Elimination of bacteria from human semen during sperm preparation using density gradient centrifugation with a novel tube insert

J. Fourie[1], N. Loskutoff[2] and C. Huyser[1]

[1]Department of Obstetrics & Gynaecology, Steve Biko Academic Hospital, University of Pretoria; South Africa

[2]The Bill & Berniece Grewcock Center for Conservation & Research, Omaha, USA;

Short Title:

Bacteria in human semen and removal thereof

Keywords:

Bacteria, density gradient centrifugation, sperm preparation, semen decontamination

Correspondence

J. M. Fourie, Reproductive Biology Laboratory, Department of Obstetrics and Gynaecology, University of Pretoria, Steve Biko Academic Hospital, Pretoria, South Africa.

Tel.: +27 (12) 354 2410/2208

Fax: +27 (12) 329 6258

E-mail: jozef.fourie@up.ac.za

Summary

The occurrence of bacteria in sperm samples intended for *in vitro* fertilization, can compromise the outcome of assisted reproductive techniques. Effective semen processing procedures should therefore be implemented to remove bacteria from semen. Unfortunately, technique failure does occur whereby bacteria can be found in processed sperm preparations. To improve the effectiveness of semen processing, a novel centrifuge tube insert was developed to facilitate the layering of density gradients and semen, and to prohibit the re-infection of purified sperm pellets. The purpose of this study was to: 1) determine the prevalence and type of bacteria present in semen of patients participating in the Unit's assisted reproduction program, and 2) evaluate the effectiveness of density gradient centrifugation with the novel tube insert, for the elimination of bacteria and yeast from spiked human semen samples. A survey in 2007-2010 indicated that 50% of semen samples were found to have positive bacterial cultures. Semen processing by means of density gradient centrifugation with the novel tube insert, eliminated significantly more *in vitro* derived (spiked) bacteria and yeast from semen compared to processing without the insert ($P<0.004$). Therefore, it is highly recommended that the centrifuge tube insert, ProInsert^{TM}, be incorporated into assisted reproductive programs.

Introduction

Bacteriospermia, the occurrence of bacteria in semen (Keck *et al.*, 1998), is present among 54% - 57% of patients attending infertility centres (Cottell *et al*., 2000; Gdoura *et al*., 2008; Kiessling *et al*., 2008) with the presence of bacteria in semen attributed to systemic and local reproductive tract infections, or to contamination post-ejaculation (Bielanski, 2007). During assisted reproductive techniques (ART) including intra-uterine insemination (IUI), *in vitro* fertilization and intracytoplasmic sperm injection (Krissi *et al*., 2004), the natural immunological defence mechanisms present in the female reproductive tract are bypassed (Cottell *et al*., 1997). Consequently, bacteria may be introduced into the upper genital tract, or the embryo culture system, potentially leading to a compromised outcome of ART and, or infection of the female genital tract (Cottell *et al*., 2000; Cottell *et al*., 1997; Kastrop *et al*., 2007; Huyser *et al*., 1991). The frequency of micro-organism infections post-IUI is approximately 0.01% (Broder, Sims & Rothman, 2007) and infections of *in vitro* embryo culture systems, range between 0.35% and 0.68% (Kastrop *et al*., 2007; Cottell *et al*., 1996).

Patients with semen cultures positive for bacteria should undergo antibiotic treatment prior to ART. However, antibiotic treatment will be ineffective against skin contaminants present in semen. Therefore, antibiotics (penicillin, streptomycin and gentamycin) are usually added to semen processing and embryo culture media (Cottell *et al*., 1997; Kastrop *et al*., 2007; Magli *et al*., 1996; Gardner & Lane, 2007). This addition of antibiotics to culture media, however, may result in antibiotic resistant bacterial strains (Kastrop *et al*., 2007), as well as a decreased embryo cleavage rate (Magli *et al*., 1996; Lemeire, Van Merris & Cortvrindt, 2007). Semen washing, with an extra swim-up step, has been reported to be more effective in decreasing the incidence of potential pathogens in sperm samples compared to antimicrobial therapy by prescription antibiotics (Huyser *et al*., 1991).

