Semen decontamination for the elimination of seminal HIV-1

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Abstract

The risk of HIV transmission to the female partner, or potential offspring of an HIV-1 infected male can be reduced using semen decontamination procedures prior to assisted reproductive treatment (ART). The objective of this study was to determine the efficiency to decontaminate semen samples (N=186) from 95 HIV-1 sero-positive patients. Aliquots of neat semen were submitted for viral validation by qualitative and quantitative polymerase chain reaction. Semen samples were processed by density gradient centrifugation in combination with a ProInsert\textsuperscript{TM} tube where after aliquots of the processed sperm samples were analysed for the presence of HIV-1. Fifty four percent of all tested neat semen samples tested positive for HIV-1 DNA, RNA or both (13.4\%, 11.3\% and 29.0\%, respectively). From a total of 103 processed sperm samples that were submitted for viral validation, two samples tested positive for HIV-1 DNA and none for RNA. In conclusion, semen processing with the ProInsert\textsuperscript{TM} followed by viral validation of processed sperm samples should be performed when providing assisted reproductive treatment to couples where the male partner is HIV-1 sero-positive.
Key words
Density gradient centrifugation ∙ HIV-1 ∙ Semen processing ∙ Semen decontamination

INTRODUCTION
The general transformation towards a more accommodating approach to the provision of assisted reproductive treatment (ART) to human immunodeficiency virus type-1 (HIV-1) sero-positive patients, is in part due to the improved clinical care of infected patients, as well as by improvements made in risk reduction procedures during ART (Zutlevics, 2006). However, concerns regarding the treatment of patients with potential seminal pathogens during ART include: i) the probability of vertical and horizontal transmission of the specific pathogen, ii) the welfare of children with infected parents that may pass away due to the infection (Lyerly and Anderson, 2001) and iii) the possibility of nosocomial transfer of pathogens in an ART laboratory (Englert et al., 2004).

Infection with the human immunodeficiency virus (HIV) is no longer considered as life threatening to patients. Improvements made in multidrug highly active antiretroviral therapy (HAART) has resulted in infection with HIV becoming a manageable, chronic disease (Lambert-Niclot et al., 2012). However, the majority of infected individuals are in their reproductive years with the desire to have their own genetically related offspring (Sauer, 2005; Paiva et al., 2007; Kanniappan et al., 2008), and it would be unethical to deny access to ART (Englert et al., 2001; Gilling-Smith, Smith and Semprini, 2001). A recent South African HIV prevalence survey in 2012, by Shisana et al. (2014) indicated that 12.2% of the national population (6.4 million persons) were HIV+. Males in the age group of 35-39 years presented with the highest prevalence of 28.8% (31.6% of females tested positive for HIV), followed by 25.6% and 17.3 % for males aged 30-34 and 25-29, respectively. HIV-prevalence of females was 36% and 28.4% for the latter age groups. A 2009 survey investigating the sero-prevalence of HIV-1 among patients (N=303) seeking ART at the Reproductive Biology Laboratory (RBL) at Steve Biko Academic Hospital (SBAH), indicated that 14.4% of couples were sero-discordant (7.1% male positive and 7.3% female positive), and 9.3% of couples were sero-concordant (unpublished data). A total of 16.4% (9.3% + 7.1%) of male patients seeking ART at SBAH are therefore HIV-1 sero-positive.
Shedding of HIV into the semen of patients taking HAART may occur (Zhang et al., 1998; Halfon et al., 2010), possibly due to inflammation and co-infections and/or poor adherence to their HAART regimens. Halfon et al., (2010) described a 3% HIV-1 RNA for blood plasma viral load (BPVL) negative and seminal viral load (SVL) positive, with an overall rate of 7% detectable HIV-1 in semen. Similarly, 6.6% of all semen samples of patients seeking ART in 2002-2011, tested positive for HIV-1 RNA, despite undetectable BPVL in a study by Lambert-Niclot et al., (2012). Conversely, Sheth and co-workers (2009) noted a relative high cell-free virus (HIV-RNA) load in seminal plasma (48%), and is of opinion that cell-associated HIV (HIV-DNA) may also persist in semen despite HAART. A small test-sample by Zhang et al., (1998) indicated that four out of seven HIV-1+ males with undetectable BPVL, presented with positive proviral DNA in semen samples. The infectious potential of semen, even from males receiving HAART, should therefore not be underestimated. Assisted reproductive procedures combined with semen decontamination should be performed to reduce the risk of vertical and horizontal transmission of the virus (Huyser, 2010). Semen processing to harvest an optimal quality sperm sample is a well-established and effective procedure in ART laboratories. However, technique failure does occur, probably due to re-contamination of the processed sperm samples after density gradient centrifugation (Loskutoff et al., 2005), resulting in the detection of HIV in processed sperm samples (Leruez-Ville et al., 2002; Fiore et al., 2005). Risk reduction procedures performed during semen decontamination should be evaluated to ensure the effective and safe ART for HIV-1 sero-positive male patients.

