4. De Craeye S, Speybroeck N, Ajzenberg D, Dardé ML, Collinet F, Tavernier P, et al. *Toxoplasma gondii* and *Neospora caninum* in wildlife: common parasites in Belgian foxes and Cervidae? Vet Parasitol. 2011;178:64–9. https://doi.org/10.1016/j.vetpar.2010.12.016
5. Cruickshank JJ, Haines DM, Palmer NC, St Aubin DJ. Cysts of a *Toxoplasma*-like organism in an Atlantic bottlenose dolphin. Can Vet J. 1990;31:213–5.6. Van Bressem MF, Duignan PJ, Banyard A, Barbieri M, Colegrove KM, De Guise S, et al. Cetacean morbillivirus: current knowledge and future directions. Viruses. 2014;6:5145–81. https://doi.org/10.3390/v6125145
7. Duignan PJ, Van Bressem MF, Baker JD, Barbieri M, Colegrove KM, De Guise S, et al. Phocine distemper virus: current knowledge and future directions. Viruses. 2014;6:5093–134. https://doi.org/10.3390/v6125093
8. Hernández M, Robinson I, Aguilar A, González LM, López-Jurado LF, Reyero MI, et al. Did algal toxins cause monk seal mortality? Nature. 1998;393:28–9. https://doi.org/10.1038/29906
9. Barbieri MM, Kashinsky L, Rotstein DS, Colegrove KM, Haman KH, Magargal SL, et al. Protozoal-related mortalities in endangered Hawaiian monk seals *Neomonachus schauinslandi*. Dis Aquat Organ. 2016;121:85–95. https://doi.org/10.3354/dao03047
10. Robinson SJ, Barbieri MM, Murphy S, Baker JD, Harting AL, Craft ME et al. Model recommendations meet management reality: implementation and evaluation of a network-informed vaccination effort for endangered Hawaiian monk seals. Proc Biol Sci. 2018; 285:20171899. https://doi.org/10.1098/rspb.2017.1899

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Increased Likelihood of Detecting Ebola Virus RNA in Semen by Using Sample Pelleting

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Ebola virus RNA can reside for months or years in semen of survivors of Ebola virus disease and is probably associated with increased risk for cryptic sexual transmission of the virus. A modified protocol resulted in increased detection of Ebola virus RNA in semen and improved disease surveillance.

During 2013–2016, Ebola virus (EBOV; family *Filoviridae*, genus *Ebolavirus*, species *Zaire ebolavirus*) caused an unprecedented outbreak of Ebola virus disease (EVD) that began in Guinea and subsequently affected Liberia, Sierra Leone, and, to a much lesser degree, several other countries in West Africa. Due in part to the lack of medical infrastructure and response preparedness in these countries, the outbreak ultimately involved 28,652 human infections and 11,325 deaths (*1*,*2*).

The large number of EVD survivors enabled detailed studies, such as the Partnership for Research on Ebola Virus (PREVAIL) III study (*3*), which aimed at characterizing potential EVD sequelae and EBOV persistence in a cohort of 1,144 EVD survivors in Liberia over the course of 5 years. An unexpected observation of these studies was the persistence of EBOV RNA and sometimes-replicating EBOV in the brain, eyes, and semen of survivors (*4*). EBOV RNA persistence in semen of EVD survivors, measurable up to 40 months (*3*,*5*), has been associated with rare events of sexual EBOV transmission and EVD outbreak flareups (*6*).

Assuming a causal relationship between EBOV RNA and EBOV presence in semen, we collaborated with the overseas response team to initiate an ongoing (and unpublished) trial, PREVAIL IV, to counter sexual EBOV transmission from survivors through reduction of viral RNA concentrations in semen by using the candidate medical countermeasure remdesivir. However, interpretation of data obtained in studies such as PREVAIL IV is crucially dependent on the sensitivity of EBOV RNA detection in semen samples.

