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Persistence Of Ebola Virus In Survivors & Risks Of Sexual Transmission: A Systematic Review

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ABSTRACT

Background: Longitudinal cohort studies of Ebola virus disease (EVD) survivors from the 2014-2016 West African outbreak have found evidence of Ebola virus (EBOV) and EBOV RNA persistence in the bodily fluids of survivors, particularly in semen. This new evidence has raised the possibility of sexual transmission of EBOV by EVD survivors. The current interim guidance issued by the World Health Organization (WHO) recommends safer sex practices for at least 12 months after acute disease onset (ADO). However, based on new evidence, these recommendations may require revision.

Objective: The main aim of this article is to present and evaluate evidence on the persistence of EBOV in genital fluids, as determined by RT-PCR or viral isolation. In addition to determining the length of persistence in these genital fluids, the relation of persistence to sexual transmission of EBOV is also examined.

Design: We conducted a systematic review of viral persistence in semen, vaginal, and rectal fluids, and assessed evidence of the potential transmissibility of persistent EBOV via sexual transmission from survivors.

Results: We identified 42 published original studies presenting results on EBOV persistence or reporting on suspected sexual transmission of EBOV from survivors. EBOV RNA has been detected in the seminal fluids of an EVD survivor for up to 40 months post-EVD onset. From a cohort of nearly 2,000 male survivors, we estimate an average length of EBOV RNA duration of 370 days. EBOV has also been detected by viral isolation for up to 82 days. Finally, we report that age is a potential determinant of EBOV persistence, with older age associated with a higher likelihood of EBOV RNA detection in seminal fluid.

Conclusion: On the basis of the evidence reviewed, we conclude that persistence of EBOV RNA is related to an increased risk of sexual transmission of EBOV, though the evidence remains mixed on whether detectable EBOV RNA necessarily signifies the presence of infectious virus. Due to reports of intermittent detection of EBOV RNA, especially among survivors who experience EBOV persistence for over a year, we recommend that at least two negative RT-PCR results be received before declaring the survivor's seminal fluid to be cleared of EBOV RNA.

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INTRODUCTION

Ebola virus (EBOV) and Ebola virus disease (EVD)

Since the Ebola virus (EBOV) was first discovered in 1976, the 2014—2016 Ebola virus disease (EVD) outbreak in West Africa was the largest and most complex Ebola outbreak ever seen. This outbreak caused more cases and deaths than all previous outbreaks combined. The ongoing outbreak in the Democratic Republic of the Congo (DRC, formerly the Republic of Zaire), which has been responsible so far for a total of 823 cases and 517 deaths as of 12 February 2019,[1] is currently the largest outbreak of Ebola in the country's history. This current outbreak, in combination with the West African epidemic, has generated a cohort of survivors of unprecedented size. Due to the high case fatality (CFR) of EVD and the limited number of survivors generated by far smaller epidemics prior to 2014, little is known about the long-term health impacts of EBOV infection on survivors. Furthermore, the recognition that EBOV can persist within various immune-privileged sites of the body, and on occasion be transmitted after long periods of time following "recovery" from acute disease onset (ADO), has significant public health implications. Although EBOV transmission via persistently-infected individuals is most often chronicled by partners engaging in sexual activity, the potential for EBOV resurgence from such events, after successful EBOV control has been declared within a country, is of significant concern.

Transmission & Evidence of Persistence of EBOV RNA in Bodily Fluids

Fruit bats of the *Pteropodidae* family are thought to be natural reservoirs of filoviruses, including EBOV. However, the evidence for a bat reservoir for EBOV is limited to the identification of viral RNA in tissues obtained from several bat species.[2]

The initial introduction of EBOV into humans, prior to subsequent human-to-human transmission, is believed to occur from interspecies transmission following exposures to "bushmeat" through butchering or consumption of infected tissues.[3] Subsequent human-to-human EBOV transmission requires close contact with patient blood, vomitus, feces, and fomites (e.g. surfaces and materials such as bedding) contaminated with these fluids.[3] During acute infection, EBOV RNA is also present in sweat, saliva, and tears.[4]

EBOV was first isolated in 1976 from seminal fluids of a victim of a laboratory accident. The virus could be isolated by viral culture from a semen specimen until day 61 after ADO.[5] However, EBOV failed to be isolated after day 76 post EVD-onset.

There is an increasing amount of evidence indicating that EBOV can persist for more than 9 months after clearance of viremia in a number of bodily sites, leading to shedding of the virus in bodily fluids. Notably, EBOV has been detected by viral isolation or RT-PCR in specimens from immune-privileged bodily sites such as the testes, the eye, or the central nervous system, which means that the presence of antigens can be tolerated in these sites without triggering an immune response. This new evidence provokes the possibility of transmission during the long convalescent period.[6-9] Since 2015, ongoing survivor cohort studies in the three most affected countries of the West African outbreak (Guinea, Sierra Leone, and Liberia) have been investigating the persistence of EBOV RNA and EBOV by isolation in semen and other bodily fluids in asymptomatic EVD survivors.

Studies have demonstrated that EBOV can be isolated from semen up to 82 days after symptom onset,[8] and recent viral persistence studies have used nucleic acid amplification tests such as reverse transcriptase polymerase chain reaction (RT-PCR), to detect genetic material (RNA) from EBOV up to 965 days (32 months) after ADO.[10] These outliers are well beyond the period that EBOV can be detected in the blood of EVD survivors and long after recovery from illness. The

persistence of viral genetic material months after symptom onset may reflect the presence of live and potentially transmissible EBOV.

Although EBOV RNA has been detected by RT-PCR in vaginal fluid from one woman 33 days after symptom onset,[8] live virus has never been isolated from vaginal fluid samples. Research on viral persistence that was conducted after the 2014-2016 EVD outbreak has been unable to demonstrate the persistence of EBOV in vaginal fluids. With such limited data, it remains unknown for how long EBOV can typically persist in vaginal fluids.

Evidence of EBOV Sexual Transmission

During the 1967 outbreak of the Marburg filovirus, a close relative of EBOV, a single instance of heterosexual transmission was documented from a male survivor to female partner, suggesting that sexual transmission of EBOV could also be possible.[11] Since the 2014-2016 outbreak of EVD in West Africa, male-to-female transmission of EBOV following exposure to the infected semen of the survivor has been reported or suspected in over 15 instances.[12,13] In one such instance from March 2015, EBOV RNA was detected in the semen of a male EVD survivor 199 days after ADO when his partner fell ill with acute EVD without a reported exposure to another acute EVD case. Matching of genetic sequences strongly suggested that the route of transmission was sexual.[14,15] Female to male transmission of EBOV is theoretically possible, but seems to be less probable, given the limited evidence of persistence of EBOV in vaginal fluids.

Den Boon *et al.* defined viral persistence-derived transmission of EBOV as person-to-person transmission from an EVD survivor to another person that occurred more than 21 days.[16] The 21-day period was chosen to reflect the upper limit of EVD's incubation period.[3] This definition of viral persistence-derived transmission of EBOV was used in this review.

Objectives

For EVD survivors, viral persistence in bodily fluids has potentially significant consequences for public health guidance. The current interim guidance issued by the World Health Organization (WHO) recommends safer sex practices for at least 12 months after ADO and apparent recovery.[17] The recommended safer sex practices include abstinence from all types of sex or the correct and consistent usage of a latex condom during any sexual activity. However, these recommendations were based on studies or reports that had been published up to April 2016, and since then, multiple research and national semen testing programs have produced additional evidence that must also be considered. A comprehensive search on both existing literature and unpublished resources can yield important data on viral persistence in bodily fluids related to sexual transmission that can be used to inform decisions on public health recommendations for survivors of EVD. The WHO defines an EVD survivor as a person with a confirmed positive result by RT-PCR testing for EBOV from any bodily fluid specimen who has subsequently recovered.[18] The definition of an EVD survivor can also include someone who is IgM and/or IgG positive for serological testing of EVD, but has not been vaccinated against EVD. The term "convalescent" is also used in EBOV literature to describe EVD survivors. In this review, the terms "convalescent" and "survivor" are used interchangeably.

The primary aim of this article is to present a systematic review on the existing literature surrounding the persistence of EBOV in bodily fluids related to sexual transmission, particularly semen, vaginal, and rectal fluids. In addition to determining the length of persistence in these genital fluids, the relation of persistence to sexual transmission of EBOV is also examined. The ultimate goal of this systematic review is to provide evidence-based recommendations for revisions of current WHO guidelines on condom use for EVD survivors.

METHODS

Searches

Searches were performed in Medline, Embase, Pubmed, Scopus, and CAB Global Health, as well as in the grey literature sources ClinicalTrials and the WHO International Clinical Trials Registry Platform. The searches were conducted from 3 to 5 April 2019, with no restrictions on date, language, or limitations related to study design or geographic location.

The search strategy included medical subject headings (MeSH) and key words for Ebola, in combination with MeSH and key words for sexual transmission, barrier method contraception, and body fluids, including semen, rectal, and vaginal secretions. A separate search strategy was developed to answer three questions relating to EBOV persistence, condom usage, and sexual transmission; these strategies are presented in *Supplementary Table 1*. The main search terms were used in different combinations, using the Boolean operators ("AND" and "OR") and wildcard variants. The search strategies were developed in coordination with information scientists at two universities. The search terms were adapted to suit the syntax of each database, and searches with these defined key words were limited to the title, keywords, or abstract of the article.

Inclusion Criteria

Since the aim was to find published and unpublished primary data on EBOV persistence in body fluids and the relation of persistence to sexual transmission, we excluded commentaries, editorials, protocols, and news reports. Eligible studies included randomized controlled trials (RCTs), non-randomized controlled trials, controlled before and after studies, interrupted time series, cohort studies, and case reports. Laboratory, animal, and modelling studies were analyzed separately when appropriate.

Titles and abstracts were screened for inclusion criteria by a single reviewer. If the abstract was unavailable, the full text of the article was only assessed if the title included at least one of the following key words or its variations: *survivor*, *convalescence*, *fluids*, *persistence*, *semen, vaginal*, *condom, contraception*, or *sexual transmission*. Following the screening of titles and abstracts, the full texts of relevant articles were then examined by the same reviewer. Papers that met the aforementioned inclusion criteria were included in the final review. To ensure that primary data points were not duplicated, articles that reported on the same patient results were grouped together.

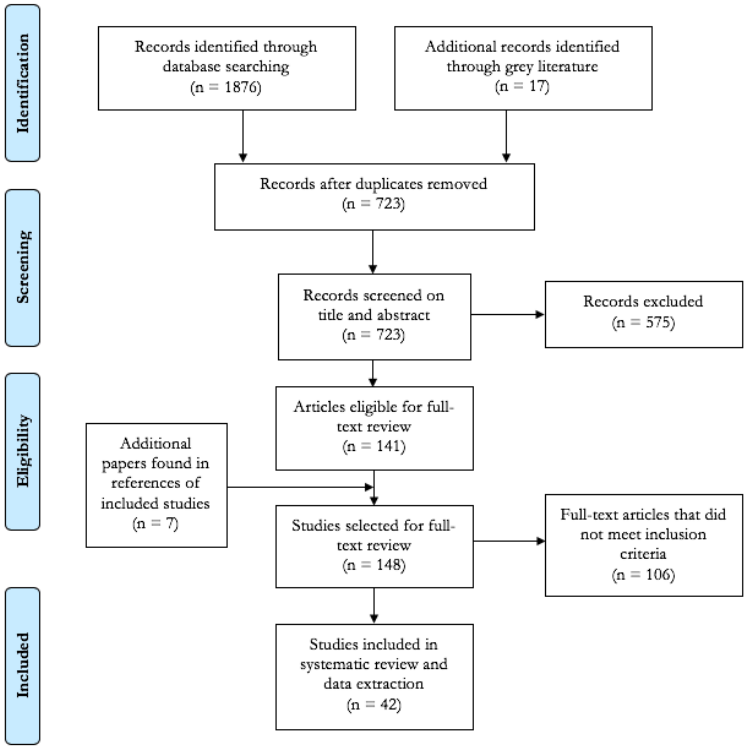
Each included study was validated through the creation of a validation assessment table *(Supplementary Table 2)*. Cases of EBOV infection that were deemed relevant to this review necessitated laboratory confirmation of a positive EBOV result via viral culture or RT-PCR assay. The presence of antibodies and other post-disease markers in body fluids were not considered eligible by themselves, as they only confirm a prior exposure or recovery from acute EBOV infection. Prior studies have shown that antibodies are widespread in regional populations, including those with no relevant clinical history.[19-21] Each study was assessed based on whether its assay methods were appropriate and validated, samples were duplicate-tested or compared to controls, and that samples were collected and stored for a relatively short period of time prior to testing (less than 2 weeks in order to minimize risks of specimen degradation, unless stored at -80°C or in dry ice or liquid nitrogen).

