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Development and validation of a pentaplex assay for the identification of antibodies against common viral diseases in cattle

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Abstract

Animal welfare and economic implications of infectious diseases in cattle demand an efficient surveillance as the foundation for control and eradication programmes. Bovine respiratory syncytial virus (BRSV), Parainfluenza virus type 3 (PI3V), Bovine herpes virus-1 (BoHV-1), Bovine viral diarrhoea virus (BVDV), and Enzootic bovine leukosis virus (EBLV) cause common and often underdiagnosed diseases in cattle that are endemic in most countries [1]. A hallmark of individual exposure to a viral pathogen is the presence of antibodies directed towards that virus. The aim of this study was to develop and validate a pentaplex assay to simultaneously detect and quantify antibodies against BRSV, PI3V, BoHV-1, BVDV and EBLV in serum, as an efficient tool to yield epidemiological data. Monoplex assays were initially developed using either complete BRSV or BoHV-1 viral lysates, or recombinant proteins for BVDV, EBLV or PI3V as capture antigens. In addition, 125 serum samples from unvaccinated cattle, which were classified as positive or negative for each of the viruses by commercial ELISA kits, were used for validation. Conditions established for the Luminex monoplex assays were adopted for the pentaplex assay. The accuracy, determined by the area under the ROC curve, was greater than 0.97, and assay diagnostic sensitivities and specificities were over 95 and 90%, respectively, for all antigens. Intra (r) and interassay (R) coefficients of variation were under 10 and 20%, respectively. Selectivity towards target viruses was shown by binding inhibition assays where unbound viruses reduced fluorescence intensities. Diagnostic agreement for samples analysed simultaneously in the monoplex and multiplex assays was almost perfect. In conclusion, a highly sensitive pentaplex assay was validated for the simultaneous identification of antibodies directed against BVDV, BoHV-1, PI3V, BRSV and EBLV in serum. The developed pentaplex assay complies with performance characteristics established by international guidelines for diagnostic tests and may be used as a tool for the implementation of epidemiological surveillance.

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Abbreviations: AOAC, Association of Official Analytical Chemists; ATCC, American Type Culture Collection; AUC, area under the curve; BacToBac, baculovirus expression system; BamH1, Bacillus amyloliquefaciens restriction endonuclease type II; BLAST, Basic Local Alignment Search Tool; BoHV-1, bovine alphaherpesvirus type 1; BRSV, bovine respiratory syncytial virus; BVDV, bovine viral diarrhoea virus; °C, degrees celsius; CV, coefficient of variation; DH10BAC, competent cells to generate recombinant bacmids by transposition; DNA, deoxyribonucleic acid; EBL, enzootic bovine leukosis; EBLV, enzootic bovine leukosis virus; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; FEUM, Pharmacopoeia of the Mexican United States (by its acronym in Spanish Farmacopea de los Estados Unidos Mexicanos); g, relative centrifuge force; µg, micrograms; gE, glycoprotein E2 of BVDV; gp51, glycoprotein 51 of bovine leukaemia virus; h, hours; HN, hemagglutinin neuraminidase; H7N3, hemagglutinin 7 neuraminidase 3; IBR, infectious bovine rhinotracheitis syndrome; IgG, gamma immunoglobulin; µl, microliter; LR+, positive likelihood ratios; M, molar; MFI, mean fluorescent intensity; ml, milliliter; Mono NC, negative monovalent control; Mono PC, positive monovalent control; NaCl, sodium chloride; NaN3, sodium azide; NCBI, National Center for Biotechnology Information; nm, nanometer; NSB, non-specific binding; PBS, phosphate buffer solution; PCR, polymerase chain reaction; PE, streptavidin-phycoerythrin; pH, potential of hydrogen; PI3V, parainfluenza virus type 3; Poly C, polyvalent positive control; ROC, receiver operating characteristics; r.p.m., revolutions per minute; RT-PCR, reverse transcription polymerase chain reaction; Sf9, spodoptera frugiperda cell line IPLB-Sf-21-AE; TCID50, median tissue culture infectious dose 50; U ml -1, units per millilitre; USPC, United States Pharmacopeial Convention; WOAH, World Organisation for Animal Health; xMAP, multi-analyte profiling.

