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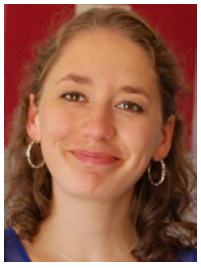
ARTICLE

Validation of a SARS-CoV-2 RT-PCR assay: a requirement to evaluate viral contamination in human semen



BIOGRAPHY

Hélène Chabrolles (PharmD, PhD in virology) specializes in virus and host interactions and currently works as a researcher biologist at the University Hospital Center and the Faculty of Medicine of Clermont-Ferrand. Part of her research focuses on SARS-CoV-2 infection, including viral dissemination in human semen.

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KEY MESSAGE

This study validated for the first time a highly reliable RT-PCR method with high sensibility, repeatability and reproducibility for SARS-CoV-2 detection in human seminal fluid and spermatozoa fractions. Semen features and cryoprotectant media used to freeze spermatozoa did not affect the performance of the assay.

ABSTRACT

Research question: Is it possible to validate an accurate and reliable method for direct detection of SARS-CoV-2 by reverse transcription polymerase chain reaction (RT-PCR) in human semen fractions?

Design: This qualitative improvement study aimed to provide a prospective validation of SARS-CoV-2 detection in male semen. The SARS-CoV-2 genome was detected by multiplex real-time RT-PCR on patient samples that underwent routine semen analyses for infertility at the Center for Reproductive Medicine at the University Hospital of Clermont-Ferrand. Samples comprised surplus semen collected for treatment with assisted reproductive technology. Seminal fluid and spermatozoa fractions were isolated with density gradient centrifugation and cryopreserved. Positive samples were prepared with a standard of inactivated SARS-CoV-2 particles.

Results: The analytical method was validated in both seminal fluid and spermatozoa fractions. In both semen fractions, the assay was repeatable, reproducible and showed high sensitivity with a limit of detection of 0.33 SARS-CoV-2 genome copies/µl. The limit of quantification was 1 copy of the SARS-CoV-2 genome/µl. The method was effective regardless of semen quality (normal and altered sperm parameters), number of spermatozoa or the cryoprotectant media used to freeze spermatozoa.

Conclusion: This validated RT-PCR assay provided accurate and reliable screening of SARS-CoV-2 in seminal fluid and spermatozoa fractions. This method is essential to ensure protection against viral contamination in the cryobanking process.

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KEYWORDS

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INTRODUCTION

The SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) pandemic has raised major concerns about the safety of semen samples for cryobanking. SARS-CoV-2 uses ACE2 (host angiotensin-converting enzyme 2) and cellular cofactors such as TMPRSS2 to enter target cells (*Hoffmann et al., 2020*). Transient viraemia during SARS-CoV-2 infection and expression of ACE2 and TMPRSS2 in the testis and accessory glands (*Massarotti et al., 2020*; *Ren et al., 2020*) raise the possibility of SARS-CoV-2 shedding in the male reproductive tract. Although SARS-CoV-2 may not replicate in the male reproductive system, specific male cells might act as viral reservoirs after a systemic SARS-CoV-2 infection. In addition, semen samples may become contaminated with the virus during sperm collection. The possible presence of SARS-CoV-2 in cryopreserved semen samples is a major concern for the safety of patients undergoing treatment with assisted reproductive technology (ART) and fertility preservation. To our knowledge, four studies have reported the presence of the SARS-CoV-2 genome in semen samples (*Delaroche et al., 2021*; *Gacci et al., 2021*; *Li et al., 2020*; *Machado et al., 2021*). One should be interpreted with caution, because the authors did not explain how the semen was collected, or describe the method of viral detection (*Li et al., 2020*). The other three used commercial reverse transcription polymerase chain reaction (RT-PCR) assays whose performances have not been evaluated for semen specimens (*Delaroche et al., 2021*; *Gacci et al., 2021*; *Machado et al., 2021*). All other studies did not detect SARS-CoV-2 in semen collected during an acute infection or after patients recovered from SARS-CoV-2 infection (*Best et al., 2021*; *Burke et al., 2021*; *Donders et al., 2022*; *Fraietta et al., 2022*; *Guo et al., 2021*; *Gupta et al., 2021*; *Holtmann et al., 2020*; *Kayaaslan et al., 2020*; *Pan et al., 2020*; *Paoli et al., 2020b*, *2020c*; *Rawlings et al., 2020*; *Ruan et al., 2021*; *Song et al., 2020*; *Tur-Kaspa et al., 2021*; *Yang et al., 2020*; *Zhang et al., 2020*). However, these studies included a small number of subjects and they did not detect the SARS-CoV-2 genome with a validated method for semen samples. To investigate the presence of SARS-CoV-2 in human semen, most studies used commercial RT-PCR assays only approved for respiratory samples, and targeting two or three viral genes in accordance with WHO guidelines (https://www.who.int/publications/i/item/diagnostic-testing-for-sars-cov-2). Three studies used a RT-PCR assay targeting only one viral gene (*Delaroche et al., 2021*; *Holtmann et al., 2020*; *Kayaaslan et al., 2020*). *Rawlings et al. (2020)* validated a digital droplet PCR (dd-PCR) for detecting the SARS-CoV-2 genome with a good level of detection (0.05 viral genome copies/µl). However, the specimen type used for the validation process was not specified and does not appear to be a semen sample. The limit of detection (LOD) in this study should be confirmed for semen samples. Although dd-PCR is a sensitive method, it is not routinely used in laboratories for diagnosis, in contrast to the RT-PCR assay. *Donders et al. (2022)* mentioned the use of a validated 'SpermCOVID test' but described a method for the detection of the SARS-CoV-2 genome in respiratory samples, not in semen samples. However, sample type is of great importance in the performance of a RT-PCR assay, especially for specimen types that have not been evaluated by the manufacturer.