In this study, the effectiveness of semen processing by discontinuous density gradient centrifugation in combination with the ProInsert™ (Nidacon International, Mölndal, Sweden) for the removal of HIV-1 from semen samples of HIV-1 sero-positive patients was evaluated.

**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).
Participants
HIV-1 sero-positive patients that participated in the semen decontamination program at RBL were required to provide the Unit with recent (<3 months old) blood viral validation results (HIV-1 RNA and CD4 count). Leukocytes expressing the CD4 receptor are the major target cells for HIV, and decreased CD4 counts are correlated with a suppressed immune response (Lawn et al., 2006). Patients with CD4+ lymphocyte counts below 300 cells/µL were therefore excluded from the program, and were referred to a virologist for antiviral therapy. Patients could only enter the ART program once the CD4+ lymphocyte count increased to above 300 cells/µL, and semen decontamination was successfully performed. The semen decontamination procedure and potential risks involved in using purified sperm from HIV-1 positive men were discussed with patients. Participants were then required to sign an informed consent form providing the Unit permission to:

i) Perform the semen decontamination procedure.

ii) Submit a neat diagnostic semen sample and purified sperm sample for HIV-1 validation.

iii) Cryopreserve and store purified sperm samples.

iv) Utilize sperm samples for potential ART use, should these samples present with HIV-1 loads below the lowest limit of detection (LLD).

A unique identification code was assigned to each participant in the program to ensure anonymity.

Collection of semen samples
From 2008 to 2012, 95 HIV-1 infected men that enrolled in the semen decontamination program were requested to provide two semen samples by masturbation per week (Monday and Wednesday, or Tuesday and Thursday), for one to two consecutive weeks, depending on the quality of the processed sperm samples. Most patients therefore were required to provide the Unit with 4 semen samples. However, in cases where sperm yield was too low for a specific ART procedure, additional semen samples were required from the patient. The first semen samples delivered each week served as diagnostic samples and those delivered on the second visit of each week were processed for therapeutic use during ART. Patients were instructed to seal the semen containing sample cup using a re-sealable zipper storage bag, placed in a polystyrene cup and
covered with a lid. Upon receiving semen samples from the patients, the samples were placed in a dedicated warming oven at 37°C and allowed to liquefy for 30 minutes.

**Evaluation of neat semen samples**

Procedures were performed by, or under the supervision of experienced scientists. Protective clothing covering the operator’s arms was worn, with face masks, protective eye wear and double gloves for the handling of samples. All procedures were performed in a laboratory dedicated to the handling of semen samples from patients known to be HIV-1 sero-positive (Mucciaccia *et al*., 2007), in a Class II biological safety cabinet (Labotec, South Africa, Midrand, Gauteng). Standard macroscopic and microscopic semen analyses were performed on all samples according to the World Health Organization’s guidelines on semen evaluation (WHO, 2010).