DATA SUMMARY

Data underlying this research can be retrieved using the following link on Figshare: https://doi.org/10.6084/m9.figshare.22341601.v1 [2]. This file has three worksheets with: (1) the mean fluorescent intensities for positive and negative samples used to estimate sensitivities and specificities in the ROC analyses. (2) Mean fluorescent intensities of positive and negative quality controls in the pentaplex assay, and their inter- and intra-assay coefficients of variation. (3) Correspondence analysis of sample status when analysed in either the monoplex or pentaplex formats.

INTRODUCTION

Infectious diseases in cattle have significant animal welfare and economic implications that threaten the sustainability of productive enterprises and affect the quality, quantity and availability of animal products. In addition, lack of specific treatment for viral diseases contribute to the increased use of antibiotics, as they are often associated with secondary microbial infections.

Bovine respiratory syncytial virus (BRSV), Parainfluenza virus type 3 (PI3V), Bovine herpes virus-1 (BoHV-1), Bovine viral diarrhoea virus (BVDV) and Enzootic bovine leukosis virus (EBLV) cause common and often underdiagnosed infectious diseases in cattle that are endemic in most countries according to the WOAH [1]. Bovine respiratory syncytial virus is a paramyxovirus with cytopathic effects, clinical signs of infection are limited to the respiratory system and vary from inapparent to severe [3]. Control is achieved by modified live or inactivated virus vaccines. Morbidity tends to be high (prevalence of antibodies in the US ranges from 60–80%) and mortality rate spans from none to 20% [4, 5]. Parainfluenza virus type 3 (PI3V) is also a paramyxovirus that is widely spread in cattle and other ruminant populations, which by itself causes subclinical to mild respiratory signs. However, PI3V infection predisposes the respiratory tract to secondary bacterial pneumonia. Inactivated and modified live vaccines are available for its control [6]. Bovine herpes virus-1 (BoHV-1) infection is associated with multiple disease syndromes in cattle including infectious bovine rhinotracheitis syndrome (IBR), conjunctivitis, pustular vulvovaginitis, balanoposthitis, abortion, encephalomyelitis and mastitis. Adult cattle are the principal reservoir, with high prevalence that indicates a wide distribution [7, 8]. The IBR syndrome, caused by BoHV-1 infection, is rarely fatal in mature cattle unless complicated by secondary bacterial infection of the lung. Despite the low mortality, IBR causes considerable economic losses, and an IBR-free status is required for semen and embryo trade in the European Union [7]. Bovine viral diarrhoea is caused by a pestivirus producing infections that range from subclinical to mucosal disease. It also associates with repeat breeding, abortion, foetal mumification, congenital defects, immunotolerance and persistent infections. Nonetheless, its most important effect is immunosuppression that facilitates the synergistic infection with other pathogens [9]. Enzootic bovine leukosis (EBL) is caused by a retrovirus. An overt cause of economic loss for the producer of EBLV infected cattle is the decommission of carcases affected by lymphomatous tumours. However, more difficult to quantify is the immunosuppression that facilitates secondary infections, which cause suboptimal performance, and a diminished response to vaccination and to natural infections [10, 11]. The economic toll caused from these diseases for bovine producers in North America and Brazil has been calculated in several millions of dollars annually [6, 12].

The implicit animal welfare and economic consequences of infectious disease occurrence, due to decreased productive performance, increased mortality rates, possible reproductive failure, as well as preventive and treatment costs [7, 8, 13]; have led many countries to implement surveillance, control and eradication programmes. Further, as control programmes advance, more rigorous regulations are enacted for commercialization of gametes, live animals and animal products at regional, national and international levels [7] to avoid an increase in prevalence in endemic areas and potential outbreaks in disease-free countries.

Control and eradication programmes require an ongoing intervention through surveillance of pathogen exposure in hosts and susceptible individuals. Initial tests for establishing prevalence of a disease often rely on serological antibody identification, since the antibody registry unequivocally indicates previous or current contact of the population to an infectious agent. Moreover, the development and validation of diagnostic platforms that allow for the detection of antibodies associated to several diseases of interest in parallel (multiplex) in a single sample, potentially provide a detailed snapshot of individual exposure and allow for determination of prevalence rates. Later serological surveillance could confirm the success of vaccination programmes.

