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Development of a multiplex assay for antibody detection in serum against pathogens affecting ruminants

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Abstract

Numerous infectious diseases impacting livestock impose an important economic burden and in some cases also represent a threat to humans and are classified as zoonoses. Some zoonotic diseases are transmitted by vectors and, due to complex environmental and socio-economic factors, the distribution of many of these pathogens is changing, with increasing numbers being found in previously unaffected countries. Here, we developed a multiplex assay, based on a suspension microarray, able to detect specific antibodies to five important pathogens of livestock (three of them zoonotic) that are currently emerging in new geographical locations: Rift Valley fever virus (RVFV), Crimean-Congo haemorrhagic fever virus (CCHFV), Schmallenberg virus (SBV), Bluetongue virus (BTV) and the bacteria complex *Mycobacterium tuberculosis*. Using the Luminex platform, polystyrene microspheres were coated with recombinant proteins from each of the five pathogens. The mix of microspheres was used for the simultaneous detection of antibodies against the five corresponding diseases affecting ruminants. The following panel of sera was included in the study: 50 sera from sheep experimentally infected with RVFV, 74 sera from calves and lambs vaccinated with SBV, 26 sera from cattle vaccinated with *Mycobacterium bovis*, 30 field sera from different species of ruminants infected with CCHFV and 88 calf sera infected with BTV. Finally, to determine its diagnostic specificity 220 field sera from Spanish farms free of the five diseases were assessed. All the sera were classified using commercial ELISAs specific for each disease, used in this study as the reference technique. The results showed the multiplex assay exhibited good performance characteristics with values of sensitivity ranging from 93% to 100% and of specificity ranging from 96% to 99% depending on the pathogen. This new tool allows the simultaneous detection of antibodies against five important pathogens, reducing the volume of sample needed and the time of analysis where these pathogens are usually tested individually.

1 | INTRODUCTION

Infectious diseases account for more than 20% of the overall losses in livestock production worldwide, with important consequences for food security and health of both animals and humans. Many of these pathogens are vector-borne diseases, which have a zoonotic character, with serious impact in both animal and public health. Environmental and socio-economic changes, such as global warming, changes in land use, host migration and globalization, have influenced the distribution of those infectious diseases transmitted by vectors, increasing their potential to spread to previously unaffected regions (Bett et al., 2017; Brand & Keeling, 2017; Dash, Bhatia, Sunyoto, & Mourya, 2013; Weaver & Reisen, 2010). This is the case for pathogens such as Crimean-Congo haemorrhagic fever virus (CCHFV), Rift Valley fever virus (RVFV), Schmallenberg virus (SBV), Bluetongue virus (BTV) or the bacteria complex *Mycobacterium tuberculosis* (Estrada-Peña, Ruiz-Fons, Acevedo, Gortazar, & de la Fuente, 2013; Maltezou & Papa, 2010; Rolin, Berrang-Ford, & Kulkarni, 2013; Samy & Peterson, 2016). Moreover these viruses, with the exception of SBV, as well as the bacteria complex Mycobacterium tuberculosis are listed as notifiable pathogens by the OIE (World Organization for Animal Health) and thus, their identification is crucial to disease management (OIE, 2020). Taken together, these factors are necessary for better preparedness, surveillance and control of these and other pathogens, in order to reduce the social and economic consequences of these diseases.

The panel of pathogens for this study include three viruses of the *Bunyavirales* order, namely CCHFV, RVFV and SBV. CCHFV is one of the most widespread tick-borne zoonotic viruses (Bente et al., 2013) and while causing an asymptomatic infection in infected animals (domestic and wild ruminants), it can cause severe haemorrhagic fever in humans, resulting in high case fatality rates. More recently, some human cases have been reported for the first time in South-Eastern Europe (Mertens, Schmidt, Ozkul, & Groschup, 2013) and Spain (Negredo et al., 2019). The transmission to humans occurs through tick bites or exposure to blood or other body fluids of an infected animal or human (Bente et al., 2013). RVFV is a mosquito-borne virus infecting both livestock and humans (Wright, Kortekaas, Bowden, & Warimwe, 2019) with infections causing a high rate of abortions in pregnant domestic ruminants, and high mortality rates among newborns, causing devastating socio-economic impacts (Sindato, Karimuribo, & Mboera, 2012). In addition, numerous outbreaks have been reported in humans especially in Africa and Middle East countries (Aradaib et al., 2013; Hassan, Ahlm, Sang, & Evander, 2011; Himeidan, Kweka, Mahgoub, El Rayah, & Ouma, 2014). Although not yet detected in Europe, RVFV introduction is a real concern due to the presence of competent arthropod vectors (Brustolin et al., 2017).

SBV and BTV, the later belonging to the *Reoviridae* family of RNA viruses, are among the most important pathogens affecting different species of ruminants such as cattle, goats and sheep (Rojas, Rodríguez-Martín, Martín, & Sevilla, 2019; Wernike & Beer, 2017). Biting midges are the main vector involved in the transmission of these two viruses. Clinical signs associated with SBV are typically non-specific in adult cattle (such as fever, reduced milk yield, diahrrea) and rare in adult sheep and goat, but infection of pregnant females is associated with congenital malformations and stillbirths (Hoffmann et al., 2012). For BTV, infection in adult cattle and goat is generally subclinical but is more prounonced in adult sheep (such as fever, congestion of nasal and oral mucosa and can cause hemorrhages in the nose, lips, and tongue that can result in death) and infection of pregnant sheep can be associated with congenital malformations and stillbirths (Rojas et al., 2019). Outbreaks of these two diseases have significant economic impact, due to loss of productivity and restrictions of animal movement and trade (Häsler, Alarcon, Raboisson, Waret-Szkuta, & Rushton, 2015; Rushton & Lyons, 2015).

Mycobacterium bovis, a member of the *M. tuberculosis* complex, is a highly pathogenic mycobacterium that causes disease mainly in cattle, but also in other species of ruminants and poses a high zoonotic risk due to possible transmission to humans which usually occurs after close contact with infected animals or consumption of unpasteurized and contaminated dairy products (El-Sayed, El-Shannat, Kamel, Castañeda-Vazquez, & Castañeda-Vazquez, 2016; Mathema, Kurepina, Bifani, & Kreiswirth, 2006). The disease has an impact on productivity of animals and consequently is linked to economic losses (Ejeh et al., 2014).

In the present work, we describe the development of a multiplex serological assay for the simultaneous detection of antibodies in ruminant serum, against CCHFV, RVFV, SBV, BTV and *M. bovis* for surveillance purposes. The nucleocapsid (N) protein of CCHFV, RVFV and SBV was selected as target antigens (Bente et al., 2013; Fafetine et al., 2007; Walter & Barr, 2011). For BTV, the viral protein, VP7, one of the two major structural proteins of the BTV core, was chosen (Wade-Evans et al., 1996). Finally, the MPB83 of *M. bovis*, which is one of the two major antigens highly expressed by *M. bovis*, was used as target antigen (Wiker, 2009).

Initially, to set up this multiplex test, several panels of sera from ruminants (cattle, goat and sheep) experimentally infected with the different pathogens, or serum from field animals, were used. To determine the diagnostic specificity of the test, a total of 220 sera from Spanish farms free of these five diseases were included in the studies. All the sera were previously characterized by individual enzyme-linked immunosorbent assays (ELISAs) specific for each pathogen and considered in this study as our reference technique.