Data were extracted from the included studies by the same reviewer. Details extracted from each article included the following: author, year, study setting, reports of sexual transmission from survivors of EBOV infection, and length of EBOV persistence in body fluids of interest, as evidenced by RT-PCR or viral isolation.

RESULTS

Following de-duplication, 706 unique articles were found in Medline, Embase, Pubmed, Scopus, and CAB Global Health *(Fig. 1)*, and 17 items were found from grey literature on clinical trials. Of these 723 references included in the title and abstract screening, 148 references were chosen for full-text review. Seven of these were articles that were cited by relevant studies but not found during the database search were thus also included in full text review. These articles typically did not show up in the initial database search because they did not cite the genital fluids of interest in their titles or abstracts, as screening of genital fluids of EBOV was not the main objective of these studies. After excluding articles that did not meet the inclusion criteria (n=106), 42 studies were included in the study. Data were extracted from these articles, and a validity assessment was performed on each study *(Supplementary Table 2)*.

Figure 1. PRISMA Flow Diagram of Search



Part 1: Evidence of EBOV Persistence in Genital Fluids

Data on the persistence of EBOV in the genital fluids of convalescents have been amassed over four EBOV outbreaks, including a 1976 laboratory accident in the United Kingdom, the 1995 outbreak in Kikwit, Democratic Republic of the Congo (DRC), the Sudan EBOV outbreak in 2000 in Gulu, Uganda, and the 2014-2016 West African outbreak. Published data relevant to this review does not yet exist from the May 2018 outbreak in Équateur province, DRC, or for the current outbreak in the North Kivu and Ituri provinces of the DRC. Rectal samples were also considered in this study, as the anus may also be involved in sexual activity.

A total of 25 studies reported data on EBOV persistence in genital and rectal fluids; these results are summarized in *Table 1*. While a number of studies were case reports of a single patient, 11 studies reported on large cohort studies of at least 100 EBOV survivors from Sierra Leone, Guinea, and Liberia during the 2014-2016 West African outbreak. The most commonly used assay to detect persistence of EBOV in genital fluids was RT-PCR, and most studies defined a positive test as one with cycle thresholds (Ct) of less than 40 for both viral targets NP and VP40. A result was considered indeterminant if only one viral target was detected, with the exception of Fischer *et al.*, who considered any result to be positive if either gene target was detected.[22] A prior study by Fischer *et al.* validated the use of RT-PCR as an assay for detecting EBOV RNA in body fluid samples.[23]

Evidence of EBOV Persistence in Semen

The average duration of time for which EBOV RNA was detectable in semen, which was measured as the number of days to the last positive semen sample, was 370.3 days (standard deviation = 345.1, n = 448). The persistence of EBOV RNA in semen was calculated as a weighted average of studies that had provided either individual data or information on mean time between disease onset and last positive sample *(Supplementary Table 3)*. Some studies reported the duration of EBOV RNA

persistence as days since discharge from an Ebola treatment center instead of days from ADO.[24-26] To standardize for this discrepancy during analysis, 14 days were added to these measures to account for the period of time between ADO and recovery from EVD. Since no estimate for the average length of recovery from EVD currently exists, the 14 day-estimate was determined using evidence from several EVD case studies from the 2014-2016 West African outbreak.[27-29]

Out of 1,926 total convalescents from 25 studies who provided semen specimens, 315 (16.4%) had at least one positive semen specimen. The longest duration of detection of EBOV RNA was roughly 40 months, as reported by the Partnership for Research on Ebola Virus in Liberia (PREVAIL III) cohort study.[30] In the PREVAIL III cohort study, 267 male EVD survivors provided a total of 2,416 semen sample. The time from ADO to collection of the first sample ranged from 233 to 1,173 days (median = 551 days). EBOV RNA was detected in at least one semen sample for 81 men (30.3%).[30]

Other large cohort studies similarly detected EBOV RNA in seminal fluids of survivors for over a year after EVD onset *(Fig. 2)*. A study of EVD survivors enrolled in the National Semen Testing Program in Liberia detected EBOV RNA in the semen of 57 survivors of the 210 survivors (27.1%) who provided samples.[6] The last positive semen sample in the PREVAIL III study was reported at 470 days post-recovery. Another cohort study of male Liberian survivors detected at least one positive EBOV RNA semen sample in 13 out of 149 survivors (8.7%), with the longest duration of persistence at 965 days after ADO.[22] Similarly, the Postebogui survivors' cohort study in Guinea found that only 15 of 188 survivors who provided semen samples (8.0%) had at least one semen sample positive for EBOV RNA.[24] The last positive semen sample from the Postebogui cohort was collected 548 days after the survivor's recovery.[25,31] The Sierra Leone Ebola Virus Persistence Study (VPS) found that 15 out of 120 survivors (12.5%) tested positive for EBOV RNA in at least one semen sample, with the longest duration of persistence at 406 days after ADO.[32]

Table 1. Summary of EBOV Persistence Studies

Reference	Sample Type	Assay	No. of Patients	No. of Patients with Positive Sample (%)	No. of Total Samples	Mean Time Between Disease Onset and Last Positive Sample	Latest Day After Disease Onset: Positive Sample	Earliest Day After Disease Onset: Negative Sample
Abel et al (2017)[24]	Semen	RT-PCR	188	15 (8.0)	409	190.4 ± 155.1*	497*	518*
Barnes et al (2017)[33]	Semen	RT-PCR	1	1 (100.0)	5		110	180
Barnes et al (2017)[33]	Semen	Viral isolation	1	1 (100.0)	5		37	
Bausch et al (2007)[4]	Semen	RT-PCR	1	1 (100.0)	2		40	45
Bausch et al (2007)[4]	Semen	Viral isolation	1	1 (100.0)	2		40	
Christie et al (2015),[14] Mate et al (2015)[15]	Semen	RT-PCR	1	1 (100.0)	1		199	231
Christie et al (2015)[14]	Semen	Viral isolation	1	0 (0)	1		--	
Deen et al (2017)[6]	Semen	RT-PCR	210	57 (27.1)	210	Not reported	470*	100*
Diallo et al (2016)[7]	Semen	RT-PCR	1	1 (100.0)	Not reported		531	Not done
Emond et al (1977)[5]	Semen	Viral isolation	1	1 (100.0)	5		61	76
Etard et al (2017)[25]	Semen	RT-PCR	188	10 (5.3)		Range: 29-548*	548*	Not done
Etard et al (2017)[25]	Vaginal	RT-PCR	191	0 (0)	191		--	
Fallah et al (2016)[34]	Semen	RT-PCR	76	28 (36.8)	76-304	Not reported	488	Not reported
Fischer et al (2017)[22]	Semen	RT-PCR	149	13 (8.7)		771.9 ± 100.9	965	
Green et al (2016)[35]	Semen	RT-PCR	1	1 (100.0)	1		114*	
Green et al (2016)[35]	Rectal	RT-PCR	17	0 (0)	17		--	
Green et al (2016)[35]	Vaginal	RT-PCR	21	0 (0)	21		--	
Knust et al (2016)[32]	Semen	RT-PCR	120	15 (12.5)		Not reported	406	

Table 1. Summary of EBOV Persistence Studies

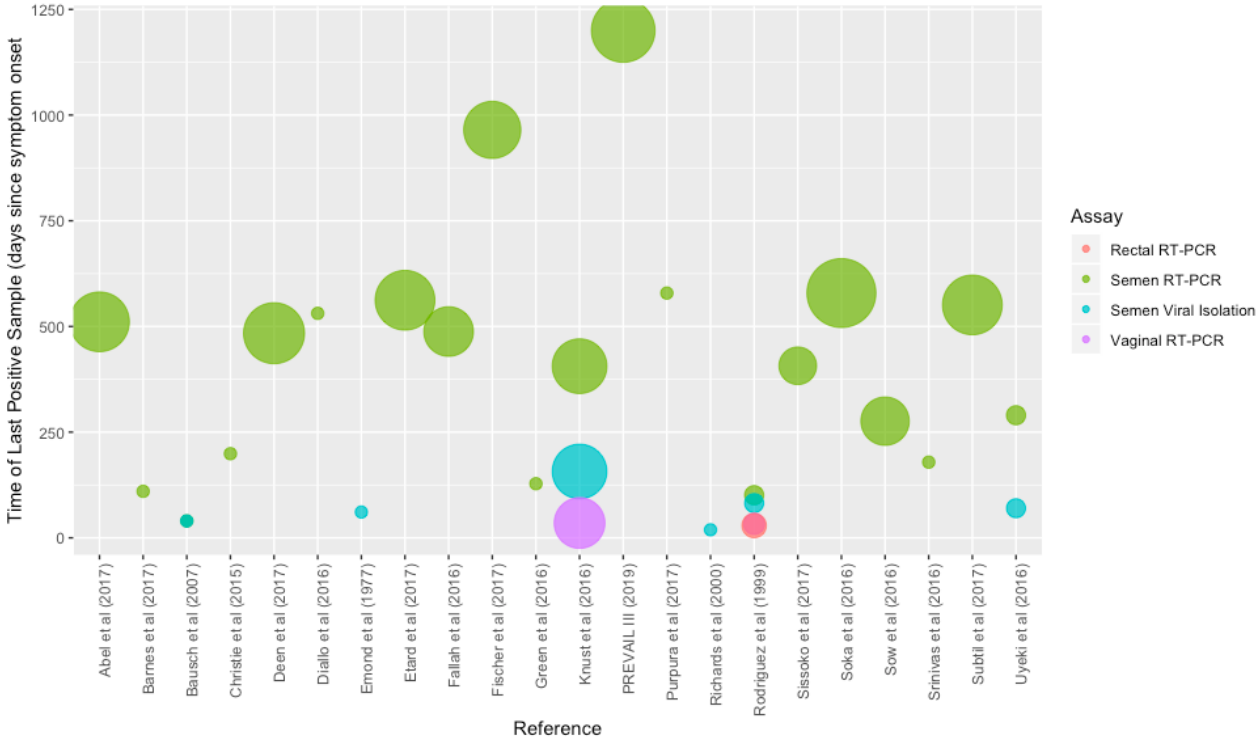
Reference	Sample Type	Assay	No. of Patients	No. of Patients with Positive Sample (%)	No. of Total Samples	Mean Time Between Disease Onset and Last Positive Sample	Latest Day After Disease Onset: Positive Sample	Earliest Day After Disease Onset: Negative Sample
Knust et al (2016)[32]	Semen	Viral isolation	120	4 (3.3)		157		
Knust et al (2016)[32]	Vaginal	RT-PCR	84	1 (1.2)			35	
PREVAIL III Study Group (2019)[30]	Semen	RT-PCR	267	81 (30.3)	2411	Not reported	40 months (~1200 days)	
Purpura et al (2017)[36]	Semen	RT-PCR	1	1 (100.0)			565*	603*
Richards et al (2000)[37]	Semen	Viral isolation	1	1 (100.0)	1		19	
Rodriguez et al (1999),[8] Rowe et al (1999)[38]	Semen	RT-PCR	5	4 (80.0)	11	85.8 ± 17.2	101	62
Rodriguez et al (1999),[8] Rowe et al (1999)[38]	Semen	Viral isolation	5	1 (20.0)	11		82	
Rodriguez et al (1999)[8]	Vaginal	RT-PCR	6	1 (16.7)	15		33	
Rodriguez et al (1999)[8]	Vaginal	Viral isolation	6	0 (0)	15		--	
Rodriguez et al (1999)[8]	Rectal	RT-PCR	8	1 (12.5)	19		29	33
Rowe et al (1999)[38]	Vaginal	RT-PCR	19	0 (0)	44		--	
Rowe et al (1999)[38]	Vaginal	Viral isolation	19	0 (0)	44		--	
Sissoko et al (2017)[9]	Semen	RT-PCR	26	19 (73.1)	130	149.6 ± 91.2	407	
Sissoko et al (2017)[39]	Semen	RT-PCR	1	1 (100.0)			Sep 15, 2015	Oct 7, 2015
Soka et al (2016)[40]	Semen	RT-PCR	429	38 (8.9)		Not reported	565*	
Sow et al (2016)[41]	Semen	RT-PCR	68	8 (11.8)	98	118.9 ± 79.9	276	