Multiplexing technologies have been increasingly used in human health diagnoses, since rapid assessment of several causative pathogen infections and/or differential identification of diseases that have similar symptoms are frequently necessary [14, 15]. In veterinary sciences, multiplexing has predominantly relied on custom assays developed in open platforms that have been mainly tailored to solve particular research demands [14, 16–18], but that have the potential to significantly increase epidemiological resources. The xMAP technology is a microsphere-based assay where beads have unique emission profiles for individual fluorescent wavelength identities that allow users to construct assays with multiple analyte sets. It is a flow-cytometry-like system that allows for each individual microsphere to be queried, and multiple readings are taken per microsphere-set, providing statistical validity and robustness to the data (for details see [19]). The aim of this study was to develop and validate a pentaplex assay, using the Luminex x-MAP platform, to detect and quantify serum antibodies directed against five viral pathogens (BRSV, PI3, IBR, BVD and EBL), as a tool to yield epidemiological data in cattle production units.

METHODS

Viruses were procured from the *American Type Culture Collection* (ATCC) (http://www.atcc.org/): BRSV, Iowa strain (FS1-1), Catalogue No. VR-1485; BVDV, NADL strain, Catalogue No. VR-1422; BoHV-1, Colorado-1 strain, Catalogue No. VR-864; PI3V, SB strain, Catalogue No. VR-739; and EBLV, BL3.1 cells. Catalogue No. CRL–2306, RRID:CVCL 3455. Viruses were grown in Madin–Darby bovine kidney cells (NBL-1; RRID: CVCL_0421) cultured at 37°C for 6 days in Minimum Essential Medium Eagle with 10% fetal calf serum, supplemented with penicillin (100 U ml^{-1})/ streptomycin (100 mg ml^{-1})/ amphotericin B (0.25 mg ml^{-1}). Infected cell cultures were then subjected to two freeze–thaw cycles and later centrifuged at 4000 ***g*** for 10 min to remove cellular debris. Protein concentrations were subsequently measured in the supernatant with a spectrophotometer at 260 nm. Viral titres were obtained by end-point dilution $TCID_{50}$ using the Reed–Muench method [20].

Viral antigens

Complete BRSV and BoHV-1 were used as antigens in their respective monoplex assays, as well as in the pentaplex assay. In addition, recombinant antigens were produced for the detection of antibodies against BVDV, PI3V and EBLV in both platforms. Bovine viral diarrhoea virus and PI3V virus RNA was isolated from their respective cell cultures and DNA was purified from EBLV-infected cells. Genes encoding viral antigens were amplified by PCR or RT-PCR. Antigen coding viral gene name and size, as well as primer sequences used for amplification are shown in Table 1 (GenBank access data for the reference genes are: EBLV: AY078387; BVDV: AJ133738; PI3V: AF178655). Primers were designed to include BamH1 sites for cloning and a sequence encoding for six histidines at the 3´ end, for affinity purification. The amplified viral genes were cloned into the baculovirus mobilization vector pFasTBacHa (BacToBac, Invitrogen), following manufacturer recommendations (https://tools.thermofisher.com/content/sfs/manuals/bactobac_topo_exp_system_man.pdf). Briefly, the recombinant pFastTBacHa DNA clones (Thermofisher.com) were used to transform competent DH10BAC cells to generate recombinant bacmids by transposition. The recombinant bacmid DNA was then isolated and employed to transfect SF9 cells to obtain infectious recombinant baculovirus. For recombinant antigen production, the recombinant baculovirus were grown on SF9 insect cells that were cultured for 3 days, and subsequently pelleted by centrifugation at 4000 r.p.m. for 10 min. Pelleted cells were later lysed with 0.1 M Tris at pH 7.5, with 0.15 M NaCl and 1% Triton X-100, incubated for 30 min on ice, homogenized, and subsequently centrifuged at 4000 r.p.m./10 min. Finally, the supernatant was recovered, and protein content quantified by spectrophotometry at 260 nm.