This new approach could be used as a high throughput screening tool to assess the presence and prevalence of antibodies

against 5 different highly pathogenic agents and identify regions at risk of infection. Moreover, these pathogens are often reported in ruminant populations which can cause huge economic losses to farmers in developing countries. These infections can routinely be detected using serological or microbiological methods which can be reinforced or replaced by multiplex assays, where detection of different pathogens is done in a single reaction. In addition, simultaneous testing of these pathogens reduces the volume of sample needed compared to performing the different ELISAs. This information will help to minimize the spread and further transmission of those pathogens within the human population.

2 | MATERIALS AND METHODS

2.1 | Recombinant proteins

cDNAs designed to express the full length nucleoproteins of CCHFV Baghdad 12 strain (GenBank accession CAD61342.1, but with conservative substitutions T111I, R195H and H445D) and RVFV (GenBank accession X53771.1) were generated synthetically (Genewiz). cDNA of the nucleoprotein of SBV (GenBank accession AGC93538) was available at INGENASA. The three sequences were cloned into the pET-28a-6His-SUMO plasmid (Thermo Fisher Scientific) for bacterial expression of corresponding fusion proteins with the small ubiquitin-like modifier (SUMO) and $6\times$ histidine-tag at their amino termini. Resulting plasmids were verified by sequence analysis and transformed into *Escherichia coli* BL21 (DE3) Rosetta2 (Novagen). The recombinant proteins were purified using Ni-NTA affinity chromatography followed by size exclusion chromatography as described by Carter, Barr, and Edwards (2012). Briefly, the expression of the three N proteins was induced with 500 µM isopropyl-β-D-thiogalactoside overnight (o/n) at 18°C. The cells were harvested by centrifugation and lysed with lysozyme (1 mg/ml) and sonication. The soluble fraction was separated from the cell debris by centrifugation, after which the $6\times$His-SUMO-N proteins were purified from the soluble fraction using Ni-NTA resin (ABT) and eluted with increasing concentrations of imidazole. The purified fusion proteins were cleaved o/n using SUMO protease (produced in-house at the University of Leeds), and the $6\times$ histidine and SUMO tags were removed using a second nickel column, followed by a final size exclusion chromatography step with a HiLoad® 26/600 Superdex® 75 pg column (GE Healthcare) using an Akta prime (GE Healthcare).

The VP7 of BTV serotype 10 (GenBank accession No. YP_052967) was produced in insect cells from a recombinant baculovirus already available in the laboratory. Briefly, Sf9 insect cells (from *Spodoptera frugiperda*) were cultured in complete Grace´s medium and infected with the corresponding recombinant baculovirus. The cells were lysed and the VP7 was purified from the soluble fraction by affinity chromatography using a specific monoclonal antibody (MAb) to BTV VP7 (INGENASA). The glutathione S-transferase (GST)-tagged MPB83 was expressed in insect cells

and was already available in the laboratory (Fresco-Taboada et al., 2019). Briefly, Sf9 cells were grown in Sf-900™ II SFM (Thermo Fisher Scientific) without serum and infected with the recombinant baculovirus containing the MPB83-GST gene. The cells were then centrifuged and the MPB83-GST was purified from the culture medium by affinity chromatography with GST Gravitrap™ (GE Healthcare) according to the manufacturer's instructions.

Protein gel electrophoresis followed by Coomassie staining was used to assess the purity and molecular size of each protein. The proteins were transferred to nitrocellulose membrane and Western blot analysis was performed using specific MAbs to each of the target proteins (1F8 for RVFV N protein, 1D5D12 for SBV N protein, 83CA3 for MPB83-GST, 2G10 for CCHFV N protein, and 2D7 for BTV VP7, all produced at INGENASA) to confirm their immunogenicity.

2.2 | Production and characterization of the monoclonal antibodies

The animal research was approved by the Consejería de Medio Ambiente, Administración Local y Ordenación del Territorio (Department of Environment, Local Administration and Territorial Planning) from the Comunidad de Madrid (Community of Madrid, Spain) reference PROEX nº024/18.

Female BALB/c mice aged from 9 to 12 weeks, weight range from 20 g to 40 g were housed in temperature-controlled, pathogen-free rooms with access to pelleted food and water ad libitum. The mice were maintained under standard animal housing conditions in an authorized centre (INGENASA, Community of Madrid registration number ES280790000095). Originally, the mice were commercially obtained from Harlan Interfauna Ibérica (Spain) and are now bred internally by the Animal Department of INGENASA.

The mice (4 per group, one group per antigen) were injected intraperitoneally (IP) with the purified RVFV NP and CCHFV NP produced in *E. coli*. For the first immunization, 25 µg of antigen emulsified in complete Freund's adjuvant (Sigma-Aldrich) was used. For the following seven immunizations (one every 2 weeks), the antigen was emulsified in incomplete Freund's adjuvant (Sigma-Aldrich). As a control, one mouse was immunized with phosphate-buffered saline (PBS) instead of antigen. Mice were bled by the tail 7 days after each immunization to detect the antibody titre in serum. Mice sera were obtained by allowing the blood to coagulate for 30 min at room temperature (RT) and further centrifugation at 1,500 *g* for 10 min at 4°C. The antibody titre was checked by indirect ELISA with the CCHFV N and RVFV N proteins. Previously to obtaining the hybridomas, three immunizations on three consecutive days were done with antigen emulsified in PBS.

The mice were euthanized by inhalation of CO_2 and their spleen was collected. To obtain hybridomas producing specific MAbs, fusions were done between the B cells of the mice spleen and NP3 myeloma cells (Sanz, García-Barreno, Nogal, Viñuela, & Enjuanes, 1985). The supernatants of hybridoma clones were screened by indirect

ELISA with the corresponding N protein and a negative antigen (the N protein of CCHFV for the hybridomas producing MAbs to RVFV N protein and vice versa) and were subsequently subcloned to obtain single hybridoma producing a specific monoclonal antibody. The supernatants of hybridoma containing the monoclonal antibodies were further purified using rprotein A/protein G GraviTrap columns (GE Healthcare) following the manufacturer's instructions. After assessing the purity of each monoclonal antibody by gel electrophoresis, the MAbs were further labelled with peroxidase according to the method described by Nakane and Kawaoi (1974). The specificities of the MAbs were determined by indirect ELISA and Western blot analysis using the corresponding recombinant N protein as antigen. The isotypes of the MAbs were determined by ELISA, using specific anti-mouse subtype antisera (Sigma). A competition ELISA between the different MAbs was performed in order to detect whether they mapped in the same or different antigenic areas.

Monoclonal antibodies against the N protein of SBV, the VP7 of BTV and the MPB83 of *M. bovis* were already available in INGENASA and produced the same way as the MAbs against the N proteins of CCHFV and RVFV.