**Viral validation of neat diagnostic semen samples**

Semen aliquots were decanted into 2.5 ml Cryo Tubes® (Nalge Nunc International, USA, Rochester, NY) and were submitted for viral validation. Viral validation was performed by qualitative and quantitative polymerase chain reaction (PCR) for HIV-1 DNA and RNA, respectively. A minimum semen volume of 500 µl was required by the pathology laboratory for viral validation to ensure maximum sensitivity of the method (Pasquier *et al.*, 2006a). Samples were transported to Lancet Laboratories (Richmond, Johannesburg, Gauteng, South Africa) on ice in a sealed polystyrene box at ± 4°C. Commercial validation assays were used to determine HIV-1 RNA viral load (COBAS® Ampliprep/COBAS® TaqMan® HIV-1 version 2, Roche Diagnostics, Indianapolis, USA) and to evaluate proviral DNA qualitatively (Amplicor HIV-1 DNA, version 1.5 Roche Diagnostics). With a LLD of 20 viral copies/ml semen, the COBAS® Taqman® test is a sensitive and commonly used method to determine seminal viral loads (Pasquier *et al.*, 2012).

Samples were treated according to the standard operating procedures of Lancet laboratories (personal communication Dr. J. Trusler, Lancet Laboratories). For RNA analyses, semen samples were diluted twofold by adding a lysis buffer and Proteinase K (COBAS® Amplirep/COBAS® Taqman® pre-extraction reagent). The samples were then incubated at 60°C for 60 minutes and centrifuged at 1800 x g for 10 minutes. Thereafter, negative human plasma was added to yield a 1:4 dilution of the semen
samples, changing the LLD to less than 80 viral copies/ml. The addition of negative human plasma to semen samples has been reported to reduce the effect of PCR inhibitors (Bourlet et al., 2003; Pasquier et al. 2006a). The diluted semen samples were then analysed for the presence of HIV-1 RNA, and if a validation error occurred, semen samples were diluted further with negative human plasma to yield a final dilution of 1:10 (LLD of less than 200 viral copies/ml).

For DNA analyses, semen samples were originally extracted on the MagnaPure 32 (Roche, Japan) using the TNA High Performance kit (Roche, Germany) as per the manufacturer’s instructions. To overcome the sperm sample volume limitation, the Promega Maxwell system was utilised. Two hundred microliters of semen was incubated with 200 µl lysis buffer and 20 µl proteinase K at 56°C, and centrifuged for 10 minutes at 200 x g. The lysed sample mixture was transferred to the Viral Maxwell extraction kit (Promega, UK) cartridges and total nucleic acid extracted on the Maxwell automated nucleic extraction platform (Promega, UK) and eluted in 100 µl Nuclease-free water. The elute was incubated with the Roche kit Internal Control at 100°C for 30 minutes and amplification and detection performed using the Amplicor HIV-1 DNA, version 1.5 Roche Diagnostics kit, as per the manufacturer’s instructions. Samples testing positive for HIV-1 or within the grey zone (indeterminate – optical density 0.2 to 0.8) were re-processed and tested in duplicate for confirmation. Viral validation results were received using a secure email address from Lancet Laboratories within 72 to 96 hours after the samples were submitted.