Positive and negative quality control sera

One hundred and twenty-five samples (expecting *a 95% diagnostic sensitivity with 5% error* [21, 22]), from unvaccinated cattle were tested with commercial ELISA monoplex kits (BVDV 99 4400; IBR Ab 99–41459; PI3 P00652-2; BRSV P00651-2; EBLV; P02110-5; Idexx Laboratories, Westbrook, Maine, USA) to establish the negative or positive antibody status for each disease. Later, for validation of monoplex assays in the Luminex x-MAP platform, the ELISA defined positive sera for a single disease, and negative samples for at least the disease being tested, were designated as positive (monoPC) and negative monovalent controls (monoNC), respectively. Seven serum samples resulted positive for all five diseases, these were pooled and used for the validation of the pentaplex assay as a polyvalent positive control (polyC). Since none of the samples tested negative for all five diseases, foetal bovine serum (FBS) was used as the negative control for development and validation of the pentaplex assay.

Pentaplex assay development and validation

For the development and validation of the pentaplex immunoassay, each individual antigen (recombinant gE/BVD, HN/PI3, gP51/EBLV, and BoHV-1 and BRSV complete viruses) was firstly tested and standardized in monoplex indirect ELISAs to determine the appropriate working concentrations. These were set at 2 µg for recombinant proteins HN (PI3V), gp51 (EBLV) and gE (BVDV). As for BRSV and BoHV-1, 5 µg and 2 µg of complete viruses were respectively used. The concentration of the biotin conjugated secondary antibody (anti-bovine IgG; Jackson Inmunoresearch 101-065-165; RRID: AB_2337297) was set at 1:10000 dilution for all monoplex ELISA assays.

Table 1. Enzootic Bovine Leukosis virus (EBLV), Bovine viral diarrhoea virus (BVDV) and Parainfluenza virus type 3 (PI3V) encoding gene names and sizes, and primer sequences used for amplification

Virus	Gene (size)	Primer sequences
BVDV	**E2** (1165pb)	E2DVBF 5' ATGGATCCGCTGATAACAGGRGYGCAAGG 3'; E2DVBRhis 5' AT GGATCCTTAGTGGTGGTGGTGGTGGTGTG CTGATAAGACCATGTATGTRACCAG 3'
PI3V	**HN** (1719pb)	HNBPI3F 5' ATGGATCC GATGGAATATTGGAAACACAC 3'; HNBPI3Rhis 5' ATGGATCCTTAGTGGTGGTGGTGGTGGTG GCTGCAGTTTTTCGGAAC 3'
EBLV	**gp51** (1570pb)	BLVF 5' ATGGATCC GATGCCYAAAG AACGACGGTCCCG 3'; BLVRHis 5' ATGGATCCTCAGTGGTGGTGGTGGTGGTG GGGCAGGGTCGRAGGTTGATGTAATCG 3'

Regarding antibody detection using the xMAP technology from Luminex, five distinctive fluorophore-coded microspheres (1.25×10^6 microspheres per antigen; BioRad Laboratories, Magplex-C Microspheres, MC10051-01) were covalently bound [23] to the complete viruses or to the recombinant viral antigens, using the carbodiimide method. The antigen–antibody interaction when present was therefore specific and revealed by a second antibody (anti-bovine IgG) labelled with biotin, as an anchor for streptavidin-phycoerythrin (PE) (BioRad Laboratories, 171304501) [23, 24]. The Luminex system allows for the microspheres to be read individually using two laser diodes. The first laser (635 nm) identifies the microsphere code (specific for each virus or viral antigen) and the second laser (523 nm) excites the PE when an antibody is bound to the microsphere-linked antigens [19, 23]. The optimum amount of inclusion of sera in the assay was determined by testing sample dilutions from 1:2 to 1:32768. The best antibody binding for all antigens was obtained between 1:8 and 1:20 dilution. The detection limits of antibody quantification were estimated by serial dilutions of the polyC and reported as the highest dilution that differed from non-specific binding values (NSB) (AOAC 2002).