2.3 | Serum samples

In this study, the panel of serum samples consisted of 334 positive sera including experimental and positive field samples and 220 negative field sera. For the detection of antibodies to RVFV, 56 serum samples from sheep experimentally inoculated with RVFV were provided by INIA-CISA, some of them previously characterized (Lorenzo, López-Gil, Ortego, & Brun, 2018; Lorenzo, López-Gil, Warimwe, & Brun, 2015). For detection of antibodies to SBV, 33 serum samples from calves and 41 serum samples from lambs experimentally infected with SBV were available at INGENASA, from a previous internal project. For detection of antibodies to *M. bovis*, 29 serum samples from cattle vaccinated intramuscularly with *M. bovis* were available at INGENASA, from the European project WildTBVac (Call Identifier: FP7-KBBE-2013-7; Grant agreement n° 613799). For detection of antibodies to CCHFV, 16 field serum samples from cattle, 10 from sheep and 4 from goats naturally infected by CCHFV were provided by the Faculty of Veterinary Medicine in Skopje (USCM, North Macedonia). For the detection of antibodies to BTV, 73 serum samples from cattle experimentally infected with BTV were available at INGENASA, obtained from previous projects (PROFIT, Ref. CIT-010000-2005-81 and Comunidad de Madrid, Ref. 27/2007) and 15 serum samples from SBV infected animals, also positive to BTV. Finally, a collection of 220 negative field samples (87 from cattle, 67 from goats and 66 from sheep) from Spanish farms were evaluated.

2.4 | Coupling of target antigens to beads

The coupling of antigens to beads was described recently by Aira et al. (2019). Briefly, the five target proteins were covalently coupled to five different regions (regions #12, #18, #21, #25 and #30) of carboxylated magnetic microspheres (Luminex) using the *xMAP® Cookbook* by Angeloni et al. (2016). Briefly, 1×10^6 microspheres, identified individually by a unique fluorescence ratio, were activated by addition of sulpho-N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride), which is based on a two-step carbodiimide reaction (Hermanson, 2013). Once activated, the beads were incubated with different amounts of the corresponding protein ranging from 12.5 µg to 50 µg per one million beads in a final incubation volume of 500 µl, and incubated for 2 hr with rotation in dark. RVFV N protein was coupled to region #12, SBV N protein to region #18, MPB83-GST to region #21, CCHFV N protein to region #25 and BTV VP7 to region #30. After washing, the beads were resuspended in 1 ml of storage buffer (PBS with 1% bovine serum albumin (BSA) and 0.05% azide) and were kept in the dark at 4°C. The concentration of the beads was determined by counting on a Neubauer plate.

Serial dilutions of monoclonal antibodies specific to each protein were used to perform a confirmation assay, in order to assess the coupling efficiency. All the MAbs were produced at INGENASA: 1F8 for RVFV N protein, 1D5D12 for SBV N protein, 83CA3 for MPB83-GST, 2G10 for CCHFV N protein and 2D7 for BTV VP7.

2.5 | Bead-based assay for antibody detection in ruminant sera

The five respective antigen-coupled microspheres were resuspended by vortex and sonication in order to perform the five-plex assay. A mix of beads was prepared mixing the five bead regions in assay buffer (PBS with 1% BSA) to a final concentration of 25 beads/µL for each bead region. As the beads are light-sensitive, they were protected from the light. To 50 µl of individual ruminants' serum samples, diluted at 1/100 in assay buffer, was added 50 µl of the bead mixture. This mix was incubated for 1 hr at RT and 650 rpm in a mini-shaker PSU-2T (Biosan). Ninety-six-well plates (Stripwell™ Microplate Medium binding Polystyrene, CoStar), previously blocked for 30 min with StabilZyme® SELECT Stabilizer (Surmodics), were used for the assay. After every incubation step, a magnetic plate separator (Luminex) was used to wash the plate twice with washing buffer (PBS with 1% BSA and 0.05% Tween 20). Then, 50 µl of the monoclonal anti-ruminant antibody EG5 labelled with biotin (INGENASA) was added to each well, at a final concentration of 0.5 µg/ml in assay buffer, for 1 hr at RT and 650 rpm. Next, 50 µl of Streptavidin R-phycoerythrin (Life Technologies) at 2 µg/ml in assay buffer were added per well and incubated for 30 min at RT and 650 rpm. Finally, the beads were washed twice with washing buffer and resuspended in 100 µl/well of washing buffer. The results were read out using MAGPIX® reader (Luminex). To be measured, the median fluorescence intensity (MFI) had to count a minimum of 50 events of each bead region.

One well per assay was incubated with GIBCO™ foetal bovine serum (Thermo Fisher Scientific) as background signal. ELISA-confirmed positive sera to each antigen, used as positive controls

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TABLE 1 Summary of the antigens used in the multiplex assay

Family	Virus/Bacteria	Target antigen	Expected molecular weight (kDa)	Expression system	Vector
Nairoviridae	Crimean-Congo haemorrhagic fever virus	N protein	54	*E. coli*	pET-28a-6His-SUMO
Phenuiviridae	Rift Valley fever virus	N protein	27.2	*E. coli*	pET-28a-6His-SUMO
Peribunyaviridae	Schmallenberg virus	N protein	26	*E. coli*	pET-28a-6His-SUMO
Reoviridae	Bluetongue virus	VP7	38.5	BES	pAcYM1
Mycobacteriaceae	*Mycobacterium bovis*	MPB83-GST	46.3	BES	pAcSECG2T

for the targeted bead region and negative controls for the other beads, were included in every plate to assess the performance of the test.

2.6 | Data processing and statistical analysis

To statistically analyse the data obtained, the MedCalc® 10 software was used. Receiver operating characteristic (ROC) curves analysis were made to establish the optimal cut-off value for each bead region. Prior to the analyses, the samples were classified into positive or negative based on five commercial ELISAs which were used as the reference techniques in this study: INGEZIM Schmallenberg Compac 2.0 (13.SBV.K3, INGENASA) for detection of specific antibodies against SBV, INGEZIM BTV Compac 2.0 (12. BTV.K3, INGENASA) for detection of specific antibodies against BTV, INGEZIM Tuberculosis DR (10.TB*.K0, INGENASA) for detection of specific antibodies against *M. bovis*, ID Screen® Rift Valley Fever Competition Multi-species (IDvet) for detection of specific antibodies against RVFV and ID Screen® CCHF Double Antigen Multi-species (IDvet) for detection of specific antibodies against CCHFV. To measure the inter-rater reliability between the reference ELISAs and the bead-based assay, Cohen's kappa coefficient was calculated.