Table 1. Summary of EBOV Persistence Studies

Reference	Sample Type	Assay	No. of Patients	No. of Patients with Positive Sample (%)	No. of Total Samples	Mean Time Between Disease Onset and Last Positive Sample	Latest Day After Disease Onset: Positive Sample	Earliest Day After Disease Onset: Negative Sample
Srinivas et al (2016)[26]	Semen	RT-PCR	1	1 (100.0)	8		165*	
Srinivas et al (2016)[26]	Semen	Viral isolation	1	0 (0)	1		--	
Subtil et al (2017)[31]	Semen	RT-PCR	188	15 (8.0)	409	231.5 (min 29 - max 551)	551	
Uyeki et al (2016)[42]	Semen	RT-PCR	5	5 (100.0)	25	184.6 ± 75.3	290	222
Uyeki et al (2016)[42]	Semen	Viral isolation	5	3 (60.0)	18	59.3 ± 10.1	70	

Note: * denotes that this value was measured as days after discharge from an Ebola treatment center (ETC)

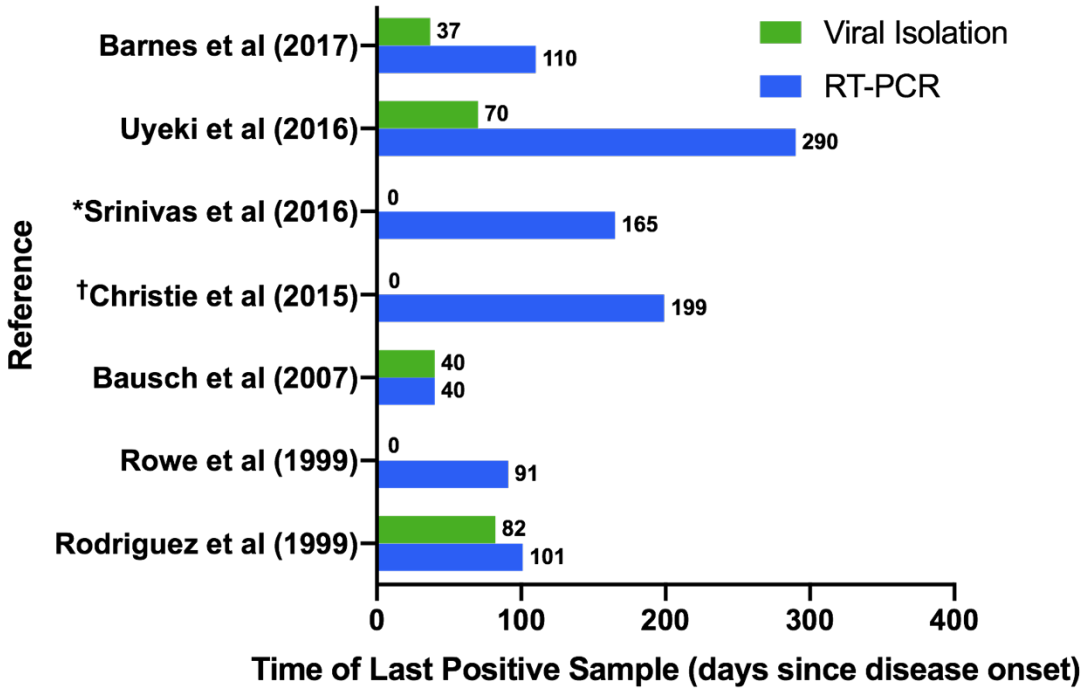
Figure 2. Bubble chart depicting average duration of EBOV persistence, as measured by days after acute disease onset (ADO). Bubble size is proportional to the sample size of each study.



Detection of EBOV by RT-PCR vs. Viral Isolation in Semen

Though many studies have reported persistence of EBOV RNA in the body fluids of survivors, the presence of EBOV RNA does not necessarily imply the presence of infectious virus. In order to establish an association between detection of EBOV RNA by RT-PCR and the presence of infectious virus, a few studies have attempted to detect EBOV by both RT-PCR and viral isolation by culture *(Fig. 3)*.

Figure 3. Bar chart comparing duration of EBOV persistence between viral isolation and RT-PCR methods, for studies that attempted both assays on the same samples
Note: * denotes that this value was measured as days after discharge from an ETC; † denotes that this reference includes both Christie *et al* (2015) and Mate *et al* (2015), which describe the same patient



Only five studies were successful in isolating EBOV by viral culture for samples that were positive for EBOV RNA by RT-PCR.[4,5,8,33,42] From these viral culture assays, the longest duration of persistence of EBOV in seminal fluids of survivors was found to be 82 days after ADO.[8] In comparing the duration of EBOV persistence detectable between these two assays, three studies found that EBOV RNA could be detected by RT-PCR longer than EBOV could be detected by viral culture (101 days by RT-PCR vs. 82 days by viral culture,[8] 290 days by RT-PCR vs. 70 days by viral culture[42], and 110 days by RT-PCR vs. 37 days by viral culture[33]). The fourth study found the same duration of EBOV persistence by both RT-PCR and viral culture,[4] and the last study only used viral culture to detect EBOV.[5] An abstract from the VPS cohort stated that of the four semen specimens that yielded

EBOV isolates by viral culture, the longest duration post-EVD onset that viable EBOV was detected was 157 days.[32] This study result was not reported in any other full-text article. Several other studies also attempted to detect EBOV by viral culture; however, these studies were unsuccessful.[14,15,26,38]

Evidence of EBOV Persistence in Vaginal & Rectal Specimens

A total of 321 female survivors of EBOV across 5 studies provided vaginal fluid specimens via vaginal swabs. Only 2 survivors (0.6%) tested positive for EBOV RNA by RT-PCR,[8,32] and the last positive vaginal fluid sample was collected 35 days after ADO *(Fig. 2)*.[32] Viral cultures were attempted from vaginal fluid specimens from 25 survivors up until day 33 after ADO, none of which were successful.[8,38] Out of 25 patients for which rectal specimens were collected,[43] only one female survivor had specimens that were positive by RT-PCR until day 29 after symptom onset, but negative by day 33.[8] Viral isolation was not attempted on this sample.

Persistence in Semen by Age

A longitudinal cohort study of EVD survivors in Monrovia, Liberia followed 149 male survivors who donated semen samples from 260 to 1016 days after ADO.[22] The study observed that older male survivors were significantly more likely to have detectable EBOV RNA in seminal fluids (median age 41.8 vs 31.2 years, $p = 0.0004$). Similarly, the Liberia Men's Health Screening Program, which provides semen testing services to EVD survivors, found that survivors over the age of 40 comprised 50% of participants with at least one semen sample testing positive for EBOV RNA, despite accounting for only 23% of the male survivor population in this cohort.[40] In one study of male survivors in Guinea, whose semen specimens were first tested between one to twelve months after EVD onset, EBOV persistence in semen was detected in eight out of 68 survivors (11%).[41] Of

these 8, the duration of EBOV persistence in seminal fluids averaged 225 days post disease onset for men over the age of 40, compared to 67.8 days for men under 40.

However, a relation between the duration of persistence and survivor age has not been universally identified. In a longitudinal study of 26 participants in Guinea, the duration of EBOV persistence in men older than 40 and in men younger than 40 differed by only 12 days, with older men experiencing longer persistence.[9]

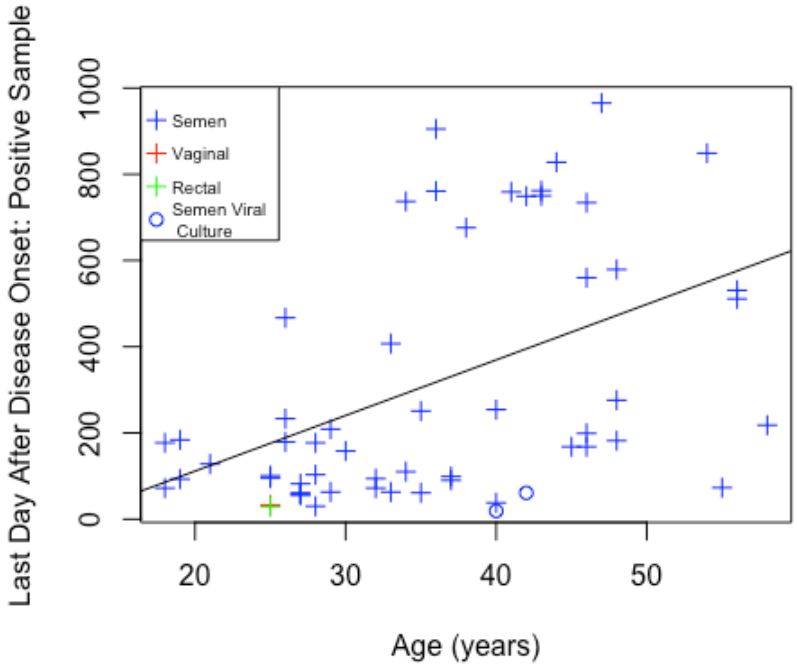
In order to elucidate the relation between persistence of EBOV RNA and survivor age, we extracted data on the participants' ages at ADO and the number of days between EVD onset and the last positive EBOV RNA RT-PCR in seminal samples. Eighteen of the 25 studies reported on these two variables, supplying a total of 54 data points for analysis *(Supplementary Table 4)*. Linear regression indicated that age was a statistically significant predictor for the duration of EBOV RNA persistence in semen ($p = 0.0003$, $n = 54$), with a positive correlation between age and the duration of persistence *(Fig. 4, Table 2)*. Survivor age accounted for 21.0% of the explained variability in duration of persistence.