In order to contribute to the establishment of a standardized protocol for the detection of SARS-CoV-2 RNA in semen fractions used for ART, this study aimed to improve the reliability of SARS-CoV-2 RNA detection in semen samples by validating a high-performance RT-PCR assay in cryopreserved seminal fluid and spermatozoa specimens. In addition, it assessed the effectiveness of the method according to different semen features.

MATERIALS AND METHODS

Ethics

The protocol for this study was approved by the French Research Ethics Committee on July 7th 2020 (IRB reference CPP Ile de France VIII, ref 20 06 29, trial registration number: EudraCT 2020-A01409-30). The trial was conducted in compliance with the principles of the Declaration of Helsinki and good clinical practice and in accordance with regulatory requirements.

Semen collection

Surplus semen was acquired from samples that had been collected between July 2020 and March 2021, from patients that underwent routine semen analyses for infertility, regardless of age, body mass index or clinical criteria, at the Reproductive Medicine Center at the University Hospital of Clermont-Ferrand, France. In accordance with the pre-analytical requirements of the medical centre, patients were systematically questioned before carrying out their exam as part of their medical care. No patient had fever or symptoms related to COVID-19 (cough, nasal congestion, asthenia, anosmia, ageusia, headache, vomiting, diarrhoea, abdominal pain, conjunctivitis, rash, muscle or joint pain or body aches) or had been in contact with anyone having COVID-19 symptoms. Written informed consent was obtained for the inclusion of semen samples in the present study. Briefly, after a period of 2–7 days of sexual abstinence, semen was collected by masturbation and ejaculation into sterile containers. Conventional semen analyses were performed, in accordance with the WHO 2020 guidelines (*World Health Organization, 2020*). All spermatozoa counts represent the mean of two independent readings. Leukocytes were detected with the LeucoScreen Plus kit (FertiPro NV, Beernem, Belgium). To assess the proportion of viable spermatozoa (vitality), samples were stained with eosin-nigrosine supplied in the VitalScreen kit (FertiPro NV). Morphology was evaluated according to strict criteria (*Menkveld et al., 1990*). The surplus semen samples were processed with two-stage (90/45%) discontinuous density gradient centrifugation at 750*g*, for 20 min at room temperature. Fresh gradient medium was used, which consisted of Puresperm® medium (NidaCon International, Mölndal, Sweden) diluted with equilibrated Sperm Preparation Medium (Origio, Målov, Denmark). The top layer of gradient medium, which contained seminal fluid, was collected and frozen in sealed cryotubes (CBS™, Cryo Bio System, L'Aigle, France). The high-density fraction was then washed by centrifuging in Sperm Preparation Medium at 750*g* for 8 min at room temperature. The pellet was resuspended in the washing medium, and samples were mixed at a 1:1 ratio (v/v) with CryoSperm™ medium (Origio, France) or at a 1:0.7 ratio (v/v) with SpermFreeze™ medium (FertiPro NV), according to the supplier's recommendations. The precision assay was assessed with the two cryoprotectant media. Next, spermatozoa samples were frozen in high-security straws (Cryo Bio System) in a Nano-Digitcool programmable freezer (Cryo Bio System). Spermatozoa straws and

cryotubes of seminal fluid were stored in a liquid nitrogen tank, according to the supplier's recommendations, in the GERMETHEQUE biobank.