3 | RESULTS

3.1 | Production of the target antigens of the different pathogens

Recombinant viral proteins were prodecued in different expression systems, as described in M& M section. A summary of the different target antigens is shown in Table 1. The expression and purification of the proteins was followed by gel electrophoresis and Coomassie blue staining, revealing the recombinant proteins with the expected molecular weights of 54 kDa, 27.4 kDa, 26.2 kDa, 38.5 kDa and 46.3 kDa for CCHFV N protein, RVFV N protein, SBV N protein, VP7 of BTV and the MPB83-GST of *M. bovis*, respectively (Figure 1, lane 1–5). A faint band probably corresponding to a degradation product of the BTV VP7 (approximately 28 kDa) and a faint band corresponding to the size of the GST (26 kDa) were
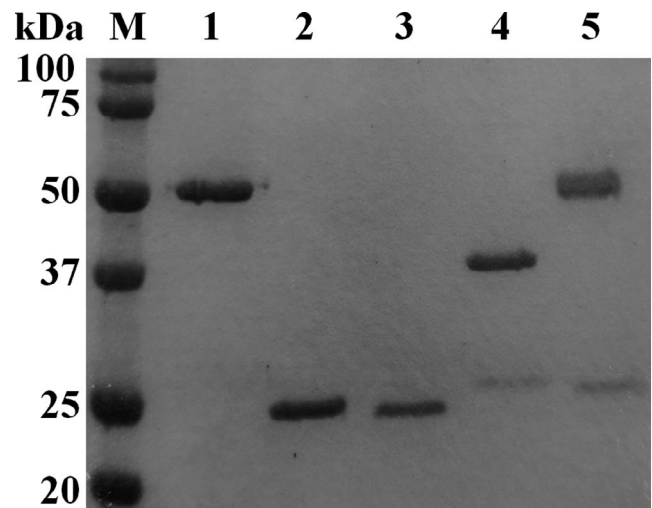

FIGURE 1 Coomassie Blue staining of the five purified proteins used in the multiplex assay. From left to right: CCHFV N protein (lane 1, MW: 54 kDa), RVFV N protein (lane 2, MW: 27.2 kDa), SBV N protein (lane 3, MW: 26 kDa), BTV VP7 (lane 4, MW: 38.5 kDa) and MPB83-GST (lane 5, MW: 46.3 kDa)

observed in lanes 4 and 5, respectively. Western blot analysis using specific MAbs to each of the target proteins was performed to confirm their immunogenicity. A specific band corresponding to each antigenic protein was observed, while neither of the faint bands observed in Figure 1 (lanes 4 and 5) were observed in the Western blot (data not shown).

3.2 | Production and characterization of the monoclonal antibodies

As described in Materials and Methods, monoclonal antibodies to CCHFV N protein and to RVFV N protein were produced and characterized. Three MAbs against CCHFV N protein and 4 MAbs against RVFV N protein were obtained. The MAbs obtained reacted specifically with the N proteins of CCHFV and RVFV as expected, with no detectable cross-reaction with the other N protein. Finally, a competition ELISA between the MAbs was performed to identify whether they mapped in the same or in different antigenic areas. A summary of the characterization of the MAbs for CCHFV and RVFV is shown in Table 2.

TABLE 2 Summary of the characterization of the MAbs anti-CCHFV N protein and anti-RVFV N protein

| | Name | Class | Reactivity in western blot | Competition for the same antigenic area | | | | | | |
				3G7	2G10	1D6	1E9	1F8	2B12	2D10
MAb anti-CCHFV N protein	3G7	IgG1	+++	■						
	2G10	IgG1	+++		■	■				
	1D6	IgG1	+++		■	■				
MAb anti-RVFV N protein	1E9	IgG1	+				■			
	1F8	IgG1	+++					■		
	2B12	IgG1	++						■	
	2D10	IgG1	+++							■

Monoclonal antibodies against the N protein of SBV, the VP7 of BTV and the MPB83 of *M. bovis* were already available in INGENASA and produced the same way as the MAbs against the N proteins of CCHFV and RVFV and characterized in previous work (not shown).

3.3 | Development and optimization of the multiplex assay

Firstly, the optimization of the coupling concentration of each antigen to each bead region was determined individually with the specific monoclonal antibodies produced as described in Materials and

Methods (data not shown). The optimal protein coupling concentration was established as the highest MFI obtained with the minimum amount of protein. The following quantities were used to coat 1×10^6 beads for each bead region: 25 µg of RVFV N protein (region #12), 25 µg of SBV N protein (region #18), 25 µg of MPB83-GST (region #21), 25 µg of CCHFV N protein (region #25) and 50 µg of BTV VP7 (region #30).

Well-characterized serum samples for each pathogen were used to establish the optimal assay conditions for the screening. A mix of the 5 bead regions coupled to the target antigens was incubated with serial dilutions of specific reference serum samples against each pathogen and the assay was performed as described in Materials and
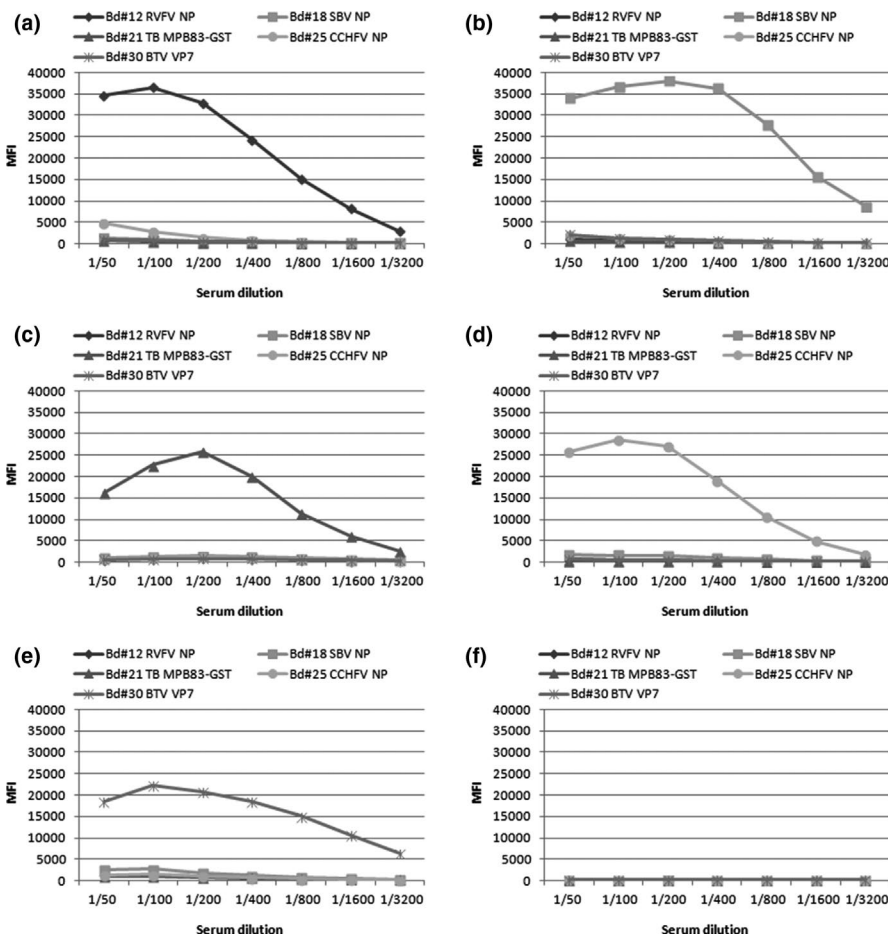

FIGURE 2 Determination of the screening conditions for the bead-based assay. The median fluorescence intensity (MFI) for each bead region is given for different serum dilutions of one experimental reference serum. (a) RVFV experimental serum; (b) SBV experimental serum; (c) TB experimental serum; (d) CCHFV field positive serum; (e) BTV experimental serum; (f) foetal bovine serum

TABLE 3 Serum classification with the ELISAs used as reference techniques for the different antigens

	Reference technique	RVFV ID Screen® RVF Competition Multi-species (IDvet)	SBV INGEZIM Schmallenberg Compac 2.0 (13. SBV.K3)	TB INGEZIM Tuberculosis DR (10.TB*. K0)	CCHFV ID Screen® CCHF Double Antigen Multi-species (IDVet)	BTV INGEZIM BTV Compac 2.0 (12. BTV.K3)
Experimental samples	Positive in ELISA	50	74	26	0	88
	Negative in ELISA	6	14	46	0	0
Total number of experimental samples		56	88	72	0	88
Field samples	Positive in ELISA	0	36	3	30	13
	Negative in ELISA	220	184	133	220	207
Total number of field samples		220	220	136	250	220

Methods. The same pattern of results was obtained for each, with an increase in the MFI until a serum dilution of 1:200 or 1:400, and then a decrease in the signal detected, without any signal for the other beads (Figure 2a-e). Neither of the specific samples for each disease gave cross-reactivity with any of the other bead regions, corresponding to the other pathogens. Finally, the foetal bovine serum used as background for the different infectious agents did not show any reactivity with any of the five beads (Figure 2f). For screening purposes a dilution of the serum at 1:200 was selected (corresponding to a sample volume of 0.5 µL), since this was the dilution showing the highest signal to most of the five beads regions, with no cross-reactivity with the non-targeted antigens.