Table 2. Linear Regression Output for Age & Duration of Persistence

Duration of persistence = -146.47 + 12.90(Age)					
	Estimate	Standard Error	T-value	Pr(>|t|)	
Intercept	-146.47	124.85	-1.17	0.25	
Age	12.90	3.32	3.88	0.000296	***

Residual standard error	256.8 on 52 degrees of freedom
Adjusted R-squared	0.2096
F-statistic	15.05 on 1 and 52 DF, p-value: 0.000296

Figure 4. Linear Regression of Age & Duration of Persistence



Intermittent Detection of EBOV RNA in Semen

Several studies have reported intermittent detection of EBOV RNA in semen, with samples fluctuating between negative and positive results when survivors provided additional samples for testing over several weeks or months *(Fig. 5a, 5b)*. The Postebogui cohort study in Guinea found that of the 15 men who had at least one semen sample positive for EBOV RNA, 8 men (53.3%) experienced fluctuating negative and positive results over multiple days.[24,31] For instance, one man tested negative on day 397 after recovery, positive on day 453, and negative once again on day 463. Another cohort study of survivors in Liberia found that 8 out of 13 men (61.5%) with positive results demonstrated intermittent detection of EBOV RNA in seminal fluids, with a positive PCR result preceded by at least one negative result.[22] Of these eight, one participant initially had two negative samples before a third sample tested positive for EBOV RNA. Similarly, in the PREVAIL III cohort study, intermittent detection of viral RNA was observed in 78 of the 252 men who provided a semen

sample (31.0%); 36 of these participants (14.2%) also had two negative PCR tests followed by a positive. A case study of an EVD survivor from Liberia also reported two negative PCR tests followed by a positive result.[36]

Each study also used different brands of RT-PCR assays. Of the studies that reported intermittent results, the RealStar Filovirus Screen RT-PCR,[24,31] the Cepheid Xpert Ebola RT-PCR,[22] and the CDC's Ebola Virus Real-Time RT-PCR[36] assays were used. Of the studies that did not report intermittent results, the EZ1 Real-Time RT-PCR[33] and the RealStar Zaire EBOV RT-PCR[9] assays were used. Christie *et al.* and Mate *et al.* did not report on which RT-PCR assay was used.[14,15]

Figure 5a. Survivors with multiple semen samples demonstrating intermittent detection of EBOV RNA by RT-PCR. Green boxes represent positive tests, while red boxes represent negative tests. The numbers in each cell indicate the day post-EVD onset on which the sample was collected and tested. Survivors were included in this figure if individual-level data on RT-PCR results were provided by the study authors. Authors who provided this data include Abel *et al.* (2017), Fischer *et al.* (2017), and Purpura *et al.* (2017).

Survivors with Intermittent Detection of EBOV RNA											
Abel *et al.* (2017)			Fischer *et al.* (2017)								Purpura *et al.* (2017)
1	2	3	4	5	6	7	8	9	10	11	12
397	195	42	779	725	648	657	610	643	707	650	406
453	265	76	849	737	660	676	624	657	719	692	532
463	414	128	980	779	732	725	713	734	749	750	548
	424	245		926	905	788	762	781	791	972	565
	435	327		975	954	957	910	802			603
						1005	958	922			624
								970			758

Figure 5b. Survivors with multiple semen samples that did not demonstrate intermittent detection of EBOV RNA by RT-PCR. Survivors were included in this figure if individual-level data on RT-PCR results showed that negative results were retested. Authors who provided this data include Barnes *et al.* (2017), Christie *et al.* (2015), Mate *et al.* (2015), and Sissoko *et al.* (2017).

Survivors without Intermittent Detection of EBOV RNA														
Barnes *et al.* (2017)	Christie, Mate *et al.* (2015)	Sissoko *et al.* (2017)												
1	2	4	5	6	7	8	9	10	11	12	13	14	15	16
32	199	254	168	251	177	168	184	177	57	94	72	103	38	61
66	231	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
110	234	338	270	336	255	236	254	282	243	274	268	242	225	118
180														
244														

In order to calculate an overall proportion of EVD survivors whose RT-PCR results yielded intermittent detection of EBOV RNA in seminal fluid, studies that retested negative RT-PCR samples but did not find fluctuating persistence were included alongside the studies that reported intermittent detection. In other words, if a study continued testing semen samples even after one negative sample was received, the study was included in this analysis. This choice was made because many other studies on EBOV persistence in semen stopped testing participants' samples after a single negative RT-PCR result was received; however, these studies might have missed detection of fluctuating persistence since these negative samples were not retested. Based on this criterion, 9 studies were eligible for this analysis *(Supplementary Table 5)*, with a total of 303 participants. Intermittent detection of EBOV RNA was detected in 95 survivors' seminal samples (31.4%); 38 (12.5%) experienced two negative PCR tests followed by a positive.

The samples from these 9 studies on EBOV persistence in seminal samples were then compared by the following: (1) day post-EVD onset of the last positive EBOV RT-PCR result in seminal samples, (2) day post-EVD onset of the first "true" negative EBOV result in seminal samples, (3) total duration of follow-up time for each individual (as calculated by the date of the first sample subtracted from the date of the last sample), and (4) duration of follow-up time from the last positive EBOV result to the last sample. In this analysis, a negative RT-PCR result was considered to be a "true" negative if this result was followed by a second negative RT-PCR result with an interval of at least one week between tests. Although less than 13% of individuals experienced two negative RT-PCR results followed by a positive, current WHO interim guidelines denote that a person is no longer at risk of sexual transmission of EBOV after two negative tests of semen by RT-PCR.[18] Data on these individual variables can be found in the supplement *(Supplementary Tables 6a, 6b)*. To compare samples with and without intermittent detection of EBOV RNA, Welch's two-sample t-test for difference of means was performed for each of these 4 variables.

This analysis found that the studies with intermittent detection of EBOV RNA had a statistically significant higher average day of last positive sample (639.2 ± 230.0 days) compared to participants whose samples did not exhibit intermittent detection (129.6 ± 71.4 days, p-value = <0.001). Similarly, the samples with intermittent detection had a statistically significant higher average day of first "true" negative sample (709.2 ± 261.2 days) compared to samples without intermittent detection (239.3 ± 68.1 days, p-value = <0.001). Average duration of follow-up time was significantly higher for samples with intermittent detection (260.8 ± 98.9 days) than for samples where intermittent detection was not observed (115.9 ± 58.0 days, p-value = <0.001). However, for the duration of follow-up time from the last positive result to the last sample, there was no significant difference between samples that did observe intermittent detection (159.8 ± 106.1 days) and those that did not

observe intermittent detection (109.7 ± 53.8 days, p-value = 0.154). These results are summarized in *Table 3*.

Table 3. Welch's Two Sample T-Test for Difference in Means for Intermittent Detection of EBOV RNA in Semen

	Mean ± SD					
	Intermittent Detection (n=12 survivors)	No Intermittent Detection (n=16 survivors)	95% Confidence Interval	t-value	Degrees of freedom	p-value
Day of Last Positive Sample (post-EVD onset)	639.2 ± 230.0	129.6 ± 71.4	(360.5, 658.6)	7.41	12.6	**6.15E-06**
Day of First "True" Negative Sample (post-EVD onset)*	709.2 ± 261.2	239.3 ± 68.1	(301.7, 638.2)	6.08	12.1	**5.27E-05**
Total Duration of Follow-Up Time (days)	260.8 ± 98.9	115.9 ± 58.0	(77.1, 212.5)	4.52	16.6	**0.00032**
Duration of Follow-Up from Last Positive to Last Sample (days)	159.8 ± 106.1	109.7 ± 53.8	(-21.1, 121.3)	1.50	15.2	0.154

* A negative RT-PCR result was considered to be a "true" negative if this result was followed by a second negative RT-PCR result with an interval of at least one week between tests

Modelling Studies on Duration of EBOV Persistence in Semen

A few modelling studies on the persistence of EBOV in semen have been conducted using viral persistence data from existing cohort studies. One such study fitted a negative binomial distribution to viral persistence data from a cohort study of 220 adult male survivors in Sierra Leone,[6]

combined with weekly disease incidence data from the WHO patient database for Guinea, Liberia, and Sierra Leone.[44] The fitted distribution was used to estimate the number of men in each country with EBOV RNA detectable in semen for each week, starting from mid-2014. The model projected that by January 2016, the total number of EBOV RNA semen-positive individuals would decrease from 2,255 people (95% CI: 1,945-2,495) in January 2015 to just 73 people (95% CI: 15-331) across all 3 countries by January 2016.[44] Another study applied parametric survival models to data from the Postebogui cohort study in Guinea to estimate the time elapsed between ADO and when EBOV RNA was no longer detectable in semen by RT-PCR.[31] The median time from symptom onset to a negative RT-PCR test was 46.4 (95% CI: 11-82.6) days. The probability of a survivor's semen sample testing positive by RT-PCR decreased from 31.6% at 3 months to 2.9% and 0.7% at 12 and 18 months post EVD-onset, respectively.[31]

Time-series data from a longitudinal study of 26 EVD survivors in Guinea were used to model the dynamics of EBOV persistence in semen over time.[9] The linear mixed-effect model, which used parameters for a hypothetical baseline Ct value at the time of ADO and a clearance rate of EBOV from semen, predicted that 50% of male survivors would clear EBOV RNA from seminal fluid by 115 days (95% CI: 72-160) post-disease onset and that 90% of male survivors would clear EBOV RNA from seminal fluid by 294 days (95% CI: 212-399) post-onset.[9]

Part 2: Sexual Transmission of EBOV from Convalescents and Subsequent EVD

Although detection of EBOV RNA in genital fluids was first reported by Emond *et al.*'s case study in 1977,[5] reports of likely sexual transmission of EBOV from survivors were first documented during the 2014-2016 West African outbreak. While the number of EVD survivors has increased dramatically since earlier outbreaks, data on actual sexual transmission of EBOV from EVD survivors remain sparse.

To date, 19 cases of EBOV transmission from convalescent survivors have been reported in the published literature *(Table 4)*. Of these, 14 cases involved either probable or confirmed sexual transmission from a male survivor; for the remaining five case, the route of transmission is unknown. Confirmation of sexual transmission was determined by a combination of epidemiological investigation, genomic sequencing of EBOV samples, and lack of evidence of contact with an individual with EVD-related symptoms. The longest duration of detectable EBOV RNA in reported cases of sexual transmission was found to be 531 days after ADO.[7] At the time of transmission, the survivor was 463 days post-EVD onset, which is the longest duration of persistence at the time of transmission for the cases in which transmissions dates are known.[7] In all cases of probable or confirmed sexual transmission, semen was the most probable vehicle for EBOV transmission. However, EBOV isolation by viral culture in sexual transmission studies was either not attempted or unsuccessful. For cases in which the transmission date was known or highly probable, onset of EVD usually occurred within 3 weeks of transmission from the survivor (mean = 19.8 days, n = 4). This finding is consistent with the incubation period of 2-21 days for EBOV infection through other routes of transmission (i.e. contact with infected blood).[3] All cases of sexual transmission were from a male survivor to their female partner. There were no reports of suspected sexual transmission from a male survivor to a male partner or of sexual transmission from a female survivor.

Molecular Evidence of Sexual Transmission

Advances in molecular typing through the past few decades have allowed researchers to use whole-genome sequencing data alongside traditional epidemiological methods to investigate potential sources and routes of transmission. In July 2015, the Ebola Outbreak Sequencing Support (EOSS), a collaboration between the Sierra Leone Ministry of Health, the WHO, and the US Centers for Disease Control & Prevention (CDC), was established to sequence all new EVD cases in Sierra Leone.

Researchers identified a possible instance of sexual transmission from a survivor more than 50 days after the last confirmed case in that particular district.[45] The EBOV genome from a blood sample collected from the new acute case was closely related to the EBOV genome from a male survivor who had recovered from EVD about a month earlier. Sexual contact was reported between the male survivor and the incident case. The viral genome obtained from the survivor's semen during investigations into this new cluster was identical to the viral genome of the survivor's initial blood sample, collected 2 months earlier during acute EVD,[45] which suggests that the virus was maintained in a low replicating state within the survivor, even after recovery from acute EVD.

Other studies have exploited similarities between genomic sequences of different EBOV samples to provide indirect evidence of sexual transmission of EBOV. Five studies investigated 10 new clusters of EVD that appeared months after the last reported case in the same geographic area.[16,45-49] While epidemiological investigations into these new clusters failed to identify a source of infection, whole-genome sequencing was able to link the EBOV genomes from the new clusters to genomes from either a prior circulating strain[16,46,47] or from a known survivor,[16,45] both of which suggest transmission from survivors.