**Decontamination of semen samples**

The weekly therapeutic semen samples, destined for semen decontamination, were evaluated and semen decontamination was performed by processing semen samples by discontinuous density gradient centrifugation (PureSperm® - 40 and 80%, Nidacon International) with the use of a polypropylene tube insert (ProlInsert™, Nidacon International), without an additional swim-up step. Processed sperm samples were obtained (580 µl) and microscopic evaluations were performed on 10 µl aliquots. The remaining 570 µl of sperm samples were cryopreserved and stored to be used during ART after the samples were confirmed to be free of HIV-1 DNA and RNA.
Cryopreservation of purified sperm samples
Samples were cryopreserved using SpermFreeze™ (FertiPro, Beernem, Belgium) which was added drop-wise to the processed sperm while gently mixing (399 µl SpermFreeze™ to 570 µl sperm). The sperm suspensions were then aspirated into three 300 µl high security semen cryopreservation straws (Cryo Bio System, CBS™, St-Ouen-sur-Ilton, France) using a micro-classic aspirator (Brand GMBH Co, Werthein, Germany). The high security ionomeric resin straws are equipped with sterile removable nozzles (CBS™) that prevent contamination of the outside surface of the straw during the loading process, with any pathogens that may potentially remain in the processed sperm samples. The straws were identified by inserting an identification rod into the straws (CBS™). These colour coded identification rods were labelled (Labxpert, Brady, Milwaukee, WI, USA) with the date of cryopreservation and the patient’s unique identification code. Both sides of the straws were then heat-sealed (CBS™) where after the straws were wiped with a tissue and 70% ethanol. Cryopreservation was performed by means of controlled rate cooling (Freeze Control CL-8800, Cryologic, Victoria, Australia). When a temperature of -80°C was reached during the free-fall process, the straws were plunged into liquid nitrogen. The cryopreserved straws were stored in a dedicated dewar (Thermo Electron Corporation, Bremen, Germany) that was locked for security purposes.

Viral validation of processed sperm samples
Prepared sperm samples (N=103) were submitted for viral validation, in a sealed polystyrene container on ice, if the neat semen tested positive for HIV-1 RNA and/or DNA, or if the result was inconclusive due to a validation error. Samples were not submitted for viral validation if the diagnostic semen sample tested negative for viral DNA, or if the HIV-1 RNA load was below the LLD of the detection method.

RESULTS
The BPVL and SVL of HIV-1 sero-positive patients (N=95) seeking ART at RBL, SBAH ranged between <LLD to 167,263 and <LLD to 1,443,350 copies/mL, respectively. To verify if the latter result was due to contamination during testing, a second neat semen sample was requested. A SVL of 797,528 copies/ml was reported from a sample obtained a week later. Concurrent positive seminal proviral DNA was also reported and the patient was referred for consultation with a virologist.
The HIV-1 RNA status of blood and semen is presented in Table 1. HIV-1 loads of <LLD were considered as negative and viral loads of ≥LLD were considered as positive. Six percent of patients presented with undetectable BPVLs, of which 32.7%, delivered semen samples with SVL of ≥LLD. Furthermore, the disagreement between blood and seminal HIV-1 RNA status (+ and -) was a random event, i.e. 32.7% of patients had BPVL <LLD and SVL ≥LLD, while 37.0% of patients presented with BPVL ≥LLD and SVL <LLD (Table 1).

Table 1: The numbers and (%) of patients’ blood and neat semen samples (N=95) that tested positive and/or negative for HIV-1 RNA.

<table>
<thead>
<tr>
<th>Blood HIV-1 RNA status</th>
<th>Semen HIV-1 RNA status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;LLD*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33 (67.35)</td>
<td>16 (32.65)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 (36.96)</td>
<td>29 (63.04)</td>
</tr>
<tr>
<td>Total</td>
<td>50 (52.63)</td>
<td>45 (47.37)</td>
</tr>
</tbody>
</table>

* LLD=20-200 viral copies/ml

The positivity of neat semen samples for HIV-1 DNA and RNA is provided in Table 2. Of all tested neat semen samples (N=186) from sero-positive patients, 53.7% tested positive for HIV-1 DNA, RNA or both (13.4, 11.3% and 29.0%, respectively). Results for 7.0% (N=13) of neat samples could not be obtained due to PCR validation errors. Validation errors for neat semen samples included: i) HIV-1 RNA errors (N=10), and ii) HIV-1 DNA and RNA errors (N=3). Processed sperm samples (N=103) were submitted for viral validation, results for 2% (N=2) of samples could not be obtained due to validation errors for DNA and RNA in both sperm samples.