Semen processing procedures that are effective in the elimination of bacteria from semen are required. Depending on the processing method employed, 5% - 43% of sperm samples will remain positive for bacteria post-processing (Cottell *et al*., 1997; Huyser *et al*., 1991; Knox *et al*., 2003). Procedural failure could be attributed to the contamination of the sperm pellets post-processing. A medical grade polypropylene, centrifuge tube insert (Proinsert™, Nidacon International, Mölndal, Sweden), has been developed to avoid inadvertent contamination without further washing (Loskutoff *et al*., 2005).

The purpose of this study was; 1) to determine the prevalence of bacteria in semen of men attending an infertility centre at Steve Biko Academic Hospital (SBAH), and 2) to evaluate the effectiveness of density gradient centrifugation (DGC) using a centrifuge tube insert for the removal of prevalent bacteria and yeast from spiked human semen samples.

Materials and Methods

Institutional approval for the study was received from SBAH and the Medical Research Council's Ethics Committee, University of Pretoria (protocol number 37/08).

Prevalence of bacteria in semen

The prevalence of bacteria in semen samples (n=1,210) from men (n=1,038) participating in the ART program at SBAH were surveyed in 2007-2010.

Patients were requested to sexually abstain for three days. Guidelines to deliver semen samples for diagnostic evaluation (World Health Organization, 2010), were verbally discussed with patients (Boucher *et al*., 1995). These guidelines were also available in a written format in four national indigenous languages.

Following liquefaction (37^{o}C for 30 minutes), 200µl semen aliquots were submitted to the National Health Laboratory's Microbiology Department for microscopy, culture and sensitivity evaluation according to the Unit's standard operating procedures (SOP) (Working Group, Tshwane Academic Division, National Health Laboratory Service, Department of Microbiology, University of Pretoria, 2006).

Semen processing for the elimination of bacteria and yeast from spiked semen samples

Semen from donors (n=5) were collected, pooled and gram-stained to ensure the absence of micro-organisms according to SOP. The pooled sperm concentration was adjusted to 40 x 10^6 spermatozoa/ml by dilution with PureSperm Wash (PSW-100, Nidacon International). Subsequently, 1 ml aliquots of the pooled semen sample were inoculated with bacteria, or yeast commonly found in semen. *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (in-house strain), *Enterococcus faecalis* (ATCC 29212), Coagulase-negative staphylococci (in-house strain), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 90028), were individually added to the semen aliquots (in duplicate) at concentrations of 1 x 10^3, 10^4, 10^5 and 10^6 colony forming units /ml (CFU/ml). The inoculated semen samples were processed using DGC (PureSperm® 40 & 80%, Nidacon International) with and without the use of the polypropylene centrifuge tube insert (ProInsert™, Nidacon International) (Figure 1), without an additional swim-up step. Bacteria and yeast quantifications were performed by inoculating Mac-Conkey and blood agar plates with 10 µl aliquots of the processed sperm samples. The numbers of colony forming units present were macroscopically counted following a 24 hour incubation period at 37^{o}C. Non-spiked semen samples served as negative controls and unprocessed spiked semen samples were included as positive controls.