3.4 | Multiplex assay validation using experimental and field serum samples

After establishing the screening conditions, a collection of 554 ruminants' serum samplesfrom cattle, goats and sheep, previously classified as positive and negative by the ELISAs used as reference, were tested in the five-plex assay. A total of 56 RVFV experimental serum samples (50 positives and 6 negatives), 88 SBV experimental serum samples (74 positives and 14 negatives), 72 *M. bovis* experimental serum samples (26 positives and 46 negatives), 30 CCHFV field positive serum samples, 88 BTV experimental serum samples (73 positives from experimentally infected cattle and 15 positives from the SBV experimental sera) and 220 field serum samples from ruminants were included in this study. Out of the 220 field sera, all were classified as negative for RVFV and CCHFV. Twelve cattle sera, 1 sheep serum and 23 goat sera were classified as positive for SBV and 13 cow sera were classified as positive for BTV. Finally, out of these 220 field sera, the sheep and goats' sera were assayed in the INGEZIM Tuberculosis DR, according to the manufacturer's instructions, and 3 were classified as positive for *M. bovis* (1 sheep and 2 goats). The serum sample volume needed to perform the five ELISAs used as references was 144 µL per animal. A summary of this classification is shown in Table 3.

After this classification, the complete panel of 554 ruminant serum samples were assayed in the five-plex assay and the results were statistically analysed by comparison with the results obtained with the reference techniques. Thus, a cut-off value was determined for each antigen using the MedCalc software. Based on these cut-offs, the performance characteristics of the multiplex assay for each pathogen was determined.

The sensitivity values ranged from 93.1% for TB and BTV to 100% for CCHFV, and the specificity ranged from 96% for SBV to 99.4% for TB. For RVFV, most of the positive experimental sera gave a high MFI for its corresponding bead and out of the 220 negative field samples and the 6 negative experimental samples, 96.9% (219/226) were detected as true negatives (Figure 3a). Concerning SBV, all the experimental sera were true positive and 96% of the negative field samples (190/198) were classified as true negatives (Figure 3b). With regard to TB, the 26 experimental sera were considered true positive with one positive field sample (1/3) considered as true positive (Figure 3c). Moreover, 99.4% of the negative field samples (175/176) were classified as true negatives. Regarding CCHFV, all the positive field sera were true positives and 2.3% of the negative field samples (5/220) were considered false positive (Figure 3d). Finally, regarding BTV, all the experimental sera were true positive (Figure 3e) and 99.0% of the negative field samples were true negatives (205/207). These results are summarized in Table 4.

Finally, some of the positive experimental sera tested in the five-plex showed some reactivity with other bead regions than the targeted one: 8 RVFV experimental sera, 39 SBV experimental samples, 24 CCHFV positive field sera and 22 BTV experimental sera (Table 5).

3.5 | Statistical analysis

Using the Medcalc software, Cohen's kappa coefficient was calculated to examine the agreement between the results obtained in the ELISAs and in the multiplex assay. For SBV, κ = 0.894 with a 95% confidence interval [0.842;0.946]. Cohen's kappa coefficients were ranging from 0.894 for SBV to 0.939 for TB, corresponding to an almost perfect agreement between the ELISAs used as reference and the multiplex assay.
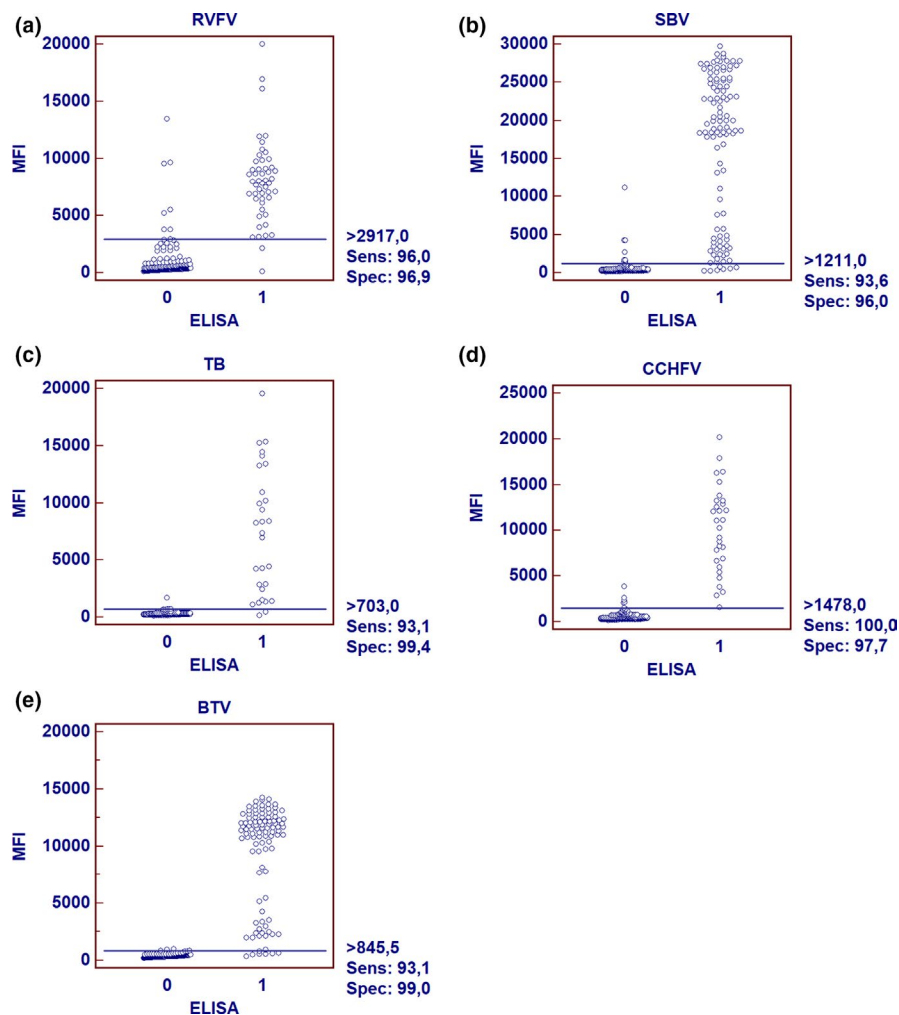
FIGURE 3 Validation of the bead-bead assay. Dot plot diagrams where each dot represents an individual sample: results obtained for RVFV N protein coupled to bead #12 (a), SBV N protein coupled to bead #18 (b), MPB83-GST coupled to bead #21 (c), CCHFV N protein coupled to bead #25 (d) and BTV VP7 coupled to bead #30 (e). The horizontal solid line corresponds to the cut-off values in each assay, according to the MedCalc software. X-axis shows the positive (1) or negative (0) classification of samples according to the ELISA used as reference technique in this study and Y-axis shows Median Fluorescence Intensity (MFI) obtained in the developed assay [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Correlation between the bead-based assay and the ELISAs used as reference for the different antigens