For monoplex and pentaplex assays in the Luminex platform, 400 antigen-bound microspheres were added per well in a total volume of 50 µl of blocking buffer (PBS 0.01M -Sigma P3813- with 1% albumin -Sigma 7906- and 0.05% of sodium azide [NaN_3] -Sigma 199931, pH 7.4), for each viral antigen studied. Fifty microlitres of the positive or negative control samples (diluted 1:10) were then added to the reaction and plates were incubated for 2 h. Wells were subsequently washed with a PBS solution with 0.05% tween 20 (Sigma P1379), and 50 µl of biotin-bound anti-bovine IgG were added at 1:10000 dilution and incubated for a further 2 h. After a second wash, antigen–antibody complexes were unveiled by the addition of 50 µl of 1:100 PE in blocking buffer. Beads were subsequently rinsed and resuspended in 100 µl wash solution. Mean fluorescent intensity (MFI) for independent antigens was measured and recorded simultaneously by the Luminex platform (Bioplex 100/200, BioRad laboratories). All assays were conducted at 25°C, with plates protected from light and under constant agitation at 300 r.p.m. throughout.

For validation of the pentaplex assay, antigens were initially evaluated within the Luminex platform as monoplex assays, using the respective monoPC and monoNC to establish cut-off points. Once the monoplex assays were standardized, according to international guidelines (AOAC 2002 [25]), validation of the pentaplex assay was conducted.

Cut-off points (measured in MFI), diagnostic sensitivity and specificity, and positive likelihood ratios (*LR+ >10*) for monoplex and pentaplex assays were determined by the receiver operating characteristics (ROC) curve method (Prism 8 GraphPad Prism – software; RRID:SCR_002798) in triplicate. Cut-off points for each disease were selected for diagnostic sensitivity and specificity values greater than 90%. The likelihood ratios for the positive results were selected at values above 10 [26].

Pentaplex assay precision was determined by repeatability (r) and reproducibility (R) parameters. For the former, polyC and FBS samples were analysed within the same assay in five replicates. An intra-assay coefficient of variation below 20% was considered adequate according to international specifications (AOAC 2002 [25]; *Norma Oficial Mexicana NOM-177-SSA1-2013* 2013 [27]; USPC 2019 [28]). For the latter, polyC and FBS were evaluated in quintuplicate on three independent assays. An inter-assay coefficient of variation below 20% was deemed as adequate (AOAC 2002 [25]; *Norma Oficial Mexicana NOM-177-SSA1-2013* 2013 [27]; USPC 2019 [28]).

Selectivity, shown by antibody binding to its particular antigen [29], was evaluated for BVDV, BoHV-1, and PI3V by binding inhibition monoplex assays. Soluble viral antigens at neat and 1:2 dilutions were added to the reaction to inhibit sample antibody binding (monoPC) to the microsphere-linked antigens. In addition, baculovirus expressing haemagglutinin antigen form the H7N3 influenza virus were added as a negative control. These assays were performed in triplicate and the results were expressed as binding percentages.

Agreement on dichotomic results (positive or negative sample status) in monoplex and pentaplex assays was evaluated in simultaneously ran tests. Sixty samples for BoHV-1, BVDV, PI3V and EBLV, as well as 44 samples for BRSV were used, and the agreement between monoplex and pentaplex assays was estimated by corrected Cohen's Kappa coefficient [30, 31].

RESULTS

Antibody quantification, which differed from non-specific binding values, was achieved for all antigens tested at sample dilutions between 1:2 to 1:1024 in both monoplex and pentaplex Luminex assays. To establish optimum true positive rate (sensitivity) and false positive rate (1-specificity) parameters, ROC curves were plotted for mono and pentaplex assays. The area under the curve (AUC) was greater than 0.97 for all antigens (Fig. 1). Mean fluorescent intensities for the positive and negative control samples and optimized cut-off points for viral antibodies are shown in Fig. 2. Diagnostic sensitivity and specificity were over 95 and 90%, respectively, with positive likelihood ratios greater than 10 in all cases (Table 2).

All assays were deemed as having an excellent precision according to Eurachem [32] and FEUM guidelines, with intra-assay coefficients of variation (r) below 10%, and inter-assay coefficients of variation (R) lower than 20% (Table 2).