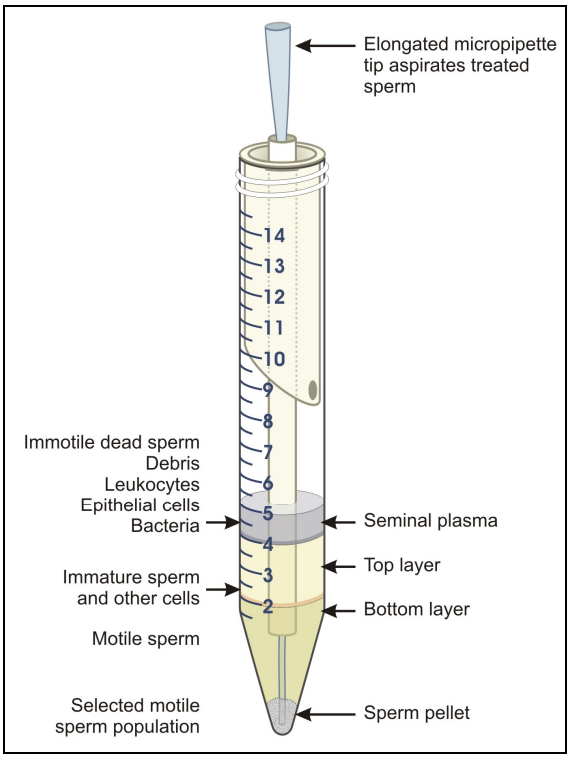

Figure 1: Centrifuge tube containing medical grade polypropylene insert (ProInsert™, Nidacon International, Mölndal, Sweden)

Statistical analysis

Stata Statistical Software: Release 10 (StataCorp., 2007) was used to perform a two factor analysis of variance (ANOVA) to compare the numbers of bacteria and yeast colony forming units present in sperm samples post-processing, either with or without the insert, and at two spiking concentrations [log ($1x10^5$ and $1x10^6$) CFU/ml].

Results

Prevalence of bacteria in semen

The prevalence of bacteria in semen is indicated in Table 1.

Table 1: Prevalence of bacteria in semen samples (n= 1,210) of patients participating in an ART programme at SBAH surveyed in 2007-2010

Organism	Gram	No.	(%)
No Growth		611	50%
Coagulase-negative staphylococci	+	280	23%
Escherichia coli	-	53	5%
Staphylococcus aureus	+	45	4%
α-haemolytic streptococci	+	45	4%
Enterococcus spp.	+	39	3%
Enterococcus faecalis	+	38	3%
β-haemolytic streptococci	+	16	2%
Klebsiella pneumoniae	-	17	1%
Enterococcus feacium	+	11	1%
Enterobacter cloacae	-	9	1%
Streptococcus spp.	+	9	1%
Staphylococcus spp.	+	12	1%
Ureaplasma spp.	Null	145	12%
Mycoplasma spp.	Null	57	5%

Pseudomonas spp., *Citrobacter* spp., *Haemophilus* spp., *Micrococcus* spp., *Neisseria* spp., *Bacillus* spp., *Acinetobacter* spp., *Enterococcus* spp. and *Aeromonas hydrophila* were present in <1% of semen samples.

Semen processing for the elimination of bacteria and yeast from spiked semen samples

Sperm pellet retrieval using the novel ProInsert™ eliminated recontamination, and removed significantly more micro-organisms (96%) from semen compared to processing without the insert [$P<0.004$ with respect to mean log(cfu)]. Treated sperm pellets remained clear of micro-organisms below the spiking concentration of 1 x 10^5 cfu/ml. Bacterial and yeast concentrations (cfu/ml) present after processing with and without the insert are illustrated in Table 2.

Table 2: Number of bacterial colony forming units/ml present in sperm samples post-DGC processing with and without the novel tube insert* (duplicate observations per spiking concentration)

Treatment		Micro-organisms (CFU/ml) present after processing					
Spiking Concentration (CFU/ml)		*Entero-bacter cloacae* In-house	*Entero-coccus faecalis* ATCC 29212	*Escherichia coli* ATCC 25922	*Coagulase-negative Staph.* In-house	*Staph. aureus* ATCC 25923	*Candida albicans* ATCC 90028
	Without Insert						
1×10^3	1.1	100	0	100	100	0	0
	1.2	100	0	100	400	0	0
1×10^4	2.1	100	4400	400	600	100	300
	2.2	1100	12100	600	1200	100	400
1×10^5	3.1	4000	24800	23300	2200	2500	700
	3.2	7000	26800	25200	3700	5500	900
1×10^6	4.1	25200	35000	49500	16000	15800	2200
	4.2	29600	36700	63600	23700	16700	1900
	With Insert						
1×10^3	1.1	0	0	0	0	0	0
	1.2	0	0	0	0	0	0
1×10^4	2.1	0	0	0	0	0	0
	2.2	0	0	0	0	0	0
1×10^5	3.1	0	200	100	100	400	0
	3.2	0	200	100	300	900	0
1×10^6	4.1	400	6700	100	1200	1400	200
	4.2	700	9500	100	1400	1500	200