Diagnosis in the five-plex	RVFV	SBV	TB	CCHFV	BTV
True positive	50	103	27	30	94
False positive	7	8	1	5	2
True negative	217	190	175	215	205
False negative	2	7	2	0	7
Total	276	308	205	250	308
Cut-off (Median Fluorescence Intensity)	2,917	1,211	703	1,478	845,5
Sensitivity [95% confidence interval]	96% [86.3%–99.4%]	93.6% [87.3%–97.4%]	93.1% [77.2%–99%]	100% [88.3%–100%]	93.1% [86.2%–97.2%]
Specificity [95% confidence interval]	96.9% [93.7%–98.7%]	96.0% [92.2%–98.2%]	99.4% [96.9%–99.9%]	97.7% [94.8%–99.2%]	99.0% [96.5%–99.9%]

4 | DISCUSSION

Multiplex assays, which allow the detection of different pathogens in a single reaction, could replace individual assays routinely carried out for the detection of these pathogens. Moreover, these assays reduce time, labor and sample volume requirements, allowing the testing of many samples for multiple targets simultaneously.

This would greatly help in surveillance studies, by allowing the development of one unique plan for a complex infectious disease panel. This could be the case of diseases affecting livestock, especially in cases of zoonoses (such as CCHFV, RVFV and M. bovis), to control the distribution of the corresponding pathogens and prevent future outbreaks. This surveillance is also crucial for vector-borne diseases such as CCHFV, RVFV, BTV and SBV which

TABLE 5 Cross-reactivity between the positive experimental and field sera with the four other bead regions

	Cross-reactive with other bead regions					
	Bead #12 RVFV N protein	Bead #18 SBV N protein	Bead #21 TB MPB83-GST	Bead #25 CCHFV N protein	Bead #30 BTV VP7	Number of positive sera
Positive serum samples						
RVFV experimental sera	■	0	2	0	6	50
SBV experimental sera	0	■	2	16	27	74
TB experimental sera	0	0	■	0	0	26
CCHFV field positive	0	16	11	■	19	30
BTV experimental sera	0	1	20	12	■	88

already have competent vectors in regions or countries where the infectious agents have not yet reached (Estrada-Peña, Sánchez, & Estrada-Sánchez, 2012; Medlock & Leach, 2015; Rolin et al., 2013; Sanders et al., 2019). In this study, a five-plex assay has been developed and optimized to detect antibodies against the N protein of RVFV, SBV and CCHFV, and against the VP7 of BTV and MPB83 of Mycobacterium bovis based on the Luminex platform. The multiplex assay was shown to have an almost perfect agreement with the ELISAs used as reference. Indeed, Cohen's kappa coefficients all had a values close to 1. To increase the robustness of the diagnostic sensitivity and specificity of the assay, more positive and negative field sera should be tested.

The ELISAs used as reference techniques were either competition ELISAs (for the detection of antibodies to RVFV, SBV and BTV) or double antigen sandwich ELISAs (for the detection of antibodies to CCHFV and TB), which can detect both immunoglobulins G (IgG) and immunoglobulins M. Our multiplex assay uses an anti-ruminants' IgG, and thus, it can only detect IgG. That could explain some of the false-negative results, as some of the samples could have been taken at early days post-infection or post-vaccination, giving a high signal in the ELISAs, but being negatives in the multiplex. The confirmation of these results would actually raise the sensitivity of our multiplex assay.

Regarding TB, BTV and CCHFV, the few false-positive samples (1, 2 and 5 serum samples, respectively) come from ELISA-negative field samples and are clustered close to the cut-off of our multiplex assay. The false-positive sample for TB was close to the cut-off (optical density$_{450nm}$ = 0.32) in the reference ELISA (negative if the optical density$_{450nm}$ < 0.37). The two false positives for BTV (MFI = 924 and 1,005) are close to the cut-off established by MedCalc (negative if MFI < 845.5). Finally, out of the 5 false positives for CCHFV, two are strongly positive to SBV.

This last result raises the question of cross-reactivity of our assay. Due to sample volume constraints, not all the cross-reactive samples were assessed in other reference techniques to check the cross-reactivity. A total of 8 RVFV experimental sera, 39 SBV experimental

samples, 24 CCHFV positive field sera and 22 BTV experimental sera gave some positive results with other bead regions than the one it was intended for (see Table 5). Out of the 6 RVFV experimental sera positive to BTV, three were infected with BTV-4 (personal communication) and the other three sera come from sheep from Spanish farms where the sheep could have been vaccinated against BTV as there is a ongoing BTV vaccination program in Spain or infected by BTV as it is endemic in certain parts of Spain (European Commission, 2020). The 16 SBV experimental sera giving a signal above the cut-off for CCHFV (ranging between 1,488.5 and 4,568) were tested and found to be negative in the reference CCHFV ELISA. For the BTV experimental sera, 20 sera gave a positive signal, but they were all close to the cut-off with MFI ranging between 735 and 1,469. As all these sera were from cows they could not be tested in the TB ELISA used as reference technique to validate or invalidate these results. For the CCHFV field positive sera, 16 sera gave MFI values above the SBV cut-off, between 1,304.5 and 8,863 and 7 of these were positive in the SBV reference ELISA. Some cross-reactivity could be expected between the CCHFV, RVFV and SBV beads as these three viruses belong to the Bunyavirales order, although each are classified within different family taxa, emphasizing their distant genetic relatedness. The N proteins of bunyavirales members are very immunogenic, easy to produce, well conserved within each viral species and the N proteins of CCHFV and RVFV were already used in multiplex assays (van der Wal et al., 2012; Wu et al., 2014). The use of other immunogenic proteins of these viruses such as the glycoproteins could overcome this issue (van der Wal et al., 2012).

A multiplex assay has already been developed with the Luminex platform to detect antibodies against RVFV and CCHFV but the proteins used to coat the bead regions were produced from viruses propagated in biosafety level (BSL)-3 or BSL-4 laboratories (O'Hearn et al., 2016). Here we describe a five-plex assay using recombinant proteins, produced in a BSL-2 laboratory, thus avoiding the need of high-level containment facilities to develop such an assay.

Finally, this multiplex assay is advantageous as it allows saving time and sample volume, as 96 samples can be analysed against five diseases in 4 hr, using only 0.5 μL of serum, versus 144 μl of serum that would be required to perform the five independent ELISAs used as references. Moreover, it is a flexible, open system that will allow including target antigens from other pathogens of interest (such as epizootic hemorrhagic disease or peste des petits ruminants), to broaden the range and impact of this assay.