The GeneXpert Systems (Cepheid, https://www.cepheid.com) are diagnostic platforms that implement single-use cartridges to simultaneously extract and detect RNA by using reverse transcription PCR. During PREVAIL III (*3*), the GeneXpert IV System was applied to standard processing of EBOV survivor semen

Table. Number of positive samples detected in whole semen samples versus pelleted semen samples for detection of Ebola virus RNA*

Method: replicate	No. samples	No. invalid samples	No. (%) Ebola virus RNA positive samples
Whole sample: A	1,661	66	50 (3.1)
Whole sample: B	1,661	112	45 (2.9)
Pellet sample: A	1,661	85	84 (5.3)
Pellet sample: B	1,661	69	73 (4.6)
*Also included are no. invalid samples (i.e., those that did not pass 1 or both of the controls (sample processing control and probe check control).			

samples using EBOV nucleoprotein and glycoprotein RNA-specific GeneXpert cartridges Cepheid) (7): 100 µL of semen sample was transferred directly into 2.5 mL of lysis buffer provided in the kit and incubated for 10 min, followed by a second incubation of 5 min in presence of 100 µL of 1 M dithiothreitol (Sigma-Aldrich, https://www.sigmaaldrich.com). Within 30 min of processing, 1 mL of this solution was then loaded into the cartridge, run according to the manufacturer's instructions, and analyzed with GeneXpert Diagnostic Software (8).

We sought to further increase the EBOV RNA detection sensitivity (then 3.1% and 2.9% with whole samples) of this protocol for semen. For this experiment, 1,661 EVD survivor samples and nonsurvivor controls from PREVAIL III and IV with sample volumes ≥1.6 mL were divided into 2 cohorts and processed either as described above (whole sample) or first pelleted (pellet sample) by using 2 replicate experiments each (A and B).

For pelleting, we centrifuged 300 µL of each semen sample at $10,000 \times g$ for 10 min. After pelleting, we discarded supernatants, resuspended pellets by pipetting in 100 µL of kit-provided lysis buffer and incubated for 10 min, and incubated for 5 min in presence of 100 µL of 1 M dithiothreitol. Then, we loaded 100 µL of each sample onto cartridges and processed the same way as the standard, unpelleted control sample. Samples were considered valid and positive when both the kit-provided sample processing control and probe check control passed kit criteria and EBOV nucleoprotein or glycoprotein RNA was detected.

Overall, an average of 3.0% of the whole sample-cohort was positive, compared with an average of 5.0% of the pellet-sample cohort, thereby almost doubling the detection rate (p<0.0001) (Table). We observed variability among replicates A and B (0.7% for the pellet and 0.2% for the whole sample), but this difference was not significant (p = 0.35) according to the F-test for the equality of 2 variances.

Mixed-effects logistic regression models appropriate to the study design (with random effects for specimens and random effects for replicates nested within specimens) yielded an estimated relative sensitivity of 2.24 (95% CI 1.51–2.98; p<0.0001) in favor of the pellet-based procedure. Thus, when

semen sample volumes from EVD survivors are >300 µL, we recommend pelleting 300 µL to increase the EBOV RNA detection rate and using the GeneXpert IV System.

About the Author

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References
1. Decroo T, Fitzpatrick G, Amone J. What was the effect of the West African Ebola outbreak on health programme performance, and did programmes recover? Public Health Action. 2017;7(Suppl 1):S1–2. https://doi.org/10.5588/pha.17.0029
2. Bullard SG. A day-by-day chronicle of the 2013–2016 Ebola outbreak. Springer: Cham (Switzerland); 2018 [cited 2021 Jan 1]. https://link.springer.com/chapter/10.1007/978-3-319-76565-5_8
3. Sneller MC, Reilly C, Badio M, Bishop RJ, Eghrari AO, Moses SJ, et al.; PREVAIL III Study Group. A longitudinal study of Ebola sequelae in Liberia. N Engl J Med. 2019;380:924–34. https://doi.org/10.1056/NEJMoa1805435
4. Jacob ST, Crozier I, Fischer WA II, Hewlett A, Kraft CS, Vega MA, et al. Ebola virus disease. Nat Rev Dis Primers. 2020;6:13. https://doi.org/10.1038/s41572-020-0147-3
5. Keita AK, Vidal N, Toure A, Diallo MS, Magassouba N, Baize S, et al.; PostEbogui Study Group. A 40-month follow-up of Ebola virus disease survivors in Guinea (PostEbogui) reveals long-term detection of Ebola viral ribonucleic acid in semen and breast milk. Open Forum Infect Dis. 2019;6:ofz482. https://doi.org/10.1093/ofid/ofz482
6. Schindell BG, Webb AL, Kindrachuk J. Persistence and sexual transmission of filoviruses. Viruses. 2018;10:683. https://doi.org/10.3390/v10120683