The semen decontamination procedure was effective in removing HIV-1 RNA and proviral DNA from 98.1% of semen samples of HIV-1 infected patients, as shown in
Table 2. From a total of 103 processed sperm samples that were submitted for viral validation, two samples tested positive for HIV-1 DNA and none for RNA.

Table 2: HIV-1 RNA and proviral DNA detection in semen (N=186) and purified sperm samples (N=103) from HIV-1 sero-positive men (N=95)

<table>
<thead>
<tr>
<th></th>
<th>Neat Semen Samples (N=186)</th>
<th>Processed Sperm Samples (N=103)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>DNA &amp; RNA</td>
<td>DNA &amp; RNA</td>
</tr>
<tr>
<td>DNA</td>
<td>46.24% N=86</td>
<td>98.06% N=101</td>
</tr>
<tr>
<td>RNA</td>
<td>13.44% N=25</td>
<td></td>
</tr>
<tr>
<td>DNA &amp; RNA</td>
<td>11.29% N=21</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>29.03% N=54</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

HIV may be transferred from 7.0% to 24.0% of females within the first few months after contraction of the virus by their male sexual partners (Pilcher et al., 2004). Even if HIV-1 seropositive males receive HAART and present with blood viral loads below the LLD, semen samples may still test positive for HIV-1 (Lambert-Niclot et al., 2012; Politch et al., 2012; Ferraretto et al., 2014). The use of condoms during intercourse which is recommended to couples infected with HIV, will diminish the risk of infecting their partner, but also will result in these patients being unable to conceive their own genetically related offspring (Pasquier et al., 2006b). Due to the potential high HIV-1 SVL, the rate of male to female transferral is 1.9 times more than female to male transferral, of the virus (European Study Group on Heterosexual Transmission of HIV, 1992). The SVL could be a valuable indicator of the risk of HIV sexual transmission (Pasquier et al., 2012).

The current study indicated that 53.8% of semen samples tested positive for HIV-1 DNA or RNA. Furthermore, 32.7% of patients with undetectable blood plasma HIV-1 RNA loads presented with HIV-1 RNA in their semen samples. The probability of HIV genotype specificity, weak adherence to antiviral therapy, and national policies to
supply such therapies only below a threshold CD4 level, could contribute to the rather high detectable HIV-1 in semen of males (29%) during the current study. The reported disagreement between blood and seminal HIV-1 RNA status (+ and -) was found to be a random event.

Concrete evidence for an association between sperm and HIV-1 does not exist (Pudney et al., 1999). Seminal HIV can be present as cell-free RNA, or in the form of proviral DNA, be associated with CD4 receptor-carrying lymphocytes and macrophages (Quayle et al., 1997; Savasi et al., 2013). The effective isolation of sperm from seminal plasma and white blood cells that serve as target cells for the virus, should then yield a population of sperm that is HIV-1 free and safe for use during ART. The promotion of semen washing procedures consequently results in more HIV-1 sero-discordant couples attending infertility units, rather than attempting to conceive by having unprotected intercourse (Lesage et al., 2006). Semen decontamination procedures employed at RBL consist of semen processing by discontinuous density gradient centrifugation in combination with the ProInsert™. This method of semen decontamination has previously been reported to be superior than standard discontinues density gradient centrifugation in the removal of in vitro added bacteria from semen (Fourie et al., 2012). The removal of HIV-1 from semen should therefore also be improved by utilization of the decontamination technique.