This five-plex assay could be used as a screening tool, to assess the presence and prevalence of antibodies against these five highly pathogenic agents and identify regions at risk of infection. This information will help to minimize the spread and further transmission of those pathogens within the human population.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The animal handling was done in compliance with the article 31 of the RD 53/2013 in Spain, transposition of the Directive 2010/63/EU in Europe. The animal research was approved by the Consejería de Medio Ambiente, Administración Local y Ordenación del Territorio (Department of Environment, Local Administration and Territorial Planning) from the Comunidad de Madrid (Community of Madrid) reference PROEX nº024/18.

DATA AVAILABILITY STATEMENT

The data sets generated and analysed during this work are available from the corresponding author on reasonable request.

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REFERENCES

Aira, C., Ruiz, T., Dixon, L., Blome, S., Rueda, P., & Sastre, P. (2019). Bead-based multiplex assay for the simultaneous detection of antibodies to African swine fever virus and classical swine fever virus. *Frontiers in Veterinary Science*, 6, 306. https://doi.org/10.3389/fvets.2019.00306

Angeloni, S., Cordes, R., Dunbar, S., Garcia, C., Gibson, G., Martin, C., & Stone, V. (2016). *xMAP® Cookbook* (3rd ed.). Austin, TX: Luminex Corporation.

Aradaib, I. E., Erickson, B. R., Elageb, R. M., Khristova, M. L., Carroll, S. A., Elkhidir, I. M., ... Nichol, S. T. (2013). Rift valley fever, Sudan, 2007 and 2010. *Emerging Infectious Diseases*, 19(2), 246–253. https://doi.org/10.3201/eid1902.120834

Bente, D. A., Forrester, N. L., Watts, D. M., McAuley, A. J., Whitehouse, C. A., & Bray, M. (2013). Crimean-Congo hemorrhagic fever: History, epidemiology, pathogenesis, clinical syndrome and genetic diversity. *Antiviral Research*, 100(1), 159–189. https://doi.org/10.1016/j.antiviral.2013.07.006

Bett, B., Kiunga, P., Gachohi, J., Sindato, C., Mbotha, D., Robinson, T., ... Grace, D. (2017). Effects of climate change on the occurrence and distribution of livestock diseases. *Preventive Veterinary Medicine*, 137, 119–129. https://doi.org/10.1016/j.prevetmed.2016.11.019

Brand, S. P. C., & Keeling, M. J. (2017). The impact of temperature changes on vector-borne disease transmission: *Culicoides* midges and bluetongue virus. *Journal of the Royal Society Interface*, 14(128), 20160481. https://doi.org/10.1098/rsif.2016.0481

Brustolin, M., Talavera, S., Nuñez, A., Santamaría, C., Rivas, R., Pujol, N., ... Busquets, N. (2017). Rift Valley fever virus and European mosquitoes: Vector competence of *Culex pipiens* and *Stegomyia albopicta* (= *Aedes albopictus*): European mosquitoes as vectors of RVFV. *Medical and Veterinary Entomology*, 31(4), 365–372. https://doi.org/10.1111/mve.12254

Carter, S. D., Barr, J. N., & Edwards, T. A. (2012). Expression, purification and crystallization of the Crimean-Congo haemorrhagic fever virus nucleocapsid protein. *Acta Crystallographica Section F Structural Biology and Crystallization Communications*, 68(5), 569–573. https://doi.org/10.1107/S1744309112009736

Dash, A. P., Bhatia, R., Sunyoto, T., & Mourya, D. T. (2013). Emerging and re-emerging arboviral diseases in Southeast Asia. *Journal of Vector Borne Diseases*, 8, 77–84.

Ejeh, E. F., Raji, M. A., Bello, M., Lawan, F. A., Francis, M. I., Kudi, A. C., & Cadmus, S. I. B. (2014). Prevalence and direct economic losses from bovine tuberculosis in Makurdi, Nigeria. *Veterinary Medicine International*, 2014, 1–6. https://doi.org/10.1155/2014/904861

El-Sayed, A., El-Shannat, S., Kamel, M., Castañeda-Vazquez, M. A., & Castañeda-Vazquez, H. (2016). Molecular epidemiology of *Mycobacterium bovis* in humans and cattle. *Zoonoses and Public Health*, 63(4), 251–264. https://doi.org/10.1111/zph.12242

Estrada-Peña, A., Ruiz-Fons, F., Acevedo, P., Gortazar, C., & de la Fuente, J. (2013). Factors driving the circulation and possible expansion of Crimean-Congo haemorrhagic fever virus in the western Palearctic. *Journal of Applied Microbiology*, 114(1), 278–286. https://doi.org/10.1111/jam.12039

Estrada-Peña, A., Sánchez, N., & Estrada-Sánchez, A. (2012). An assessment of the distribution and spread of the tick *Hyalomma marginatum* in the Western Palearctic under different climate scenarios. *Vector-Borne and Zoonotic Diseases*, 12(9), 758–768. https://doi.org/10.1089/vbz.2011.0771

European Commission (2020). *Map showing the location of the restriction zones for different BTV serotypes in Europe as of 9th March 2020*. Retrieved from https://ec.europa.eu/food/sites/food/files/animals/docs/ad_control-measures_bt_restrictedzones-map.jpg

Fafetine, J. M., Tijhaar, E., Paweska, J. T., Neves, L. C. B. G., Hendriks, J., Swanepoel, R., ... Rutten, V. P. M. G. (2007). Cloning and expression of Rift Valley fever virus nucleocapsid (N) protein and evaluation of a N-protein based indirect ELISA for the detection of specific IgG and IgM antibodies in domestic ruminants. *Veterinary Microbiology*, 121(1–2), 29–38. https://doi.org/10.1016/j.vetmic.2006.11.008

Fresco-Taboada, A., Risalde, M. A., Gortázar, C., Tapia, I., González, I., Venteo, Á., ... Rueda, P. (2019). A lateral flow assay for the rapid diagnosis of *Mycobacterium bovis* infection in wild boar. *Transboundary and Emerging Diseases*, 66(5), 2175–2179. https://doi.org/10.1111/tbed.13260

Häsler, B., Alarcon, P., Raboisson, D., Waret-Szkuta, A., & Rushton, J. (2015). Integration of production and financial models to analyse the

financial impact of livestock diseases: A case study of Schmallenberg virus disease on British and French dairy farms. *Veterinary Record Open*, *2*(1), e000035. https://doi.org/10.1136/vetreco-2014-000035

Hassan, O. A., Ahlm, C., Sang, R., & Evander, M. (2011). The 2007 Rift Valley fever outbreak in Sudan. *PLoS Neglected Tropical Diseases*, *5*(9), e1229. https://doi.org/10.1371/journal.pntd.0001229

Hermanson, G. (2013). Microparticles and nanoparticles. In G. Hermanson (Ed.), *Bioconjugate techniques* (3rd ed., pp. 549–587). London, UK: Academic Press.

