].
Berkas *et al.* have shown the negative impact of certain species such as Enterobacteriaceae on
248 semen motility *in vitro* [21]. Merino *et al.* found that bacterial infection can cause alteration in
seminal characteristics, such as volume, motility and viability [5]. Nevertheless, the lack of
250 association between bacteriospermia and abnormal semen parameters or the occurrence of adverse
events in IVF was also reported [13-14, 23]. However, bacterial examination of semen remains
252 necessary before IVF to avoid contamination of culture media, especially for those who do not
contain antibiotics, which is the case in our IVF center.

254 Regarding antibiotic resistance, whatever the species studied, antibiotic susceptibility testing
revealed mostly a **susceptible** phenotype. Only ten multi-drug-resistant (MDR) bacteria **isolated**
256 were recovered during the six-year period. Clinical data analysis revealed that most patients with
MDR bacteria had spinal chord injury and **consequent** paraplegia, leading to permanent urinary
258 catheter use with potential repeated antibiotic regimens.

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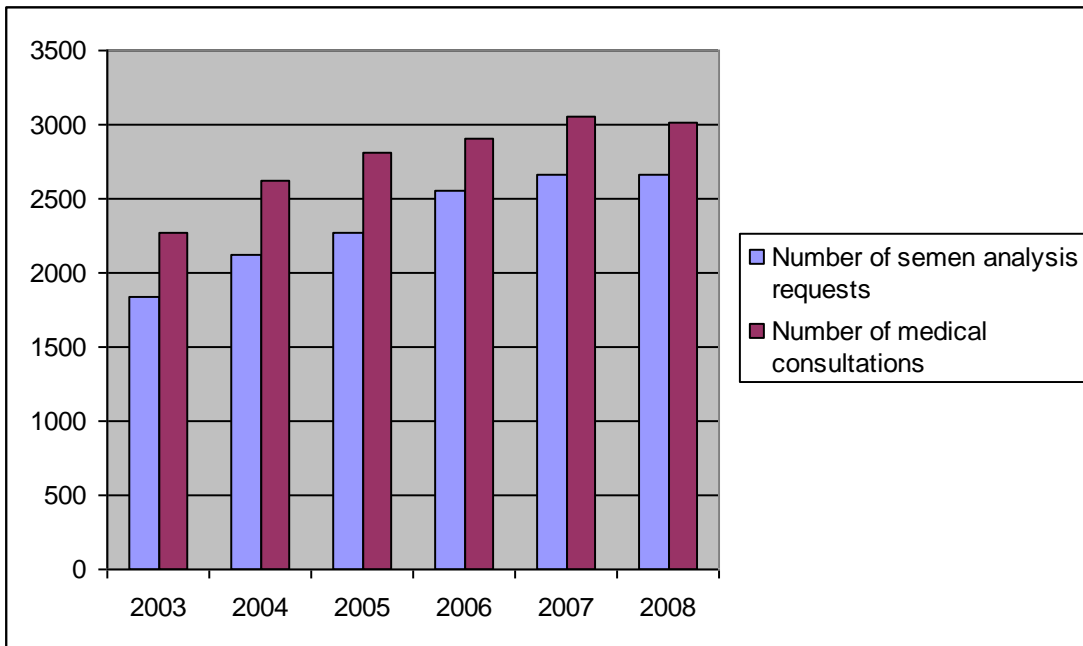
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326

328 **Figure 1 :** Evolution of the activity (number of semen analysis *versus* number of medical
330 consultations) between 2003 and 2008

330



332

334

Table 1 : Evolution and characteristics of semen culture requests, at Nantes University Hospital,