Table 4. Summary of Reports of Transmission of EBOV from a Convalescent

Reference	Country	Acute Case Date of Confirmation	Transmission Date (days post EVD-onset of survivor, if known)	Transmission Route	Most Suspected Body Fluid	Date of Recovery of Survivor	Duration of Persistence in Survivor	Total Cases	Deaths	Virus Isolation
Alpren et al (2016)[46]	Sierra Leone	Jan-3, 2016	Unknown	Unknown, but likely from persistence in a survivor (from genomic data)	Unknown	Unknown	N/A	2	>1	Unknown
Arias et al (2016)[45]	Sierra Leone	Aug-29, 2015	Aug, 2015 (14-44 days)	Sexual, probable	Semen, probable	Jul-18, 2015	>51 days	6	>1	Unknown
Blackley et al (2016)[47]	Liberia	Jun-28, 2015	Jun-1, 2015	Unknown, but likely from persistence in a survivor (from genomic data)	Unknown	Unknown	10 months (Probable cases with reported potential persistent survivor or matching sequence found, providing possible persistence lengths)	8	2	Unknown
Christie et al (2015),[14] Mate et al (2015)[15]	Liberia	Mar-20, 2015	Mar-7, 2015 (151 days)	Sexual, confirmed	Semen	Oct-7, 2014	199 days	1	1	Unsuccessful
Christie et al (2015)[14]	Liberia	*no transmission despite 3-5 occasions of unprotected vaginal intercourse (between Feb-28,2015 – Mar-15,2015)						0	0	N/A
Den Boon et al (2019)[16]	West Africa (unspecified country)	Unspecified	Unspecified	Sexual, probable	Semen	Unknown	~7 weeks (but unspecified)	1	Unknown	Unknown
Den Boon et al (2019)[16]	West Africa (unspecified country)	Unspecified	~1-3 weeks before symptom onset	Sexual, confirmed	Semen, probable	Unspecified	2 months	1	1	Unknown

Table 4. Summary of Reports of Transmission of EBOV from a Convalescent

Reference	Country	Acute Case Date of Confirmation	Transmission Date (days post EVD-onset of survivor, if known)	Transmission Route	Most Suspected Body Fluid	Date of Recovery of Survivor	Duration of Persistence in Survivor	Total Cases	Deaths	Virus Isolation
Den Boon et al (2019)[16]	West Africa (unspecified country)	Unspecified	Unknown	Unknown, but likely from persistence in a survivor (from genomic data)	Unknown	Unknown	N/A	1	Unknown	Unknown
Den Boon et al (2019)[16]	West Africa (unspecified country)	Unspecified	Unknown	Unknown, but likely from persistence in a survivor (from genomic data)	Unknown	N/A	~5 months (but unspecified)	1	1	Unknown
Den Boon et al (2019)[16]	West Africa (unspecified country)	Unspecified	Unknown	Sexual, probable	Semen, probable	Unknown	~5-6 months (but unspecified)	1	1	Unknown
Diallo et al (2016)[7]	Guinea	Mar-16, 2016	Feb-20, 2016 (463 days)	Sexual, confirmed	Semen	Nov-14, 2014	531 days	13	8	Not attempted
Dokubo et al (2018)[48]	Liberia	Nov-19, 2015	Oct, 2015 (likely, but unconfirmed) (396-456 days)	Unknown, but likely via bodily fluids or close contact	Unknown	Aug, 2014	Unknown	2	1	Unknown
Keita et al (2016)[49]	Guinea	Oct-13, 2015	Unknown	Unknown, but likely close contact with body fluids* (but sexual transmission from survivor to wife, then from wife to brother who was the index case)	Semen, probable	Dec, 2014	Unknown	2	0	Not attempted
Lee et al (2017)[50]	Liberia	Jun-29, 2015	Unknown	Sexual, probable	Unknown	Jan-16, 2015	164 days	1	Unknown	Unknown
Lee et al (2017)[50]	Guinea	Mar-17, 2016	Unknown	Sexual, probable	Semen, probable	Unknown	140 days	1	Unknown	Unknown

Table 4. Summary of Reports of Transmission of EBOV from a Convalescent

Reference	Country	Acute Case Date of Confirmation	Transmission Date (days post EVD-onset of survivor, if known)	Transmission Route	Most Suspected Body Fluid	Date of Recovery of Survivor	Duration of Persistence in Survivor	Total Cases	Deaths	Virus Isolation
Lee et al (2017)[50]	Sierra Leone	Jan-14, 2016	Unknown	Sexual, probable	Semen, probable	Unknown	123 days	1	Unknown	Unknown
WHO[51] (2015)	Sierra Leone	Sep-12, 2015	Unknown	Sexual, probable	Unknown	Unknown	Unknown	1	1	Unknown
Thorson et al (2016)[12]	Liberia	Unspecified	Unknown	Sexual, probable	Semen, probable	Unknown	Unknown	1	Unknown	Unknown
Thorson et al (2016)[12]	Liberia	Unspecified	Unknown	Sexual, probable	Semen, probable	Unknown	Unknown	1	Unknown	Unknown
Thorson et al (2016)[12]	Liberia	~Nov, 2014 (but unspecified)	Unknown	Sexual, probable	Semen, probable	Unknown	Unknown	1	Unknown	Unknown

A prior study by Rodriguez *et al.* tested the genomic stability of EBOV by sequencing a highly variable region of the GP gene, which encodes for a viral envelope glycoprotein, in multiple samples.[8] These samples were chosen from patients who were known to be within the same chain of direct human-human transmission. The authors found that samples demonstrated high sequence similarity for this highly variable region, suggesting that similarities in EBOV genomes between two different patients may be indicative of direct transmission.[8] Based on the Rodriguez *et al.* study findings, several studies that had previously identified a convalescent survivor as the source of infection for an incident EVD case through an epidemiological investigation were able to compare EBOV genome sequences between the convalescent and incident cases. Sequencing data from these studies found a high level of similarity in the EBOV genome, suggesting that sexual transmission did indeed occur.[7,14-16,39,48,49]

In transmission reports in which a specific survivor was identified as the source of infection, researchers observed reduced rates of EBOV evolution during persistent infection, despite months or even years of elapsed time between samples. For instance, the EBOV genome from one survivor's blood sample during acute infection differed from that in his semen sample by only 5 nucleotide substitutions, despite being collected 504 days prior to the collection of the semen sample.[7] The resulting evolutionary rate for this convalescent semen sample was roughly 6 times slower than that of the average evolutionary rate seen in acute human-to-human transmission in the West African outbreak.[7] This finding, along with similar results in other studies,[15,45,47,49] suggests that persistent EBOV exhibits reduced evolutionary rates in survivors.

Barnes *et al.* reported EBOV RNA detected by RT-PCR from a 34-year-old survivor from Sierra Leone, whose seminal fluid was RT-PCR positive 110 days after ADO.[33] To determine whether the EBOV in the semen sample was actively replicating within cells or if it persisted only as extracellular virions, the authors used strand-specific RNA methods to compare levels of genomic viral RNA versus viral antigenomic RNA (cRNA) and messenger RNA (mRNA). The levels of cRNA

and mRNA detected in samples from 32 days to 110 days after ADO were similar to the levels found in acute-EVD blood samples, suggesting active viral replication the survivors' semen. Barnes *et al.* not only reported high concentrations of replication-competent virus in semen, but also decreased viral diversification during persistence.[33]

Modelling Studies on EBOV Sexual Transmission by Convalescents

To quantify the importance of EBOV viral persistence-related sexual transmission in increasing the number of cases and duration of the epidemic, several studies developed models that accounted for sexual transmission from convalescent survivors. One model used weekly incidence data from EVD cases in Sierra Leone and found that, for a fixed 0.1% transmission probability per sexual contact, a 3-month duration of EBOV persistence created very few additional cases but extended the epidemic by 83 days on average.[52] They estimated that a 6-month duration of EBOV persistence extended the epidemic by 540 days, which was double the length of the 2014-2015 Sierra Leone Ebola epidemic.[52] Another modelling study found that sexual transmission by convalescents is a significant factor in determining the risk of EVD recurrence in areas that were previously declared transmission free.[53] The authors reported that public health officials may need to wait up to one year after the last EVD case before declaring the end of the epidemic, though this wait time could decrease if survivors routinely practice safer sex or sexual abstinence.[53] A modelling study on EVD intervention efficacies fit an SIR compartmental model to predictive EVD transmission patterns and found that post-recovery condom usage by all recovered patients could reduce the number of EVD cases by 26% and shift the peak of the epidemic curve earlier by 19 days.[54] Another study used daily cumulative cases from West Africa to fit a compartmental model that considered contact with infectious individuals, contact with dead bodies, and sexual transmission from convalescent survivors; the

authors found that sexual contact with convalescent patients had significant effects on increasing the basic reproduction number R_0.[55]

Animal Studies of Sexual Transmission Potential

A study on the transmission potential of persistently-infected survivors inoculated immunodeficient mice with semen samples from eight convalescents who had previously tested positive for EBOV RNA by RT-PCR.[9] Infectious virus was detected by culture in 15 out of 26 (58%) specimens that were tested in the mice, and these mice subsequently developed EVD. Another animal model study described EBOV persistence in convalescent rhesus monkeys that were experimentally infected. The authors detected EBOV RNA in eye, testicle, or brain tissues in 11 out of 112 survivors (9.8%) from samples that were collected 43 days post-exposure.[56] In contrast, EBOV RNA was not detected in liver, lymph node, or spleen tissues, which are common target tissues during acute EBOV infection. Notably, multiplex fluorescence *in situ* hybridization discovered not only the EBOV genome, but also the EBOV antigenome in the eye, epididymis, and brain of survivors, which are immune-privileged sites. Taken together, this data suggests ongoing EBOV replication at the time of sample collection.[56]

Prevention of Sexual Transmission through Condom Use by Male EVD Survivors

The use of condoms by EVD survivors during sexual activity remains inconsistent. Sixty-five percent of participants in the Postebogui survivor cohort study in Guinea (n=664) reported sexual activity without a condom since recovery, including 48% of those with a semen sample that tested positive for EBOV RNA by RT-PCR (n=491).[34] Liberia's Men's Health Screening Program found that 427 of 466 participants (92%) reported being counselled by EVD treatment unit (ETU) staff to

either abstain from sexual activity or to use condoms for 90 days post-recovery, as recommended by WHO interim guidelines for survivors at the time.[40] At the time of enrollment, which ranged from 7 days to 697 days post-EVD recovery (median = 384 days), 424 (91%) participants reported having resumed sexual activity. Of the 410 participants who reported the date that they resumed sexual activity, 363 (89%) waited at least 90 days after discharge from an ETU before resuming sexual activity. Of the 424 participants who reported resuming sexual activity, 190 (45%) reported using a condom the last time they had intercourse.[40]

DISCUSSION

This is the first systematic review on the persistence of EBOV in genital fluids and its relation to sexual transmission since Thorson *et al.* in 2016.[12] Our review captures recent evidence released by national semen testing programs and cohort studies that followed survivors from the 2014-2016 West African EVD outbreak. The WHO's interim guidelines on clinical care for EVD survivors were last revised in April 2016.[18] Currently, the WHO recommends safer sex practices for at least 12 months for EVD survivors who have not had their semen tested. The evidence reviewed here suggests that EBOV can persist in the seminal fluids of survivors for longer than 12 months, which justifies the need for a reassessment of these guidelines.