7. Loftis AJ, Quellie S, Chason K, Sumo E, Toukolon M, Otieno Y, et al. Validation of the Cepheid GeneXpert for detecting Ebola virus in semen. J Infect Dis. 2017;215:344–50.
8. Pettitt J, Higgs E, Fallah M, Nason M, Stavale E, Marchand J, et al. Assessment and optimization of the GeneXpert diagnostic platform for detection of Ebola virus RNA in seminal fluid. J Infect Dis. 2017;215:547–53.

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Polyresistant *Mycobacterium bovis* Infection in Human and Sympatric Sheep, Spain, 2017–2018

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The main etiologic agent of tuberculosis (TB) in livestock is *Mycobacterium bovis*; human TB cases caused by *M. bovis* are rare. Analysis of a TB outbreak caused by polyresistant *M. bovis* involving a human and sympatric sheep in Spain suggests local circulation of drug-resistant *M. bovis* strains among livestock.

The main etiologic agent of tuberculosis (TB) in livestock and wildlife is *Mycobacterium bovis*. This species also infects humans through inhalation or ingestion and causes TB that is clinically indistinguishable from that caused by *M. tuberculosis*.

In 2017, a case of pulmonary TB caused by *M. bovis* in a human was detected in the Vall d'Hebron Hospital in Barcelona, Spain. Bacteriological culture of clinical specimens in Löwenstein-Jensen and 7H9 media (BD Diagnostics, https://bd.com), followed by antimicrobial resistance testing (BACTEC MGIT 960; BD Diagnostics), revealed a strain resistant to 2 first-line anti-TB drugs: pyrazinamide (100 μg/mL) and isoniazid (0.1 μg/mL). A complementary analysis, performed by using the proportion method, confirmed resistance to isoniazid (0.2 μg/mL), elucidating a polyresistant case of TB (resistance to >1 first-line anti-TB drug other than both isoniazid and rifampin); the strain was also resistant to ethionamide (30 μg/mL), an antimicrobial drug specifically used to treat active multidrug-resistant TB (resistance to at least both isoniazid and rifampicin). Molecular characterization by direct variable repeat (DVR)-spoligotyping identified the isolates as *M. bovis* spoligopattern SB0124 (http://www.mbovis.org).

The patient worked as a farmer on cattle and small ruminant farms in his county. Therefore, the epidemiologic investigation included the livestock he was in contact with, particularly the herd of sheep and goats he was currently managing. In 2018, a total of 34 (25%) ewes and 3 (18%) goats had positive results to a single intradermal tuberculin test, interferon gamma release assay (IDvet, https://www.id-vet.com), or both. Animals with positive test results were slaughtered, and tissues from 23 (21 sheep and 2 goats) were examined postmortem. TB-compatible lesions were found in the lungs and thoracic, mesenteric, or ileocecal lymph nodes of 13 animals (12 sheep, 1 goat). Tissues with lesions were cultured in Löwenstein-Jensen with pyruvate and Coletsos and in 7H9 media by using BACTEC MGIT 320 (all BD Diagnostics). Culture indicated growth of *M. tuberculosis* complex in 9 sheep samples, and *M. avium* subspecies *avium* was isolated from another sheep and the goat. DVR-spoligotyping was performed for the 9 *M. tuberculosis* complex isolates, and *M. bovis* SB0124 was identified in all. This unusual spoligopattern had also been identified in a cattle herd in the same county in 2005 (Spanish Database of Animal Mycobacteriosis; https://www.visavet.es/mycodb); the patient had no known connection to that herd.

Genome sequence analysis based on assessment of single-nucleotide polymorphisms (SNPs)