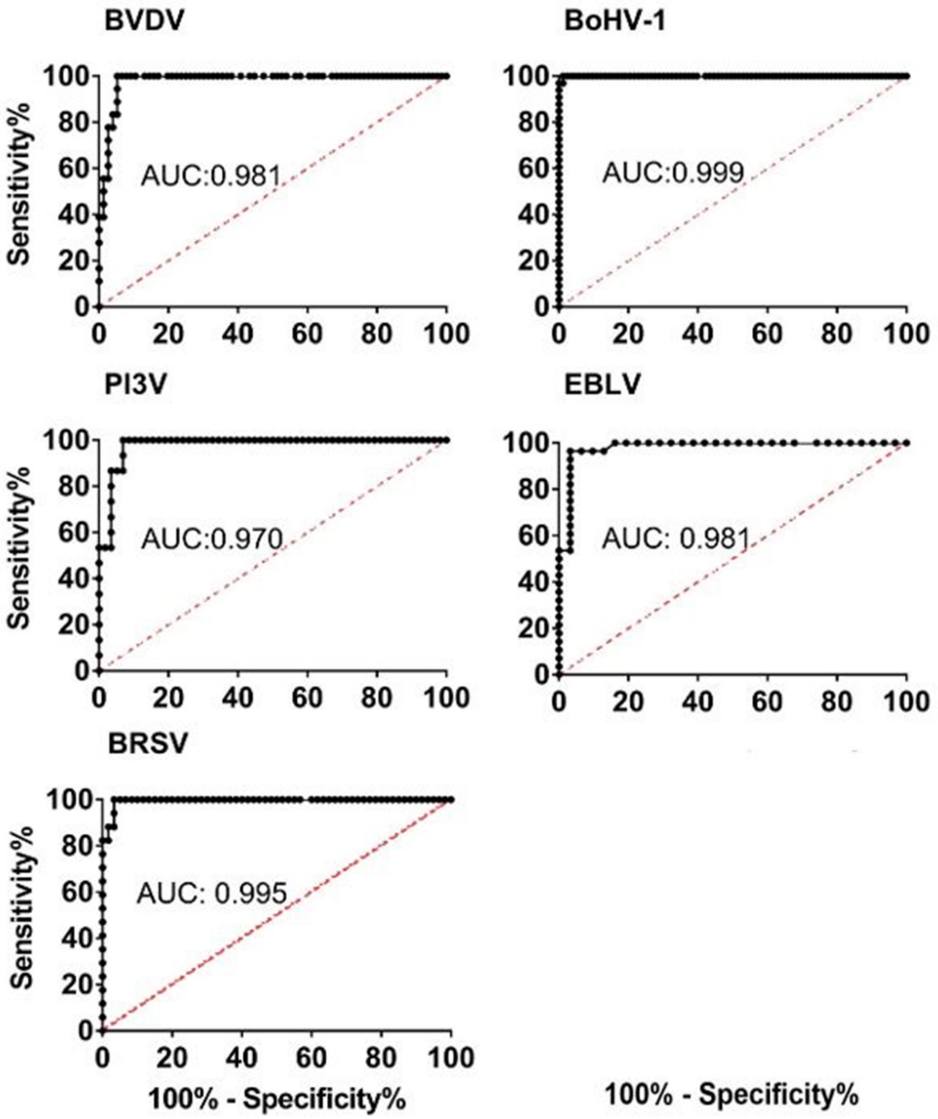

Fig. 1. ROC analyses showing diagnostic sensitivity and specificity values at different cut-off points in Luminex monoplex assays to detect serum antibodies directed against Bovine viral diarrhoea virus (BVDV), Bovine Herpes virus-1 (BoHV-1), Parainfluenza virus type 3 (PI3V), Enzootic Bovine Leukosis virus (EBLV) and Bovine respiratory syncytial virus (BRSV).

Selectivity towards the viral antigens was shown by binding inhibition assays (Fig. 3) where the addition of unbound BVDV, PI3V and BoHV-1 viruses reduced MFI values. Further, the inclusion of baculovirus expressing influenza H7N3 viral antigens to the assay reaction showed low non-specific non-specificity (<5%).

To validate the pentaplex assay, established conditions for the monoplex tests were used when all antigens were combined within the same assay (pentaplex platform). The AUCs for the pentaplex assay, which are used to determine sensitivity and specificity parameters, did not differ with those obtained for the monoplex tests (Table 2). Moreover, diagnoses agreement for samples analysed simultaneously in the monoplex and multiplex assays was almost perfect for BoHV-1, EBLV, BRSV and BVDV (>0.81) and substantial for PI3V (0.61–0.80) (Table 3).