In this review, we provide quantitative estimates of the length of EBOV persistence in semen, vaginal, and rectal fluids and report on the transmission potential of persistent EBOV. Among almost 2,000 male EVD survivors, more than 16% had at least one RT-PCR positive semen sample result during convalescence or post-recovery, indicating that viral persistence in semen is not a rare occurrence. EBOV RNA was detected in semen by RT-PCR for up to 40 months,[30] which far exceeds the WHO's 12-month recommendation for safer sex practices among EVD survivors. Across studies

included in this review, EBOV RNA persisted in semen for an average of 370 days, which also suggests the need to reassess the interim WHO guidelines. Data on viral persistence in semen is right-censored; for five studies included in this review, some participants were still RT-PCR positive for EBOV RNA in semen when the study ended.[22,30,35,41,57] This suggests that the average duration of persistence of viral RNA is likely an underestimate.

Of the more than 300 female survivors whose vaginal fluids were tested for EBOV persistence up to 35 days post-EVD onset, less than 1% were RT-PCR positive for EBOV RNA. This low proportion is consistent with previous studies of EBOV persistence in vaginal fluids.[8,38] The longest duration of EBOV RNA persistence was 35 days across the 321 vaginal fluid samples that tested positive.[32] Similarly, while 25 rectal samples were tested, the last rectal sample tested positive for EBOV RNA at 29 days.[8]

The detection of EBOV RNA in genital fluids does not necessarily imply that the sample is infectious; evidence on the transmission potential of persistent EBOV RNA in genital fluids was also evaluated. From studies that attempted viral cultures of RT-PCR positive genital fluid samples, the maximum time at which EBOV was isolated was 82 days post-EVD onset from a semen sample.[8] No EBOV has been successfully isolated from vaginal or rectal samples. Only 5 studies have successfully detected EBOV by viral culture in convalescent seminal fluids.[4,5,8,33,42] This could be related to the small number of studies that attempted viral isolation, to publication bias, or to the inactivation of the virus by standard practices of storing and freezing specimens.[8]

The most direct link between persistent EBOV RNA detection in seminal fluids and evidence of infectiousness was established by Sissoko *et al.* Seminal fluid specimens from EVD survivors, with persistent EBOV RNA detected at a median duration of 158 days after ADO, were inoculated into immunodeficient mice.[9] Infectious virus was detected in more than half of the specimens tested in

the mice. Subsequent EBOV infection of these mice indicates that EBOV RNA detected by RT-PCR in seminal fluid may be indicative of infectious viral particles.

A study by Barnes *et al.*, which used semen samples from a male EVD survivor with EBOV RNA detected up to 110 days post-EVD onset, found high concentrations of replication-competent virus in viral cultures.[33] A consistent result across this study and a number of other studies that examined genomic sequencing data was that EBOV RNA exhibited decreased viral diversity during persistence, with very few nucleotide substitutions between fluid samples that were collected months, and even years, apart.[15,33,45,47,49] In other words, in persistent EBOV RNA samples, EBOV can be maintained in a low replicating state, but with an evolutionary rate that is reduced compared to that seen during acute human-to-human transmission. To explain the reduced evolutionary rate, Blackley *et al.* and Diallo *et al.* suggest that EBOV in immune-privileged sites such as the testes might experience a reduced rate of viral replication;[7,47] persistence of the EBOV RNA could then be explained by the characteristic reduction of immune clearance seen in immune-privileged sites.[58] However, Barnes *et al.*'s discovery of high amounts of EBOV cRNA and mRNA in semen from EVD survivors strongly suggests the presence of replicating virus in seminal fluid cells.[33] Instead, because evolutionary rates are dependent on replication and mutation rates, as well as selective pressures, the reduced EBOV evolutionary rate could be related to reduced selection within immune-privileged sites such as the testes rather than reduced replication.

Diallo *et al.* indicates that one explanation for EBOV persistence that cannot be ruled out is that the survivor was sub-clinically re-infected with EBOV following recovery. However, this is unlikely because there has not yet been a documented, laboratory-confirmed case of EBOV reinfection.[7] Similarly, in every study, blood samples that were collected concurrently with semen samples from EVD survivors tested negative by RT-PCR, which indicates the lack of an acute infection. The lower-than-expected number of mutations observed in the EBOV genome from

persistent samples also renders it unlikely that this survivor is a subject of an undetected chain of human-to-human transmission among acute cases.

Genomic techniques have been implemented alongside traditional epidemiological methods to investigate clusters of EVD that cannot be temporally, geographically, or epidemiologically linked to any known existing acute EVD cases. Multiple studies have employed genome sequencing to characterize uncertain sources of infection, with direct human-to-human transmission suggested by high sequence similarities between EBOV samples from a survivor and the new, acute case.[7,14-16,39,45,48,49] In almost all cases of EBOV transmission that implicated a recovered survivor, sexual transmission via persistently-infected semen was the most suspected route. Given the link between EBOV RNA detection and infectious viral particles described in the virologic studies above, combined with EBOV genomic analyses, sexual transmission from a survivor poses a plausible risk for initiating subsequent cases of EVD. However, cases resulting from this transmission route appear to be few, as this route has been suggested in fewer than twenty reports. All of the cases of potential sexual transmission from a survivor described male-to-female transmission, with no evidence of female-to-male transmission. This is consistent with the lack of conclusive evidence of EBOV persistence in vaginal fluids.

However, it is important to note that not every sexual exposure with an EVD survivor necessarily results in sexual transmission. Christie *et al.* and Mate *et al.* described molecular evidence of sexual transmission from a male, EVD survivor, resulting in a new, acute case of EVD.[14,15] The survivor also reported multiple instances of unprotected vaginal intercourse with another woman, which took place around the same time as the survivor's contact with the acute EVD case. The other woman did not develop EVD, and serologic testing for antibodies was negative, indicating no prior EBOV infection. This inconsistency suggests that there may be other undescribed factors that place some people at a higher risk of contracting persistence-derived EVD from a survivor.

With regards to age and persistence, we found that age was a statistically significant predictor of EBOV RNA persistence in semen, which confirms the findings from two other cohort studies. Soka *et al.* and Fischer *et al.* first described a significant association between the age of the survivor and persistence of EBOV RNA in semen, with men over 40 years old more likely to have detectable EBOV RNA in seminal fluids.[22,41] In contrast, Sissoko *et al.* did not detect a significant association for age and persistence.[9] However, the small sample size of this study (n=26), in contrast to Fischer *et al.* (n=149) and Soka *et al.* (n=429), could explain this discrepancy. Moreover, the participants in Sissoko *et al.*'s study skewed towards the younger ages (median=31 years, IQR 26-40 years). These two factors suggest that the study may have had insufficient statistical power to detect a significant association between older men and persistence of EBOV RNA in semen. Taken together, these findings hint that older age is a potential determinant of EBOV persistence, and our analysis also finds that older age is significantly correlated with a longer length of persistence. While age may be a proxy for other risk factors, this finding is plausible due to age-related changes in immune functioning, as immune senescence from natural aging may allow for persistence of EBOV into immune-privileged sites such as the testes.[59]

A final concern to consider in revising clinical guidelines for EVD survivors relates to reports of intermittent detection of EBOV RNA by RT-PCR in multiple cohort studies.[23,24,30,36] From our sample of 300 survivors, for which individual RT-PCR results were provided, we estimate that over 30% of participants received a negative RT-PCR result for a semen sample followed by a positive result weeks or months later. Over 12% of participants even received two negative RT-PCR results prior to a positive result. We also found that studies that reported intermittent results observed a later average day post-EVD onset of the last positive RT-PCR sample and also followed survivors for a longer period of time, compared to studies that did not report intermittent results. The high rate of intermittent results in select studies could potentially be explained by increased difficulties in detecting

EBOV RNA as time passes, since Ct values have been shown to increase over time and approach the threshold of positivity set at Ct=40.[6] Another possible explanation is that because studies with high rates of intermittent results followed survivors for a longer period of time, they were better able to capture patterns of intermittent detection, compared to other studies that may have stopped following survivors after a single negative RT-PCR result was received. This explanation is further supported by the right-censoring observed in the data from 5 studies, who reported participants who were still positive by RT-PCR for EBOV RNA by the time the study ended.[22,30,35,41,57] For instance, Sissoko *et al.* reported 3 individuals who were still positive for EBOV RNA by their last semen sample.[9] Further testing of seminal fluids may have revealed either an intermittent pattern or an even longer duration of persistence.

Limitations

There are a few limitations to our review. Due to the constraints of having only one reviewer, selection of studies during the initial screening process may be subject to bias. For similar reasons, a validity assessment was performed in lieu of the more time-intensive GRADE process, which could mean that the inclusion of some of the final selected studies may not have been fully validated. Some of the same individuals were reported in different studies (e.g. Christie *et al.* and Mate *et al.*, or Rodriguez *et al.* and Rowe *et al.*). Where possible, we corrected for this to avoid instances of the same individual contributing multiple times to estimates such as average duration. However, there remains a small chance that a few individuals were double-counted.

Our analyses were limited by our lack of access to individual-level data. Some analyses, such as those conducted for intermittent detection of EBOV RNA and for the association between age and persistence, were limited to publications that included subject-level data. A few cohort studies that were included in this review also did not consistently report variance data for our primary outcome

of interest, the duration of EBOV persistence. For instance, Subtil *et al.* reported only a minimum and maximum for duration of EBOV persistence rather than providing data on the variance.[31]

Finally, we focused our attention on EBOV persistence in semen, vaginal fluids, and rectal samples because we were interested in the relation between EBOV persistence and sexual transmission. Contact with urine, fecal matter, or other bodily fluids not included in this review may also occur during sexual activity. Few studies estimated EBOV RNA persistence in these bodily fluids, so it is unlikely that this exclusion would have had a significant impact on our analyses.[4,5,8,35,38]

Conclusions

EBOV RNA has been detected in the seminal fluids of an EVD survivor for up to 40 months post-EVD onset. After synthesizing all published evidence from 42 studies, representing nearly 2,000 male survivors, the average duration of EBOV RNA in semen was 370 days post-EVD onset. Given these results, we suggest that the WHO consider revising their current recommendation that EVD survivors who have not had their semen tested practice safer sex for at least 12 months. Our review indicates that persistence of EBOV RNA is related to an increased risk of sexual transmission of EBOV. Due to reports of intermittent detection of EBOV RNA, especially among survivors who experience EBOV persistence for over a year, we recommend that at least two negative RT-PCR results be received before declaring the survivor's seminal fluid to be cleared of EBOV RNA. Finally, we report that age is a potential determinant of EBOV persistence, with older age associated with a higher likelihood of EBOV RNA detection in seminal fluid.

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APPENDIX

Supplementary Table 1. Key search terms used and their combinations. The search strategy described below was used for Ovid(Medline). The search terms were subsequently adapted to suit the syntax of each database.