Sample preparation

For each specimen type (seminal fluid and spermatozoa), normozoospermic samples were thawed at 4°C in one step and pooled for the tests performed for the validation of the analytical method. Each pool was then aliquoted for single use and preserved at −80°C, before testing the repeatability and reproducibility of the method. Standard controls were prepared by spiking pools of seminal fluid and spermatozoa with whole, inactivated SARS-CoV-2 particles (SARS-CoV-2 Analytical Q Panel, Qnostics, Glasgow, Scotland) at 10^3 copies or 1 copy of the SARS-CoV-2 genome/µl sample. Frozen aliquots were thawed in one step at 4°C just before RNA extraction and the RT-PCR process.

To test linearity and LOD, the pools were processed immediately after preparation. All tests were performed with multiple replicate samples. The samples and standard controls were handled by trained laboratory personnel, according to good laboratory practices, and in accordance with national recommendations from the SFM (*Société Française de Microbiologie (SFM) 2019a*).

RNA extraction and SARS-CoV-2 genome amplification

RNA was extracted from 160 µl of each sample with the MGISP-960 High-Throughput Automated Sample Preparation System and the MGIEasy Magnetic Beads Virus DNA/RNA Extraction Kit (MGI Tech, Shenzhen, China). Briefly, lysis buffer was added to samples in a 96-deep-well plate, nucleic acid absorption was performed using magnetic beads and finally RNA elution was performed after several washes. The extraction kit included an internal control, which comprised a packaged phage RNA fragment, and the internal control was spiked into each sample. Each extraction included negative and positive control samples, according to the manufacturer's instructions. Next, 10 µl of each RNA extract were reverse transcribed and the cDNA were amplified in a real-time RT-PCR assay, performed with the one-step TaqPath™ COVID-19 CE-IVD RT-PCR Kit (ThermoFisher Scientific, Waltham, MA, USA). The kit targeted the *ORF1ab* and the *N* (nucleocapsid) and *S* (spike) genomic regions of SARS-CoV-2 RNA. Reactions were run on the QuantStudio™ 5 thermocycler (ThermoFisher Scientific), according to the manufacturer's instructions. The PCR thermal profile consisted of an incubation step of 25°C for 2 min and a reverse transcription step of 53°C for 10 min, followed by 2 min at 95°C, and 45 cycles of 3 s at 95°C and 30 s at 60°C. The results were analysed with Applied Biosystems™ QuantStudio™ Design and Analysis Software 2.4.0. Analysis criteria were fulfilled when the positive and negative controls were in accordance with the manufacturer's instructions. For each assay, the cycle threshold (Ct) value of the internal control in each sample must not exceed by more than 3Ct, the Ct value of the extraction control included in each run (ΔCt(IC) <3). Adequate efficiency is defined by ΔCt(IC) <3 with Ct value ≤40. To conclude a positive detection of SARS-CoV-2 RNA, at least two viral targets with Ct values ≤40 had to be detected. The sample was retested when one viral target was detected. A negative result was concluded when no viral target was detected.

Analytical validation of the SARS-CoV-2 RT-PCR assay in sperm fractions

The flow chart of the experimental design is given in Supplemental Figure 1.

Precision assay

Precision assays were performed with three pools of seminal fluid or spermatozoa that had been spiked with 10^3, 1 and 0 copies of the SARS-CoV-2 genome/µl and stored at –80°C until analysis. The numbers of replicates performed were selected according to recommendations from *Rabenau et al. (2007)* and the SFM. To assess repeatability (intra-assay precision), each sample (i.e. each spiked pool of seminal fluid or spermatozoa) was tested in six replicates per run. The repeatability was analysed in one run. To assess reproducibility (inter-assay precision), each sample was tested in three replicates per run. The reproducibility was analysed in three independent runs (different days, different operators and different QS5 instruments). The precision was expressed as the SD of the cycle threshold value (Ct SD). Adequate repeatability is defined by Ct SD <0.2log10, i.e. 0.66Ct; reproducibility by <0.25log10, i.e. 0.83Ct (*Burd, 2010*; *Société Française de Microbiologie (SFM) 2019b*).