*Processing with the insert significantly ($P<0.004$) reduced the bacteria with respect to mean log(cfu) for all six micro-organisms.

Discussion

Presence of bacteria in semen

Bacteria were present in 50% of semen samples from men seeking ART at SBAH during the period 2007-2010. This is in agreement with results from studies that reported bacterial prevalence in neat semen samples to be between 54% and 57% (Cottell *et al*., 2000; Gdoura *et al*., 2008; Kiessling *et al*., 2008). The presence of bacteria in an *in vitro* embryo culture

system can compromise the outcome of assisted reproductive procedures, by impacting directly on sperm quality by a reduction in motility (Nunez-Calonge *et al*., 1998), by the induction of apoptosis/necrosis (Villegas *et al*., 2005), or by causing degeneration of *in vitro* fertilized oocytes (Huyser *et al*., 1991). During embryo transfer the introduction of pathogens, such as *Mycoplasma genitalium,* into the uterus can lead to intra-uterine infections that may lead to infertility (Kastrop *et al*., 2007; Grzesko *et al*., 2009). Infected sperm samples used for *in vitro* fertilization are a considerable cause (35%) of infected embryo culture systems (Kastrop *et al*., 2007). Therefore, the sterile delivery and effective preparation of sperm samples intended for use in ART should be a priority.

Urination and proper washing prior to collection of a semen sample is recommended by the World Health Organization (WHO, 2010). Appropriate washing will significantly reduce bacterial infection of semen samples (Krissi *et al*., 2004); nevertheless, micro-organisms resident within the male genital tract will continue to contaminate ejaculates (Woolley *et al*., 1992). Semen samples from patients enrolling in an ART program should be examined for the presence of micro-organisms and those patients presenting with reproductive tract infections should undergo antibiotic treatment prior to ART. However, microorganisms such as *Mycoplasma genitalium,* cannot be cultured on substrates generally used for the detection of mycoplasmas. Patients with asymptomatic undetected infections will therefore be overlooked and the infection will remain untreated (Grzesko *et al*., 2009). Due to the fact that bacterial presence in semen is mostly attributable to contamination by skin flora (Krissi *et al*., 2004; Kim & Goldstein, 1999), treatment of these patients with antibiotics will be ineffective (Huyser *et al*., 1991). Therefore, the importance of strictly adhering to the prescribed washing guidelines to deliver a semen sample must be stressed to patients (Boucher *et al*., 1995). Sufficient washing prior to the delivery of a semen sample will reduce the presence of outer

skin contaminants, but will not eliminate bacteria, since microorganisms may also be present in the anterior urethra (Kohn *et al*., 1998; Damirayakhian, Jeyendran & Land, 2006).