Questions 1 & 2	Search Terms
How long do Ebola virus and Ebola viral RNA persist in semen, vaginal, and rectal fluids, by sex? Is the persistence of Ebola viral RNA related to sexual transmission of EBOV?	1. exp Ebola virus/ OR exp Hemorrhagic Fever, Ebola/
	2. (Ebola or EBOV or ebolavirus).ti,ab,kw.
	3. 1 OR 2
	4. exp semen/ OR exp rectum/ OR exp vagina/
	5. semen.ti,ab,kw. OR seminal.ti,ab,kw. OR testes.ti,ab,kw. OR vagina*.ti,ab,kw. OR cervix.ti,ab,kw. OR cervical.ti,ab,kw. OR faeces.ti,ab,kw. OR feces.ti,ab,kw. OR fecal.ti,ab,kw. OR rectum.ti,ab,kw. OR rectal.ti,ab,kw. OR anus.ti,ab,kw. OR anal.ti,ab,kw.
	6. 4 OR 5
	7. 3 AND 6
Question 3	
Does consistent and correct condom use reduce transmission of Ebola virus?	1. exp Ebola virus/ OR exp Hemorrhagic Fever, Ebola/
	2. (Ebola or EBOV or ebolavirus).ti,ab,kw.
	3. 1 OR 2
	4. exp condom/ OR exp contraception, barrier/ OR exp safe sex
	5. condom.ti,ab,kw. OR barrier method.ti,ab,kw. OR safe* sex.ti,ab,kw.
	6. 4 OR 5
	7. 3 AND 6
Questions 4 & 5	
How long after the resolution of EVD-related symptoms does a male partner need to use a latex condom with his male or female sexual partners? For how long, following the resolution of symptoms, should a man who recovered from EVD continue to have his semen tested?	1. exp Ebola virus/ OR exp Hemorrhagic Fever, Ebola/
	2. (Ebola or EBOV or ebolavirus).ti,ab,kw.
	3. 1 OR 2
	4. exp sexually transmitted diseases/ OR exp coitus/
	5. coitus.ti,ab,kw. OR sex.ti,ab,kw. OR sexual*.ti,ab,kw. OR intercourse.ti,ab,kw. OR penetrative.ti,ab,kw. OR penetration.ti,ab,kw.
	6. 4 OR 5
	7. 3 AND 6

Supplementary Table 2. Validity Assessment of Included Studies

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Abbate et al (2016)	N/A; the study's purpose was to develop a mathematical model for studying sexual transmission from convalescent survivors	The model was fitted to data from weekly incidence of confirmed and probable cases in Sierra Leone (2014-2015) from the WHO patient database.	N/A	Yes. Aim: "to investigate the potential impact of convalescent sexual transmission on the transmission dynamics in general, and on the tail of the epidemic in particular, to understand how long that vigilance might remain critical."
Abel et al (2017)	Unclear, probably yes.	Semi-quantitative RT-PCR assay, with positive results confirmed by a second RT-PCR targeting viral nucleoprotein sequences. The Ct* cut-off for positive results was <40.	Yes, duplicate-tested	Yes. The aim was to investigate "questions about the longterm persistence of Ebola virus in semen and how long surveillance of survivors should be maintained."
Alpren et al (2016)	Unclear, probably yes, in accordance with WHO sample collection and testing standard operating protocols	Positive result by postmortem buccal swab tested by RT-PCR for deceased patient, or RT-PCR of blood samples for living patients	Unclear, but likely yes for living patients	Yes. Aim: to detail a new chain of transmission occurring in Sierra Leon 4 months after the the last reported case.

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Arias et al (2016)	Unclear, probably yes.	Collected blood samples or buccal swaps; positive if RT-PCR results had Ct values <40	Yes, duplicate-tested	Yes. Aim: to use genome sequencing to "identify unconventional transmission chains involving body fluids, including semen"
Barnes et al (2017)	Yes, serum and blood samples were collected and tested daily from day 7 to day 30 post-symptom onset, then semen samples from day 32 to day 244	RT-PCR assay, with positive results for Ct values <40; also viral isolation by tissue culture	Unclear	Yes. Aim: to "[utilize] reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and deep sequencing to determine concentration of viral RNA, replicative capacity, and viral evolution in blood and semen of a single EVD patient over 110 days of illness"
Bausch et al (2007)	Yes, samples were placed into cryovials and stored at ambient temperature for <6 hours before being stored in liquid nitrogen	ELISA antigen positive or RT-PCR positive; considered to be convalescent if they were previously a confirmed clase, but whose ELISA antigen and RT-PCR results had reverted to negative	Yes, duplicate-tested by both culture and real-time RT-PCR	Not exactly sexual transmission. Aim: "To better understand the precise modes of transmission, we sampled various clinical specimens from patients as well as from environmental surfaces"

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Blackley et al (2016)	Yes, samples were tested within 1 day	RT-PCR positive results	Unclear	Yes. Aim: "use epidemiological and genomic data to investigate the source of the second Liberian flare-up, centered in Margibi County"
Christie et al (2015)	Unclear, probably yes.	Positive result by RT-PCR	Unclear	Yes. Aim: " describes the investigation by the Government of Liberia and international response partners of the source of Liberia's latest Ebola case and discusses the public health implications of possible sexual transmission of Ebola virus"
Deen et al (2017)	Yes, specimens were refrigerated for no longer than 3 days	RT-PCR assays were performed that targeted EBOV NP and VP40 gene targets. A specimen was considered positive if the NP and VP40 gene targets were both detected within 40 cycles of replication.	Unclear	Yes. Aim: "describes the participants' characteristics at entry in the cohort of male survivors of EVD whose semen was tested by means of RT-PCR"
Den Boon et al (2019)	N/A; the study's purpose was to report on possible viral persistence derived transmission based on epidemiological data	RT-PCR positive results	N/A	Yes. Aim: "describe a series of EBOV transmission events with evidence of transmission related to viral persistence in EVD survivors"
Diallo et al (2016)	Unclear, probably yes.	RT-PCR positive results	Unclear	Yes. Aim: "report on an Ebola virus disease (EVD) survivor who showed Ebola virus in seminal fluid 531 days after onset of disease"

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Dokubo et al (2018)	N/A; the study's purpose was to investigate epidemiological links of a cluster of new cases	Positive laboratory result for Ebola virus antigen by reverse transcriptase qualitative PCR (RT-PCR) detection of virus RNA or by detection of anti-Ebola IgM antibodies	N/A	Not exactly sexual transmission. Aim: "Case investigations were done to ascertain previous contact with cases of Ebola virus disease or infection with Ebola virus."
Eggo et al (2015)	N/A; modeling study	N/A	N/A	Yes. Aim: "to estimate the current number of semen-positive men in affected West African countries"
Emond et al (1977)	Unclear, probably yes, as this person was hospitalized	Positive by viral culture	Unclear	Yes. This was a case report that also happened to describe EBOV isolated from convalescent semen specimens
Etard et al (2017)	Unclear, probably yes.	"Laboratory-confirmed EVD," likely positive by RT-PCR	Unclear	Yes. Aim: " to assess long-term clinical, psychosocial,and viral outcomes in EVD survivors in Guinea"
Fallah et al (2016)	Unclear, probably yes.	RT-PCR positive results	Unclear	Yes. Aim: "To characterize the clinical sequelae in survivors and to assess whether they can transmit infection to household members and sexual contacts"
Fischer et al (2016)	N/A; the study's purpose was to assess the efficacy of detecting EBOV in semen and to determine the	N/A	N/A	Yes. Aim: "We assessed the efficiency of detecting Ebola virus in semen samples by molecular diagnostics and the stability of Ebola virus in ex vivo semen under simulated tropical conditions"

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
	stability of EBOV in ex vivo semen			
Fisher et al (2017)	Yes, semen was tested for EBOV RNA within 2 days of collection	Samples were considered positive if either target gene (GP or NP) was detected by RT-PCR	Unclear	Yes. Aim: " describes the investigation by the Government of Liberia and international response partners of the source of Liberia's latest Ebola case and discusses the public health implications of possible sexual transmission of Ebola virus"
Green et al (2016)	Unclear, probably yes	RT-PCR assay, with positive results for Ct values <40	Samples were retested if Ct values were 37-40	Yes. Aim: "to better inform necessary protective measures for health-care providers, behavioural modification advice for survivors"
Guo et al (2016)	N/A; modeling study	N/A	N/A	Yes. Aim: "to predict epidemic trends and evaluate intervention measure efficacy following the 2014 EVD epidemic in West Africa"
Keita et al (2016)	Unclear, probably yes	Positive result by RT-PCR	Unclear	Yes. Aim: to investigate possible transmission of EBOV from an EVD survivor to another person
Knust et al (2016)	Unclear, probably yes	Positive result by RT-PCR	Unclear	Yes. Aim: "to assess the presence and duration of EBOV and viral RNA in semen and other body fuids of EVD survivors"

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Lee et al (2019)	N/A; modeling study	N/A	N/A	Yes. Aim: "to compute the probability of the end of an Ebola virus disease epidemic, accounting for sexual transmission and underascertainment of cases"
Lee et al (2017)	N/A; the study's purpose was to report on recrudescence events in West Africa	Recrudescent Ebola was defined as "reappearance of at least one confirmed case of EVD in a country where the end of EVD had been declared in advance"	N/A	Yes. Aim: "to review all known recrudescence events in West Africa occurring during the period 2014–2016"
Luo et al (2019)	N/A; modeling study	N/A	N/A	Yes. Aim: " to understand how the [West African epidemic was affected by various transmission routes which include contact with infections, contact with dead bodies, and having sex with convalescent survivors"
Martini et al (1968)	Unclear, probably yes	Viral culture	Unclear	Not specifically Ebola, but the study was a case report on sexual transmission of the related Marburg virus
Mate et al (2015)	N/A; the study's purpose was to use genomic data to demonstrate sexual transmission from a survivor	Positive result by RT-PCR	N/A	Yes. Aim: to use genomic analysis to provide evidence of sexual transmission of EBOV and evidence of persistence of infective EBOV in semen

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Moreau et al (2015)	N/A; the study's purpose was to report on two Ebola virus (EBOV) RT-PCR discordant mother–child pairs	Positive result by RT-PCR	Unclear	Not exactly from survivors. Aim: to investigate two Ebola virus RT-PCR discordant mother-child pairs to suggest the need for RT-PCR testing of breastmilk
PREVAIL et al (2019)	Unclear, probably yes	For antibody specimens: "548 enzyme-linked immunosorbent assay units (EU) per milliliter was used as the cutoff for positivity"; For semen specimens: positive result by RT-PCR	Unclear	Yes. Aim: "describes the investigation by the Government of Liberia and international response partners of the source of Liberia's latest Ebola case and discusses the public health implications of possible sexual transmission of Ebola virus"
Purpura et al (2017)	Unclear, probably yes	Positive result by RT-PCR	Unclear	Yes. Aim: " to report an EVD survivor with preexisting HIV infection, whose semen was positive for Ebola virus RNA 565 days after recovery from EVD"
Richards et al (2000)	Unclear, probably yes	EBOV isolation by viral culture, as well as using ELISA and IgM antibodies for viral antigen detection by ELISA	Unclear	Yes. Aim: "To describe the clinical manifestations of viral hemorrhagic fever, and to increase clinicians' awareness and knowledge of these illnesses"
Rodriguez et al (1999)	Unclear, probably yes	Positive laboratory result for Ebola virus antigen by reverse transcriptase qualitative PCR (RT-PCR)	Unclear	Yes. Aim: "to determine whether EBO virus is still present in body fluids of convalescent patients after clinical symptoms subside, and if so, what the duration of virus persistence is"

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
		detection of virus RNA or by viral culture		
Rowe et al (1999)	Unclear, probably yes	Positive result by RT-PCR	No, but the authors attempted to select 2 controls of the same sex as and close in age to the convalescent	Yes. Aim: "to describe the clinical course of convalescence following EHF, determine whether body fluids contain EBO virus, and monitor household contacts for evidence of secondary transmission from the convalescents"
Sissoko et al (2017)	Yes, "We processed samples of seminal fluid from participants immediately after collection at the European mobile laboratory (EMLab) unit in Coyah"	Positive result by RT-PCR	Unclear	Yes. Aim: "to use biostatistical modelling to describe the dynamics of Ebola virus RNA load in seminal fluid, including clearance parameters"
Sissoko et al (2017)	Unclear, probably yes	Positive result by RT-PCR	Unclear	Not exactly about survivors. Aim: to investigate the case of a 9-month-old infant died from Ebola virus (EBOV) disease with unknown epidemiological link. The parents may have had asymptomatic carriage