LOD for the SARS-CoV-2 RT-PCR assay

The LOD was defined as the lowest genome copy number per volume unit detected in 95% of cases. A six-point range dilution was performed, to obtain the following concentrations: 1, 0.33, 0.22, 0.11, 0.07 and 0.04 SARS-CoV-2 genome copies/µl. Each concentration was prepared in six replicates, and the LOD was analysed in one run. The LOD for spermatozoa cryopreserved in CryoSperm medium was determined by probit analysis.

Linear range and limit of quantification (LOQ) for the SARS-CoV-2 RT-PCR assay

The LOQ was defined as the lowest concentration with detectable N target in the linear range for seminal fluid and spermatozoa frozen in CryoSperm medium. Duplicate solutions of 10-fold serial dilutions were prepared from 10^2 copies of the SARS-CoV-2 genome/µl until absence of detection. Correlation (r^2) and linear regression analyses ($y = ax + b$, where a was the slope and b was the intercept) were performed. The amplification efficiency (E) was calculated as the slope, a, of the standard curve, based on the following equation: $E\ (\%) = (10 - 1/a - 1) \times 100$.

Impact of sperm quality on the efficiency of the analytical method

To assess the impact of sperm quality on the RT-PCR assay efficiency, RT-PCR results for seminal fluids from 10 patients that exhibited abnormal sperm parameters (leucospermia, hypospermia, oligoasthenoteratozoospermia) were compared with RT-PCR results for seminal fluids from 10 patients with normozoospermia. To measure inter-individual variation and to determine whether the spermatozoa number could affect viral detection, RT-PCR results for 10 spermatozoa straws with high numbers of spermatozoa (9 to 48 million per straw) were compared with RT-PCR results for 10 spermatozoa straws with low numbers of spermatozoa (0.1 to 0.5 million spermatozoa per straw).

Statistical analysis

Categorical data are expressed as the number and percentage. Continuous data are expressed as the mean and SD. The Gaussian distribution assumption was analysed with the Shapiro–Wilk test. Independent groups of quantitative data (e.g. normal and abnormal sperm