The last line of defence against seminal-derived bacterial contamination of the embryo culture system, is semen processing, utilizing strict aseptic techniques and proper changing of sterile pipette tips and tubes between the DGC and washing procedures (Nicholson *et al*., 2000). Unfortunately technical error often occurs. Bacterial contaminants, as well as sexual transmitted pathogens such as *Ureaplasma parvum* and *Ureaplasma urealyticum,* have been found in processed sperm samples (Cottell *et al*., 1997; Huyser *et al*., 1991; Knox *et al*., 2003). During standard processing methods, the supernatant is aspirated to allow access to the purified sperm pellet. Pathogens from the upper layers can adhere to the inside surface of the test tube and flow down to re-infect the purified sperm. Details of the method have been previously described (Loskutoff *et al*., 2005). The current study demonstrated that, by utilizing the ProInsert™, re-infection of the purified sperm pellets post-DGC was and significantly (96%) more bacterial colony forming units were removed from semen when compared to processing without the insert ($P<0.004$), all without a further swim-up step. Treated sperm pellets remained clear of bacteria below the spiking concentration of 1×10^5 CFU/ml. Similar semen processing methods using the ProInsert™ has also proved to be effective in the removal of human immunodeficiency virus subtype 1 (HIV-1) and hepatitis C virus from *in vitro* spiked semen (Loskutoff *et al*., 2005).

In conclusion, the high prevalence of seminal pathogens warrants the need for improved semen processing procedures. In the present study the novel ProInsert™ device facilitated discontinuous density gradient layering, retrieval of the treated sperm pellet without recontamination, and effective removal of selected seminal pathogens. Used test tubes

containing the insert after semen processing can be capped and the potential hazardous material contained within the test tube can be appropriately disposed of. The ProInsert™, therefore, allows for a cost-effective and user-friendly means to improve the effectiveness of DGC to eliminate pathogens from semen. The results of this report reflects the comments by Anderson & Politch (2003) that more attention be given to develop improved semen processing methods, particularly in developing countries.

Acknowledgements

The authors would like to express their gratitude to: Prof. P. Becker, Biostatistics Unit Medical Research Council (MRC) – Statistical evaluation; Dr. R. Lekalakala Dept of Microbiology, NHLS – Verification of data on micro-organsms; Nidacon International – provision of PureSperm® products and ProInserts™; The MRC – funding of research. The views expressed by the authors do not necessarily reflect the views of the MRC.

References

Anderson D & Politch J, (2003) Providing fertility care to HIV-1 Serodiscordant couples: A Biologists's point of view. Am J Biotech 3(1):47-49

Bielanski A, (2007) Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. *Theriogenology* 68(1):1-22.

Boucher P, Lejeune H, Pinatel MC & Gille Y (1995) Spermoculture: improvement of the bacteriological quality of samples by direct verbal counselling before semen collection. *Fertil Steril* 64(3):657-660.

Broder S, Sims C & Rothman C (2007) Frequency of postinsemination infections as reported by donor semen recipients. *Fertil Steril* 88(3):711-713.

Cottell E, Harrison RF, McCaffrey M, Walsh T, Mallon E & Barry-Kinsella C (2000) Are seminal fluid microorganisms of significance or merely contaminants? *Fertil Steril* 74(3):465-470.

Cottell E, Lennon B, McMorrow J, Barry-Kinsella C & Harrison RF (1997) Processing of semen in an antibiotic-rich culture medium to minimize microbial presence during in vitro fertilization. *Fertil Steril* 67(1):98-103.

Cottell E, McMorrow J, Lennon B, Fawsy M, Cafferkey M & Harrison RF (1996) Microbial contamination in an in vitro fertilization-embryo transfer system. *Fertil Steril* 66(5):776-780.

Damirayakhian M, Jeyendran RS & Land SA (2006) Significance of semen cultures for men with questionable semen quality. *Arch Androl* 52(4):239-242.

Gardner DK & Lane M (2007) Embryo Culture Systems: in *In Vitro Fertilization: A Practical Approach*, ed. D.K. Gardner, Informa Healthcare USA, Inc., New York, NY, pp. 221-282.

Gdoura R, Kchaou W, Znazen A, Chakroun N, Fourati M, Ammar-Keskes L & Hammami A (2008) Screening for bacterial pathogens in semen samples from infertile men with and without leukocytospermia. *Andrologia* 40(4):209-218.

Grzesko J, Elias M, Maczynska B, Kasprzykowska U, Tlaczala M & Goluda M (2009) Occurrence of Mycoplasma genitalium in fertile and infertile women. *Fertil Steril* 91(6):2376-2380.