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Soka et al (2016)	Yes, "All self-collected semen specimens submitted by the MHSP were stored and transported at –20°C or colder to the Tappita Ebola virus disease laboratory in Nimba County. Upon receipt in the laboratory, specimens were maintained at –20°C or colder until testing"	Positive result by RT-PCR	Unclear	Yes. Aim: "describe Liberia's national semen testing programme for Ebola virus, present preliminary semen testing results, and report sexual risk behaviours"
Sow et al (2016)	Unclear, probably yes	Positive result by RT-PCR	Unclear	Yes. Aim: "report new evidence of long-term persistence of Ebola virus RNA in semen of male survivors"
Srinivas et al (2016)	Unclear, probably yes, as this person was placed under quarantine	Positive result by RT-PCR or viral isolation by culture	Unclear	Yes. Aim: "report follow-up of a man who recovered from EVD and was monitored for 165 days after he was declared Ebola-free"
Subtil et al (2017)	Unclear, probably yes	Positive result by RT-PCR, with a Ct cutoff for positivity of ≤40.9	Unclear	Yes. Aim: "This study modeled the presence of Ebola virus RNA in the semen of male Ebola survivors participating in the Postebogui study in Guinea"

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Toure et al (2017)	Unclear, probably yes	Detection of antibodies to GP, NP and VP40 proteins and of EBOV RNA in semen by PCR	Unclear	Yes. Aim: "to quantify individual risk of exposure of contact persons to EVD cases; to measure the presence of antibodies to EBOV; to look for EBOV RNA in semen of adult seropositive men"
Uyeki et al (2016)	Yes. "Semen specimens were collected and transported as soon as possible or maintained at 4 degrees Celsius and shipped overnight on frozen cold packs to CDC. The majority of the specimens were processed within 0–3 days; however a few were processed 5–8 days after collection. Only one specimen that was collected 290 days post symptom onset was frozen prior to virus culture"	Positive result by RT-PCR; cycle threshold (Ct) values <40 were considered positive; viral isolation by culture was also performed	Unclear	Yes. Aim: "[to investigate] the duration of Ebola virus (EBOV) RNA and infectious EBOV in semen specimens of 5 Ebola virus disease (EVD) survivors"

Study	Acceptable time delay between sample collection & testing (< 3 months for assessment of exposures)	Case definition & method of Ebola status confirmation	Were the samples duplicate-tested?	Was the aim relevant to human-to-human sexual transmission or transmission from survivors?
Zeng et al (2016)	N/A; the study's purpose was to assess the viability of rhesus monkeys as an animal model for EBOV persistence	N/A	N/A	Yes. Aim: "to provide an animal model that demonstrates EBOV persistence is associated with ongoing replication in the presence of an inflammatory host response"

Supplementary Table 3. Data for Weighted Average Calculation for Length of Persistence of EBOV RNA in Semen

Reference	No. of Patients	No. of Patients with Positive Sample (%)	Mean Time Between Disease Onset and Last Positive Sample	Weight
Abel et al (2017)	188	15	190.4 +/- 155.1*	0.41964286
Fischer et al (2017)	149	13	771.9 +/- 100.9	0.33258929
Rodriguez et al (1999), Rowe et al (1999)	5	4	85.8 +/- 17.2	0.01116071
Sissoko et al (2017)	26	19	149.6 +/- 91.2	0.05803571
Sow et al (2016)	68	8	118.9 +/- 79.9	0.15178571
Uyeki et al (2016)	5	5	184.6 +/- 75.3	0.01116071
Barnes et al (2017)	1	1	110	0.00223214
Bausch et al (2007)	1	1	40	0.00223214
Christie et al (2015), Mate et al (2015)	1	1	199	0.00223214
Diallo et al (2016)	1	1	531	0.00223214
Green et al (2016)	1	1	128	0.00223214
Purpura et al (2017)	1	1	579	0.00223214
Srinivas et al (2016)	1	1	179	0.00223214
Weighted Average	370.3149554	Note: * denotes that the value was measured as days since discharge from ETC		
Weighted SD	345.077			
N	448			

Supplementary Table 4. Data for Analysis of Age & Duration of EBOV RNA Persistence in Seminal Fluids of EVD Survivors

Reference	Body Fluid	Assay	Age	Latest Day After ADisease Onset: Positive Sample
Abel et al (2017)	Semen	RT-PCR	56	511
Abel et al (2017)	Semen	RT-PCR	26	467
Abel et al (2017)	Semen	RT-PCR	29	209
Abel et al (2017)	Semen	RT-PCR	37	90
Barnes et al (2017)	Semen	RT-PCR	34	110
Christie et al (2015), Mate et al (2015)	Semen	RT-PCR	46	199
Diallo et al (2016)	Semen	RT-PCR	56	531
Fischer et al (2017)	Semen	RT-PCR	47	965
Fischer et al (2017)	Semen	RT-PCR	54	849
Fischer et al (2017)	Semen	RT-PCR	34	737
Fischer et al (2017)	Semen	RT-PCR	36	905
Fischer et al (2017)	Semen	RT-PCR	38	676
Fischer et al (2017)	Semen	RT-PCR	46	560
Fischer et al (2017)	Semen	RT-PCR	43	762
Fischer et al (2017)	Semen	RT-PCR	36	761
Fischer et al (2017)	Semen	RT-PCR	46	734
Fischer et al (2017)	Semen	RT-PCR	44	828
Fischer et al (2017)	Semen	RT-PCR	42	749
Fischer et al (2017)	Semen	RT-PCR	43	750
Fischer et al (2017)	Semen	RT-PCR	41	759
Green et al (2016)	Semen	RT-PCR	21	128
Purpura et al (2017)	Semen	RT-PCR	48	579
Rodriguez et al (1999)	Semen	RT-PCR	25	101
Rodriguez et al (1999)	Semen	RT-PCR	27	82
Rodriguez et al (1999)	Semen	RT-PCR	29	63
Rodriguez et al (1999)	Semen	RT-PCR	33	63
Sissoko et al (2017)	Semen	RT-PCR	40	254
Sissoko et al (2017)	Semen	RT-PCR	45	168
Sissoko et al (2017)	Semen	RT-PCR	35	251
Sissoko et al (2017)	Semen	RT-PCR	18	177
Sissoko et al (2017)	Semen	RT-PCR	33	407
Sissoko et al (2017)	Semen	RT-PCR	26	233
Sissoko et al (2017)	Semen	RT-PCR	30	158
Sissoko et al (2017)	Semen	RT-PCR	46	168

Sissoko et al (2017)	Semen	RT-PCR	19	184
Sissoko et al (2017)	Semen	RT-PCR	28	177
Sissoko et al (2017)	Semen	RT-PCR	27	57
Sissoko et al (2017)	Semen	RT-PCR	32	94
Sissoko et al (2017)	Semen	RT-PCR	32	72
Sissoko et al (2017)	Semen	RT-PCR	28	103
Sissoko et al (2017)	Semen	RT-PCR	40	38
Sissoko et al (2017)	Semen	RT-PCR	35	61
Sissoko et al (2017)	Semen	RT-PCR	18	72
Sissoko et al (2017)	Semen	RT-PCR	55	73
Sissoko et al (2017)	Semen	RT-PCR	25	95
Sow et al (2016)	Semen	RT-PCR	48	276
Sow et al (2016)	Semen	RT-PCR	28	30
Sow et al (2016)	Semen	RT-PCR	27	56
Sow et al (2016)	Semen	RT-PCR	27	61
Sow et al (2016)	Semen	RT-PCR	48	182
Sow et al (2016)	Semen	RT-PCR	19	93
Sow et al (2016)	Semen	RT-PCR	37	99
Sow et al (2016)	Semen	RT-PCR	58	218
Srinivas et al (2016)	Semen	RT-PCR	26	179

Supplementary Table 5. Studies Eligible for Analysis of Intermittent Detection Data

Reference	Number of people with fluctuating results	Number of people with 2 – 's before a + (if applicable)	Total Number of People Tested
Abel et al (2017), Subtil et al (2017)	8	0	15
Barnes et al (2017)	0	0	1
Christie et al (2015), Mate et al (2015)	0	0	1
Emond et al (1977)	0	0	1
Fischer et al (2017)	8	1	13
PREVAIL III Study Group (2019)	78	36	252
Purpura et al (2017)	1	1	1
Sissoko et al (2017)	0	0	19
Total	95	38	303
Proportions	0.313531353	0.125412541	

Supplementary Table 6a. Samples that demonstrated intermittent detection of EBOV RNA

Reference									Total Length of Follow-Up	Length of Follow-Up from Last Positive to Last Sample
Abel et al (2017)	**RT-PCR Result**	-	+	-					66	10
	Day After Disease Onset	397	453	463						
	RT-PCR Result	+	-	+	-	-			240	21
	Day After Disease Onset	195	265	414	424	435				
	RT-PCR Result	-	+	-	-	-			285	251
	Day After Disease Onset	42	76	128	245	327				
Fischer et al (2017)	**RT-PCR Result**	-	+	-					201	131
	Day After Disease Onset	779	849	980						
	RT-PCR Result	-	+	-	-	-			250	238
	Day After Disease Onset	725	737	779	926	975				
	RT-PCR Result	+	+	-	+	-			306	49
	Day After Disease Onset	648	660	732	905	954				
	RT-PCR Result	-	+	-	-	-	-		348	329
	Day After Disease Onset	657	676	725	788	957	1005			

	RT-PCR Result	+	+	-	+	-	-		348	196
	Day After Disease Onset	610	624	713	762	910	958			
	RT-PCR Result	+	-	+	-	-	-	-	327	236
	Day After Disease Onset	643	657	734	781	802	922	970		
	RT-PCR Result	-	+	+	-				84	42
	Day After Disease Onset	707	719	749	791					
	RT-PCR Result	-	-	+	-				322	222
	Day After Disease Onset	650	692	750	972					
Purpura et al (2017)	**RT-PCR Result**	(previous + tests)	-	-	+	-	-	(more - tests)	352	193
	Day After Disease Onset	406	532	548	565	603	624	758		

Supplementary Table 6b. Samples that did not demonstrate intermittent detection of EBOV RNA

Reference							Total Length of Follow-Up	Length of Follow-Up from Last Positive to Last Sample
Barnes et al (2017)	**RT-PCR Result**	+	+	+	-	-	212	134
	Day After Disease Onset	32	66	110	180	244		
Christie et al (2015), Mate et al (2015)	**RT-PCR Result**	+	-	-			35	35
	Day After Disease Onset	199	231	234				
Emond et al (1977)	**Viral Culture Result**	+	+	-	-	-	71	49
	Day After Disease Onset	39	61	76	92	110		
Sissoko et al (2017)	**RT-PCR Result**	+	-	-			84	84
	Day After Disease Onset	254	N/A	338				
	RT-PCR Result	+	-	-			102	102
	Day After Disease Onset	168	N/A	270				
	RT-PCR Result	+	-	-			85	85
	Day After Disease Onset	251	N/A	336				
	RT-PCR Result	+	-	-			78	78
	Day After Disease Onset	177	N/A	255				

	RT-PCR Result	+	-	-			68	68
	Day After Disease Onset	168	N/A	236				
	RT-PCR Result	+	-	-			70	70
	Day After Disease Onset	184	N/A	254				
	RT-PCR Result	+	-	-			105	105
	Day After Disease Onset	177	N/A	282				
	RT-PCR Result	+	-	-			186	186
	Day After Disease Onset	57	N/A	243				
	RT-PCR Result	+	-	-			180	180
	Day After Disease Onset	94	N/A	274				
	RT-PCR Result	+	-	-			196	196
	Day After Disease Onset	72	N/A	268				
	RT-PCR Result	+	-	-			139	139
	Day After Disease Onset	103	N/A	242				
	RT-PCR Result	+	-	-			187	187
	Day After Disease Onset	38	N/A	225				
	RT-PCR Result	+	-	-			57	57

	Day After Disease Onset	61	N/A	118				