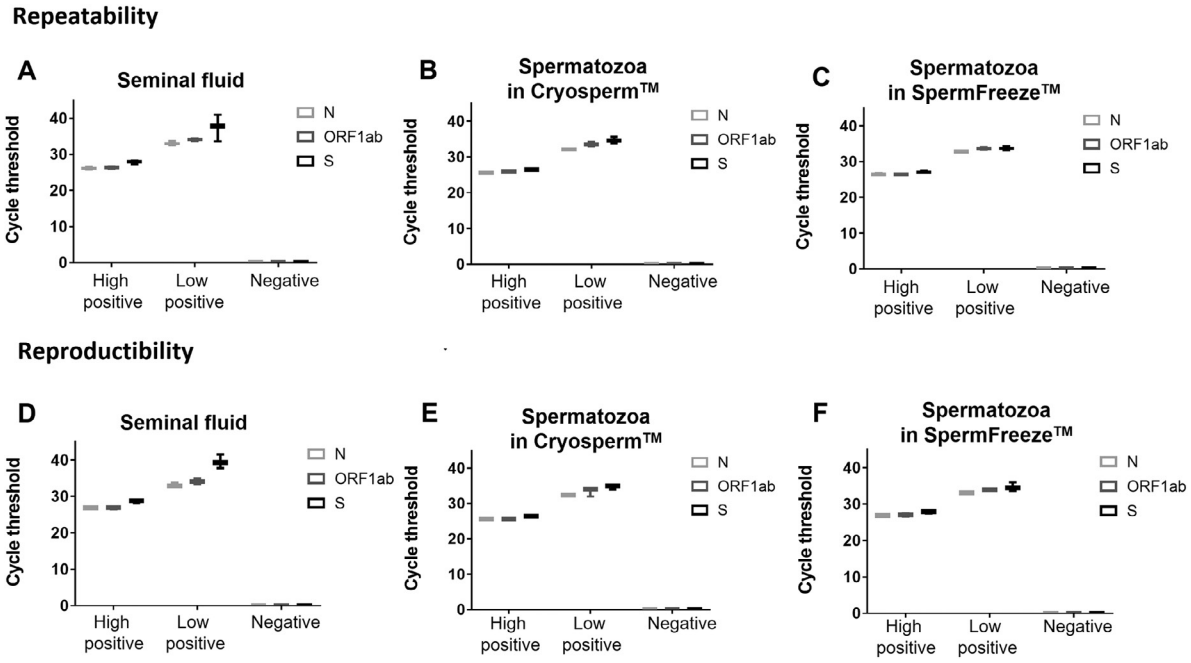

FIGURE 1 Repeatability and reproducibility of the SARS-CoV-2 RNA detection method. The assays were performed for (A, D) seminal fluid and (B, E) spermatozoa frozen in CryoSperm™ medium and (C and F) spermatozoa frozen in SpermFreeze™ medium. Each standard sample (the high positive, low positive and negative samples) was evaluated for (A–C) repeatability and (D–F) reproducibility. The cycle threshold mean and SD (whiskers) were determined for each sample for the *ORF1ab* and the *N* (nucleocapsid) and *S* (spike) genomic regions of the SARS-CoV-2 genome.

parameters or low and high spermatozoa numbers) were compared with Student's *t*-test or the Mann–Whitney test, when appropriate. The equality of variances was explored with the Fisher–Snedecor test. For data with repeated measures, specifically the comparison of the ΔCT between samples preserved in SpermFreeze and samples preserved in CryoSperm media, mixed models were used to take into account different sources of variability (such as random effects). The normality of residuals was analysed, as previously stated. To study the relationship between quantitative variables (e.g. seminal fluid and spermatozoa) for LOQ analyses, correlation coefficients were estimated, and the results are expressed as the r^2 value. The LOD was determined by probit analysis, with a 95% confidence interval. All statistical analyses were performed with Stata Statistical Software, Release 15 (StataCorp LP, College Station, Texas, USA). All tests were two-sided, with a type 1 error set at 0.05.

RESULTS

Assay precision

For *N* and *ORF1ab* viral targets, the precision of repeatability assays was below 0.66 for the high and low positive seminal fluid samples (Ct SD ≤0.34; FIGURE 1A and Supplemental Table 1). The *S* gene target was detected in the low positive seminal fluid samples with a high Ct SD of 3.00. For spermatozoa cryopreserved in CryoSperm or SpermFreeze media, the Ct SD was also below 0.66 for all positive samples and for both cryoprotectant media (Ct SD ≤0.64, FIGURE 1B and C and Supplemental Table 1). SARS-CoV-2 RNA was not detected in any negative samples.

For reproducibility, the Ct SD values were below 0.83 for the high and low positive seminal fluid samples (Ct SD ≤0.68, FIGURE 1D and Supplemental Table 1), and as expected for the *S* target in the low positive sample (Ct SD 1.54). For spermatozoa, the Ct SD values were below 0.83 for all positive samples and for both cryoprotectant media (Ct SD ≤0.48, FIGURE 1E and F and Supplemental Table 1), except for the *ORF* target in the low positive in CryoSperm media (Ct SD 1.06) and for the *S* target in the low positive in SpermFreeze media (Ct SD 1.20). SARS-CoV-2 RNA was not detected in any negative samples.

Assay precision was in accordance with expected requirements for all targets and semen specimen types for the high SARS-CoV-2 RNA concentration, and for at least two viral targets and all semen specimen types for the low SARS-CoV-2 RNA concentration. The cryoprotectant used to freeze spermatozoa did not affect the precision of the assay. Also, the *S* gene was the least sensitive viral target of the assay, while the *N* gene was the most sensitive.

Impact of sperm quality and cryoprotectant medium on analytical performance and efficiency

The study analysed whether the semen features and the composition of the cryoprotectant medium impacted the efficiency of the analytical method by comparing the ΔCt of the internal control between samples. It was found that the analytical efficiency of the method was consistent (ΔCt(IC) < 3) and similar for samples obtained from normal and abnormal semen (FIGURE 2A) and for straw samples with a high or low number of spermatozoa (FIGURE 2B). Additionally, the analytical method efficiency was similar in SpermFreeze and CryoSperm cryoprotectant media: ΔCt(IC) was below 3 and was not significantly different in the two media (FIGURE 2C). Thus, semen features and cryoprotectant media did not affect the efficiency of the detection method.