In the current study, from a total of 103 processed sperm samples that were submitted for HIV-1 validation only two samples tested positive for HIV-1 DNA and none for RNA. The relatively low SVL of these two samples (1,690 and 2,900 viral copies/ml, respectively) from patients with HIV-1 DNA in processed sperm samples suggests that SVL is not indicative of the efficiency of semen decontamination procedures. The success rate for semen decontamination achieved at RBL was 98.1% for HIV-1 DNA and 100% for RNA. The effectiveness of the decontamination method employed at RBL can be attributed to the secure method of obtaining the purified sperm pellet post-density gradient centrifugation through the ProInsert™ (Loskutoff et al., 2005). It must be noted that during this research, the centrifugation tube insert underwent various design changes by the manufacturer (Nidacon International). See Fourie and co-workers (2013) for an illustration of the ProInsert™. The changes in design allowed the operator to layer semen onto the density layers without contaminating the inner lumen of the
Samples that tested positive for proviral DNA could have been contaminated post-processing when an initial prototype of the insert was tested during the first part of the study period.

Challenges that could be encountered when assisting HIV-1 sero-discordant couples with semen decontamination procedures include the following:

i) Researchers agree that the complete removal of HIV-1 from semen cannot be guaranteed (Savasi et al., 2013). Furthermore, the PCR method utilised for viral validation’s LLD was 20 to 200 viral copies/ml, whereby false negative results are possible (Pasquier et al., 2006b). Patients should be counselled that semen decontamination is merely a risk reduction method and that risk cannot be totally eliminated (Gilling-Smith et al., 2005). Additionally patients must sign an informed consent form wherein the potential consequences of technique failure are acknowledged.

ii) The presence of seminal PCR inhibitors results in the inhibition of HIV-1 RNA amplification whereby viral validation errors are obtained (Lesage et al., 2006). Patients should be informed that in the event of PCR errors, viral validation should be repeated, whereby less sperm will be available for ART.

iii) Viral validation of neat semen and processed sperm samples is costly. According to a quotation obtained from Lancet Laboratories, the current cost (May 2013) of HIV-1 DNA and RNA validation is 78€ and 81€, respectively. This study demonstrated that seminal viral load (ranges from <LLD to 1,443,350 copies/ml semen) was no indication of the effectiveness of the semen decontamination. It is therefore recommended that only purified sperm samples, and not neat semen samples should be submitted for viral validation.

iv) To ensure that sufficient purified sperm cells were available for ART, most patients were requested to provide 4 semen samples during two consecutive weeks, where after sperm samples were cryopreserved and stored while awaiting viral validation results. Same day viral validation (Lesage et al., 2006) of purified sperm samples would require a single semen sample on the day of the assisted reproductive procedure. Purified sperm could be stored at room temperature until an aliquot of the sample is confirmed to be virus free. Hereby purified sperm samples would not be compromised during the
cryopreservation process. Same day viral validation is however not commercially available in developing countries such as South Africa.

v) Viral validation of processed sperm samples could be impossible in some developing countries due to a lack of pathology services. Under these circumstances, risk reduction procedures should be maximised to ensure the safe usage of purified, untested sperm for ART (Huyser, 2013). Semen decontamination procedures should be followed by single sperm washing and intra-cytoplasmic sperm injection (Mencaglia et al., 2005). This approach has been reported to be the safest method for HIV-1 sero-discordant couples to conceive (Garrido et al., 2004). Savasi et al. (2013) suggest that the intra-cytoplasmic sperm injection technique is the only assisted reproductive procedure that should be applied to HIV-1 sero-discordant couples where the male is positive.

In conclusion, the provision of ART to couples where the male partner is HIV-1 sero-positive, is ethically justifiable and if stringent precautionary measures are taken, reasonably safe. HIV-1 sero-positive male patients with undetectable blood plasma viral loads should receive counselling regarding the potential presence of virus in their semen samples. These patients should then be dissuaded from having unprotected intercourse in an attempt to conceive. Assisted reproductive treatment in combination with semen decontamination by discontinuous density gradient centrifugation and the ProlInsert™ is the safest reported option for these patients to conceive. Furthermore, processed sperm samples should only be utilized for ART once the samples have been confirmed to be free from virus by PCR testing, if these tests are available.

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