336 between 2003 and 2008

	2003		2004		2005		2006		2007		2008	
Number of requests	1843		2127		2269		2556		2660		2664	
Number of patients	1060		1167		1303		1359		1399		1435	
< 1000 CFU/mL	968	52.5 %	1117	52.5 %	1239	54.6 %	1519	59.4 %	1432	53.8 %	1413	53 %
> ou = 1000 et < 3000 CFU/mL	165	9 %	229	10.8 %	290	12.8 %	279	10.9 %	286	10.8 %	317	11.9 %
1 type of microbial colonies	52	31.5 %	62	27.1 %	48	16.6 %	53	19 %	59	20.7 %	82	25.90 %
2 types of microbial colonies	52	31.5 %	54	23.6 %	63	21.7 %	55	19.7 %	29	10.1 %	46	14.50 %
3 types of microbial colonies	24	14.6 %	38	16.6 %	120	41.4 %	130	46.6 %	168	58.7 %	161	50.80 %
4 types of microbial colonies	1	0.6 %	42	18.3 %	55	18.9 %	37	13.3 %	28	9.8 %	25	7.90 %
> or = 5 types of microbial colonies	36	21.8 %	33	14.4 %	4	1.4 %	4	1.4 %	2	0.7 %	3	0.90 %
> ou = 3000 CFU /mL	676	36.7 %	762	35.8 %	718	31.6 %	741	29 %	933	35.1 %	920	34.5 %
1 type of microbial colonies	118	17.5 %	160	21 %	126	17.6 %	199	26.9 %	213	22.8 %	312	33.9 %
2 types of microbial colonies	157	23.2 %	146	19.2 %	131	18.3 %	125	16.9 %	142	15.2 %	154	16.8 %
3 types of microbial colonies	76	11.2 %	94	12.3 %	207	28.8 %	243	32.8 %	386	41.4 %	279	30.3 %
4 types of microbial colonies	17	2.5 %	177	23.2 %	207	28.8 %	152	20.5 %	175	18.8 %	149	16.2 %
> or = 5 types of microbial colonies	308	45.6 %	185	24.3 %	47	6.5 %	22	2.9 %	17	1.8 %	26	2.8 %
Uninterpretable*	4	0.2 %	1	0.05 %	2	0.1 %	0	0 %	5	0.2 %	4	0.2 %
Insufficient amount	30	1.6 %	18	0.85 %	20	0.9 %	17	0.7 %	4	0.2 %	10	0.4 %

* due to the invasion of the culture by *Proteus mirabilis*

338

Table 2 : Distribution of bacterial species isolated from semen cultures, at Nantes University

340 hospital, between 2003 and 2008

	2003		2004		2005		2006		2007		2008	
	N	%	N	%	N	%	N	%	N	%	N	%
alpha-haemolytic <i>Streptococci</i>	288	34.3	187	32.6	126	28.9	144	27.1	115	21.9	258	33.7
coagulase negative <i>Staphylococci</i>	235	28	107	18.7	78	17.9	111	20.9	93	17.8	155	20.2
<i>Enterococcus</i>	159	19	60	10.5	82	18.8	105	19.8	128	24.4	136	17.7
Group B <i>Streptococcus</i>	16	1.9	27	4.7	24	5.5	20	3.8	22	4.2	25	3.3
<i>Staphylococcus aureus</i>	1	0.1	5	0.9	4	0.9	8	1.5	3	0.6	6	0.8
β-haemolytic <i>Streptococcus</i>	10	1.2	4	0.7	4	0.9	7	1.3	7	1.3	1	0.1
<i>Escherichia coli</i>	56	6.7	101	17.6	63	14.4	75	14.1	92	17.6	111	14.5
<i>Proteus/Providencia/Morganella</i>	13	1.5	22	3.8	15	3.5	16	3	20	3.8	45	5.9
Other <i>Enterobacteria</i>	29	3.5	31	5.4	27	6.2	26	4.9	32	6.1	22	2.9
<i>Pseudomonas aeruginosa</i>	1	0.1	2	0.4	4	0.9	2	0.4	1	0.2	4	0.5
<i>Corynebacterium seminale</i>	17	2	21	3.7	3	0.7	2	0.4	1	0.2	3	0.4
Others	14	1.7	6	1	6	1.4	15	2.8	10	1.9	0	0
Total	839	100	573	100	436	100	531	100	524	100	766	100

342

344

Table 3 : Evolution of number of antibiotic susceptibility testing between 2003 and 2008.

Years	Number of requests	Number of antibiotic susceptibility testing	Number of MDR bacteria
2003	1843	308	2
2004	2127	307	1
2005	2269	220	2
2006	2556	205	0
2007	2660	144*	4
2008	2664	192	1
Total	14119	1376	10

346 * In agreement with ART physicians, antibiotic susceptibility testing were stopped for *Enterococci* since the end of the year 2006.

348