Analytical sensitivity

The LOD was 0.33 SARS-CoV-2 genome copies/µl sample for the seminal fluid

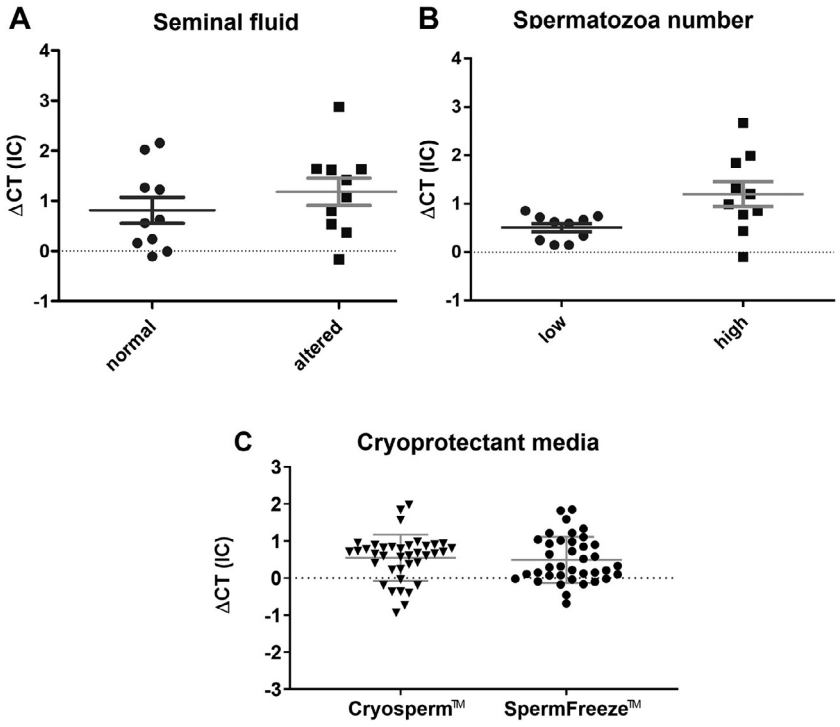

FIGURE 2 Impact of cryoprotectant media and semen features on the efficiency of the analytical method. Each sample was subjected to RNA extraction and RT-PCR to detect SARS-CoV-2 RNA. The ΔCt(IC) values were determined for: (A) seminal fluids from normozoospermic (n = 10) or altered semen parameters (n = 10) patients; (B) high (n = 10) or low (n = 10) numbers of spermatozoa issues for cryopreserved straws; (C) spermatozoa cryopreserved in CryoSperm™ or SpermFreeze™ media.

(TABLE 1). The LOD for spermatozoa was very similar, with a value of 0.23 SARS-CoV-2 genome copies/µl as determined by probit analysis (TABLE 1). This slight difference between the seminal fluid and spermatozoa fractions resulted from acceptable inter-run variability. Altogether, the LOD was not greater than 0.33 viral genome copies/µl for all semen fractions.

LOQ

In the plot of 10-fold serial dilutions, a linear fit of Ct values across three dilutions: 10^2 copies to 1 SARS-CoV-2 genome copies/µl (corresponding to 2 to 0 $\log_{10}$, FIGURE 3) showed a linear range for all semen fractions (r^2 = 0.999). The amplification efficiencies were similar for seminal fluid (110.8%) and spermatozoa (100.2%, FIGURE 3). The linear range analysis generated an LOQ of 1 viral genome copy/µl of sample.

DISCUSSION

This study validated a method for the detection of the SARS-CoV-2 genome in semen fractions, i.e. seminal fluids and spermatozoa fractions. This method showed high precision, specificity and sensitivity and a low LOD (0.33 SARS-CoV-2 genome copies/µl). The amplification method detects three viral targets, which improved the sensitivity and specificity and preserves sensitivity in case of a mutation, a common occurrence in coronaviruses. This analytical method enables the detection of all SARS-CoV-2 variants identified in April 2022. The LOQ was determined for the most sensitive target of the assay, i.e. the *N* gene. The LOQ was 1 copy of the SARS-CoV-2 genome/µl. It provides quantitative information in cases of positive viral detection. The efficiency of the extraction and amplification methods were equivalent in both seminal fluid and spermatozoa, in semen with different features (normal and abnormal parameters), and in samples issued from frozen straws containing