Huyser C, Fourie FL, Oosthuizen M & Neethling (1991) Microbial flora in semen during in vitro fertilization. *J In vitro Fert Embryo Transf* 8(5):260-264.

Kastrop PM, de Graaf-Miltenburg LA, Gutknecht DR & Weima SM (2007) Microbial contamination of embryo cultures in an ART laboratory: sources and management. *Hum Reprod* 22(8):2243-2248.

Keck C, Gerber-Schäfer C, Clad A, Wilhelm C & Breckwoldt M (1998) Seminal tract infections: impact on male fertility and treatment options. *Hum Reprod* 4(6):891-903

Kiessling A, Desmarais BM, Yin HZ, Loverde J & Eyre RC (2008) Detection and identification of bacterial DNA in semen. *Fertil Steril* 90(5):1744-1756.

Kim FY & Goldstein M (1999) Antibacterial skin preparation decreases the incidence of false-positive semen culture results. *J Urol* 161(3):819-821.

Knox CL, Allan JA, Allan JM, Edirisinghe WR, Stenzel D, Lawrence FA, Purdie DM & Timms P (2003) Ureaplasma parvum and Ureaplasma urealyticum are detected in semen after washing before assisted reproductive technology procedures. *Fertil Steril* 80(4):921-929.

Kohn FM, Erdmann I, Oeda T, el Mulla KF, Schiefer, HG & Schill WB (1998) Influence of urogenital infections on sperm functions. *Andrologia* 30(Suppl 1):73-80.

Krissi H, Orvieto R, Ashkenazi J, Gilboa Y, Shalev J, Moscovitch I & Bar-Hava I (2004) Effect of contaminated preprocessed semen on fertilization rate and embryo quality in assisted reproductive techniques. *Gynecol Endocrinol* 18(2):63-67.

Lemeire K, Van Merris V & Cortvrindt R (2007) The antibiotic streptomycin assessed in a battery of in vitro tests for reproductive toxicology. *Toxicol in Vitro* 21(7):1348-1353.

Loskutoff NM, Huyser C, Singh R, Walker DL, Thornhill AR, Morris L & Webber L (2005) Use of a novel washing method combining multiple density gradients and trypsin for removing human immunodeficiency virus-1 and hepatitis C virus from semen. *Fertil Steril* 84(4):1001-1010.

Magli MC, Gianaroli L, Fiorentino A, Ferraretti AP, Fortini D & Panzella S (1996) Improved cleavage rate of human embryos cultured in antibiotic-free medium. *Hum Reprod* 11(7):1520-1524.

Nicholson CM, Abramsson L, Holm SE & Bjurulf E (2000) Bacterial contamination and sperm recovery after semen preparation by density gradient centrifugation using silane-coated silica particles at different g forces. *Hum Reprod* 15(3):662-666.

Nunez-Calonge R, Caballero,P, Redondo C, Baquero F, Martinez-Ferrer M & Meseguer MA (1998) Ureaplasma urealyticum reduces motility and induces membrane alterations in human spermatozoa. *Hum Reprod* 13(10):2756-2761.

StataCorp. (2007) *Stata Statistical Software: Release 10*, 10th edn, StataCorp LP., College Stattion, TX.

Villegas J, Schulz M, Soto L & Sanchez R (2005) Bacteria induce expression of apoptosis in human spermatozoa. *Apoptosis* 10(1):105-110.

Woolley PD, Kinghorn GR, Bennett KW & Eley A (1992) Significance of Bacteroides ureolyticus in the lower genital tract. *Int J STD AIDS* 3(2):107-110.

Working Group, Tshwane Academic Division, National Health Laboratory Service, Department of Microbiology, University of Pretoria (2006) *Standard operating procedure; Processing om male genital tract specimens, TADM0251*.

World Health Organization (2010) *WHO* Laboratory manual for the examination and processing of human semen, 5th edn. Cambridge University Press, Cambridge.