Himeidan, Y. E., Kweka, E. J., Mahgoub, M. M., El Rayah, E. A., & Ouma, J. O. (2014). Recent outbreaks of Rift Valley fever in East Africa and the Middle East. *Frontiers in Public Health*, *2*, 169. https://doi.org/10.3389/fpubh.2014.00169

Hoffmann, B., Scheuch, M., Höper, D., Jungblut, R., Holsteg, M., Schirrmeier, H., ... Beer, M. (2012). Novel orthobunyavirus in cattle, Europe, 2011. *Emerging Infectious Diseases*, *18*(3), 469–472. https://doi.org/10.3201/eid1803.111905

Lorenzo, G., López-Gil, E., Ortego, J., & Brun, A. (2018). Efficacy of different DNA and MVA prime-boost vaccination regimens against a Rift Valley fever virus (RVFV) challenge in sheep 12 weeks following vaccination. *Veterinary Research*, *49*(1), 21. https://doi.org/10.1186/s13567-018-0516-z

Lorenzo, G., López-Gil, E., Warimwe, G. M., & Brun, A. (2015). Understanding Rift Valley fever: Contributions of animal models to disease characterization and control. *Molecular Immunology*, *66*(1), 78–88. https://doi.org/10.1016/j.molimm.2015.02.001

Maltezou, H. C., & Papa, A. (2010). Crimean-Congo hemorrhagic fever: Risk for emergence of new endemic foci in Europe? *Travel Medicine and Infectious Disease*, *8*(3), 139–143. https://doi.org/10.1016/j.tmaid.2010.04.008

Mathema, B., Kurepina, N. E., Bifani, P. J., & Kreiswirth, B. N. (2006). Molecular epidemiology of tuberculosis: Current insights. *Clinical Microbiology Reviews*, *19*(4), 658–685. https://doi.org/10.1128/CMR.00061-05

Medlock, J. M., & Leach, S. A. (2015). Effect of climate change on vector-borne disease risk in the UK. *The Lancet Infectious Diseases*, *15*(6), 721–730. https://doi.org/10.1016/S1473-3099(15)70091-5

Mertens, M., Schmidt, K., Ozkul, A., & Groschup, M. H. (2013). The impact of Crimean-Congo hemorrhagic fever virus on public health. *Antiviral Research*, *98*(2), 248–260. https://doi.org/10.1016/j.antiviral.2013.02.007

Nakane, P. K., & Kawaoi, A. (1974). Peroxidase-labeled antibody a new method of conjugation. *Journal of Histochemistry & Cytochemistry*, *22*(12), 1084–1091. https://doi.org/10.1177/22.12.1084

Negredo, A., Habela, M. Á., Ramírez de Arellano, E., Diez, F., Lasala, F., López, P., ... Sánchez-Seco, M. P. (2019). Survey of Crimean-Congo hemorrhagic fever enzootic focus, Spain, 2011–2015. *Emerging Infectious Diseases*, *25*(6), 1177–1184. https://doi.org/10.3201/eid2506.180877

O'Hearn, A. E., Voorhees, M. A., Fetterer, D. P., Wauquier, N., Coomber, M. R., Bangura, J., ... Schoepp, R. J. (2016). Serosurveillance of viral pathogens circulating in West Africa. *Virology Journal*, *13*(1), 163. https://doi.org/10.1186/s12985-016-0621-4

OIE-Listed Diseases (2020). *OIE - World Organisation for Animal Health*. Retrieved 17 May 2020 from https://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2020/

Rojas, J. M., Rodríguez-Martín, D., Martín, V., & Sevilla, N. (2019). Diagnosing bluetongue virus in domestic ruminants: Current perspectives. *Veterinary Medicine: Research and Reports*, *10*, 17–27. https://doi.org/10.2147/VMRR.S163804

Rolin, A. I., Berrang-Ford, L., & Kulkarni, M. A. (2013). The risk of Rift Valley fever virus introduction and establishment in the United States and European Union. *Emerging Microbes and Infections*, *2*(1), 1–8. https://doi.org/10.1038/emi.2013.81

Rushton, J., & Lyons, N. (2015). Economic impact of Bluetongue: A review of the effects on production. *Veterinaria Italiana*, *6*, 401–406.

Samy, A. M., & Peterson, A. T. (2016). Climate change influences on the global potential distribution of bluetongue virus. *PLoS One*, *11*(3), e0150489. https://doi.org/10.1371/journal.pone.0150489

Sanders, C. J., Shortall, C. R., England, M., Harrington, R., Purse, B., Burgin, L., ... Gubbins, S. (2019). Long-term shifts in the seasonal abundance of adult *Culicoides* biting midges and their impact on potential arbovirus outbreaks. *Journal of Applied Ecology*, *56*(7), 1649–1660. https://doi.org/10.1111/1365-2664.13415

Sanz, A., García-Barreno, B., Nogal, M. L., Viñuela, E., & Enjuanes, L. (1985). Monoclonal antibodies specific for African swine fever virus proteins. *Journal of Virology*, *54*, 8. https://doi.org/10.1128/JVI.54.1.199-206.1985

Sindato, C., Karimuribo, E., & Mboera, E. G. (2012). The epidemiology and socio-economic impact of Rift Valley fever epidemics in Tanzania: A review. *Onderstepoort Journal of Veterinary Research*, *79*(2), 1. https://doi.org/10.4102/ojvr.v79i2.467

van der Wal, F. J., Achterberg, R. P., de Boer, S. M., Boshra, H., Brun, A., Maassen, C. B. M., & Kortekaas, J. (2012). Bead-based suspension array for simultaneous detection of antibodies against the Rift Valley fever virus nucleocapsid and Gn glycoprotein. *Journal of Virological Methods*, *183*(2), 99–105. https://doi.org/10.1016/j.jviromet.2012.03.008

Wade-Evans, A. M., Romero, C. H., Mellor, P., Takamatsu, H., Anderson, J., Thevasagayam, J., ... Black, D. N. (1996). Expression of the major core structural protein (VP7) of bluetongue virus, by a recombinant capripox virus, provides partial protection of sheep against a virulent heterotypic bluetongue virus challenge. *Virology*, *220*(1), 227–231. https://doi.org/10.1006/viro.1996.0306

Walter, C. T., & Barr, J. N. (2011). Recent advances in the molecular and cellular biology of bunyaviruses. *Journal of General Virology*, *92*(11), 2467–2484. https://doi.org/10.1099/vir.0.035105-0

Weaver, S. C., & Reisen, W. K. (2010). Present and future arboviral threats. *Antiviral Research*, *85*(2), 328–345. https://doi.org/10.1016/j.antiviral.2009.10.008

Wernike, K., & Beer, M. (2017). Schmallenberg virus: A novel virus of veterinary importance. *Advances in Virus Research*, *99*, 39–60. https://doi.org/10.1016/bs.aivir.2017.07.001

Wiker, H. G. (2009). MPB70 and MPB83—Major antigens of *Mycobacterium bovis*. *Scandinavian Journal of Immunology*, *69*(6), 492–499. https://doi.org/10.1111/j.1365-3083.2009.02256.x

Wright, D., Kortekaas, J., Bowden, T. A., & Warimwe, G. M. (2019). Rift Valley fever: Biology and epidemiology. *Journal of General Virology*, *100*(8), 1187–1199. https://doi.org/10.1099/jgv.0.001296

Wu, W., Zhang, S., Qu, J., Zhang, Q., Li, C., Li, J., ... Li, D. (2014). Simultaneous detection of IgG antibodies associated with viral hemorrhagic fever by a multiplexed Luminex-based immunoassay. *Virus Research*, *187*, 84–90. https://doi.org/10.1016/j.virusres.2013.12.037