TABLE 1 LIMIT OF DETECTION

SARS-CoV-2 genome copies/µl	Seminal fluid						Spermatozoa					
	R1	R2	R3	R4	R5	R6	R1	R2	R3	R4	R5	R6
1	1	1	1	1	1	1	1	1	1	1	1	1
0.33	1	1	1	1	1	1	1	1	1	1	1	1
0.22	0	0	0	0	0	0	1	1	0	1	1	1
0.11	0	0	0	0	0	0	0	0	0	0	0	0
0.07	0	0	0	0	0	0	0	0	0	0	0	0
0.04	0	0	0	0	0	0	0	0	0	0	0	0

R = replicate; 1 = positive detection; 0 = negative detection.

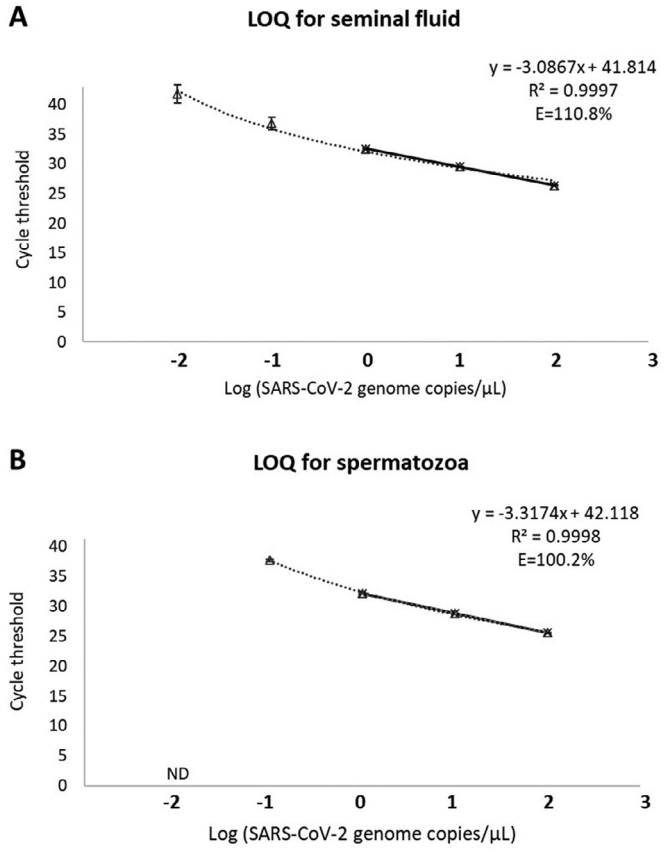

FIGURE 3 Limit of quantification for the SARS-CoV-2 RNA detection method. SARS-CoV-2 RNA was serially diluted in 10-fold steps, from 10^2 to 10^{-2} copies/µl in sperm samples (on logarithmic scale: 2 to $-2\log_{10}$ copies/µl). The coefficient of correlation (r^2), linear regression (y) and efficiency (E) were calculated over the linear range (solid line) from 1 to 10^2 copies of the SARS-CoV-2 genome/µl (i.e. 0 to $2\log_{10}$ copies/µl), in (A) seminal fluid and (B) spermatozoa frozen in CryoSperm™ media.

different spermatozoa numbers (high or low). Interestingly, the analytical method for the detection of the SARS-CoV-2 genome was equally efficient with spermatozoa frozen in different cryoprotectant media routinely used in ART laboratories.

A limited number of studies have suggested that sexual transmission is unlikely (*Tur-Kaspa et al., 2021*). *Li et al. (2020)* detected SARS-CoV-2 RNA in the seminal fluids of six men (27% positive results during acute infections, and 17% during the recovery phase). However, these results need to be confirmed, because the authors provided very little information on the semen collection protocol and the RT-PCR assay method (i.e. the extraction and amplification process, viral gene targets and Ct interpretation). The main shortcoming in studies that show the absence of SARS-CoV-2 in semen samples was that they used a non-validated method for semen specimens (*Best et al., 2021*; *Donders et al., 2022*; *Kayaaslan et al., 2020*; *Ma et al., 2020*; *Song et al., 2020*). The performance of the RT-PCR assay observed in respiratory samples might not be valid in other specimen types, because specimen properties affect kit performance, especially the LOD.

A low LOD is crucial for valid interpretation, particularly in cases of negative SARS-CoV-2 detection. This study has validated a LOD of 0.33 copies/µl, while the manufacturer specifies a LOD of 1 genome copy/µl in respiratory samples. For comparison, *Fraietta et al. (2022)* showed the absence of SARS-CoV-2 detection in human semen during acute infection, using a commercial kit approved for respiratory samples, with a LOD of 0.5 SARS-CoV-2 genome copies/µl. *Rawlings et al. (2020)* decreased to a LOD of 0.05 genome copies/µl using dd-PCR technology. dd-PCR is more accurate than RT-PCR for SARS-CoV-2 detection, especially for low viral load specimens (*Suo et al., 2020*). However, it is not currently used for routine clinical detection of the SARS-CoV-2 genome, either for respiratory or for sperm samples. In summary, the current method is accessible for diagnosis in medical laboratories while preserving very good sensitivity of SARS-CoV-2 RNA detection in sperm fractions. The present study assessed the performances in semen fraction samples of the RT-PCR assay routinely used for SARS-CoV-2 RNA detection in nasopharyngeal swabs in the study centre laboratory. Samples were processed with automated RNA extraction followed by the amplification of three viral targets. An internal control was included throughout the analytical procedure. The internal control was essential to exclude the presence of PCR inhibitors and validate the process. Internal control evaluation has rarely been specified in published studies (*Best et al., 2021*; *Guo et al., 2021*; *Holtmann et al., 2020*; *Li et al., 2020*; *Ruan et al., 2021*; *Song et al., 2020*).

Previous studies assessed the presence of the SARS-CoV-2 virus in native semen or seminal fluid (*Burke et al., 2021*; *Gacci et al., 2021*; *Li et al., 2020*; *Paoli et al., 2020a*). Only *Holtmann et al. (2020)* and the present study tested semen fractions after seminal fluid was isolated with density gradient centrifugation and spermatozoa were selected, in accordance with ART standard procedures for men with HIV or hepatitis infections. Viral safety for ART is an important topic that has been extensively studied, with respect to the evaluation of viral contamination (*Zafer et al., 2016*). Laboratories have had to develop manual techniques or adapt commercial kits for use with semen specimens (*Maertens et al., 2004*). Concerning SARS-CoV-2, all available commercial kits are designed for use with respiratory samples. Even though many studies have explored the presence of SARS-CoV-2 RNA in semen samples with commercial kits developed for respiratory samples, it is mandatory to use a validated process specifically for semen samples. Because the present study was aimed at validation of a clinical method for the detection of the SARS-CoV-2 viral genome in semen, it seemed obvious to apply the ART standard procedure for men with viral infections (*Anifandis et al., 2020*, *2021*). Moreover, scientific committees, such as the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM), have recommended the cryopreservation of spermatozoa for

fertility preservation for urgent oncological patients during the pandemic period. The objective was also to validate an accurate method of detecting SARS-CoV-2 in the seminal fluid fraction and/or the equivalent of a single semen straw, to limit the loss of useful spermatozoa straws for the patient. To achieve this, the efficiency of the method for detecting SARS-CoV-2 in frozen–thawed samples from straws that contained very high or very low (under 100,000 spermatozoa) counts of spermatozoa was tested, to mimic the different qualities of straws used in ART procedures. The results clearly showed that cryopreserved spermatozoa could be efficiently tested for SARS-CoV-2 contamination, regardless of cell numbers or semen parameters. Nevertheless, to reproduce the performances presented in this study, it is crucial to use the same extraction and amplification systems.

This study was the first to validate a method for the detection of SARS-CoV-2 RNA in seminal fluid and cryopreserved spermatozoa. The method was effective, irrespective of the semen specimen type or features of the sperm specimen. The validation assessment showed that the method had a LOD of 0.33 viral genome copies/µl and a LOQ of 1 viral genome copy/µl, in both seminal fluid and spermatozoa fractions. This validated method will be crucial for detecting viral contamination in cryopreserved semen samples intended for ART. Consequently, this method guarantees safety for semen donors and for patients who have benefited from fertility preservation techniques during the SARS-CoV-2 pandemic.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2022.09.004.

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