DISCUSSION

A highly sensitive pentaplex assay was validated for the identification of serum antibodies directed against BVDV, BoHV-1, PI3V, BRSV and EBLV. All antigens developed were specific and recognized by antibodies of samples that tested positive by commercial ELISA kits. In addition, a binding inhibition assay proved the selectivity of the antigens to particular viruses or viral antigens.

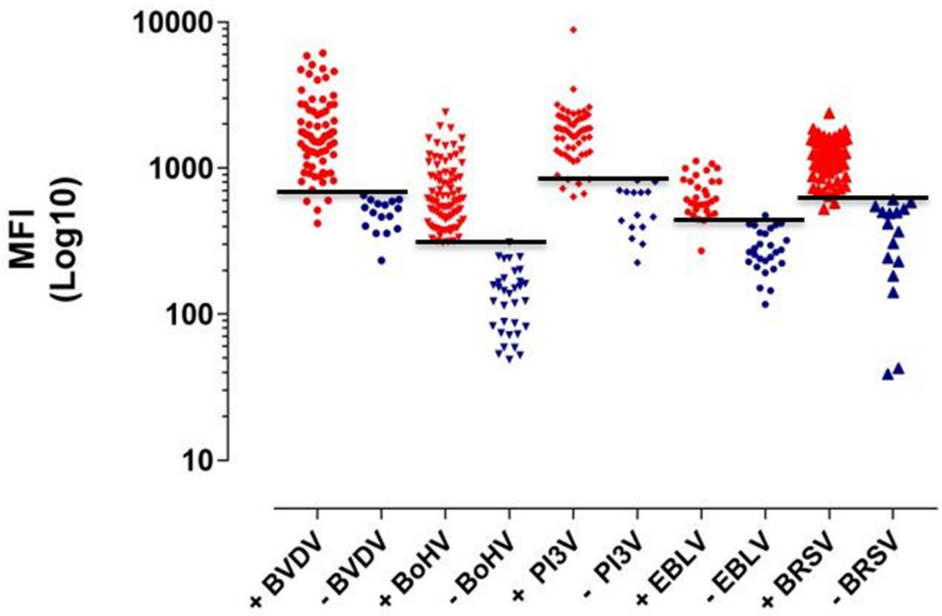

Fig. 2. Mean fluorescent intensities for positive (red) and negative (blue) control samples in monoplex assays standardized in the Luminex platform. The horizontal lines show the selected cut-off points for Bovine respiratory syncytial virus (BRSV), Parainfluenza virus type 3 (PI3V), Bovine Herpes virus-1 (BoHV-1), Bovine viral diarrhoea virus (BVDV), and Enzootic Bovine Leukosis virus (EBLV). (+=positive control, -=negative control for each disease tested).

Table 2. Performance characteristics of the Luminex Pentaplex assay for Bovine viral diarrhoea virus (BVDV), Bovine Herpes virus-1 (BoHV-1), Parainfluenza virus type 3 (PI3V), Enzootic Bovine Leukosis virus (EBLV), and Bovine respiratory syncytial virus (BRSV), antibody detection. Cut-off points values are shown in mean fluorescent intensity units (MFI). Sensitivity (Se), specificity (Sp), accuracy (AUC), positive (LR+) and negative (LR-) likelihood ratios, and coefficients of variation (CV) for reproducibility (R) and repeatability (r) of at least three assays are shown

Antigen	Cut-off (MFI)	Se (%)	Sp (%)	AUC 95%	LR+	CV (%)	
						R	r
gE2 rBVDV	683.6	100	94.7	0.981 (0.95–1.00)	19.00	11.03	4.28
BoHV-1	313.1	100	98.8	0.999 (0.99–1.00)	83.00	10.84	6.39
HN rPI3V	833.1	100	93.1	0.970 (0.95–1.00)	14.50	20.00	3.25
gP51 rEBLV	430	96.43	96.8	0.981 (0.95–1.00)	29.89	4.93	5.10
BRSV	621	100	96.67	0.995 (0.98–1.00)	30.0	9.63	3.86

Further, identification of positive and negative samples by monoplex and pentaplex assays had an almost perfect agreement. The detection limit for all diseases was effective up to a 1:1024 sample dilution.

For an equivalent number of determinations, multiplex assays have several advantages over monoplex assays. The simultaneous analyses for different antigens, with the reduction in the amount of sample, reagents and time needed for diagnosis are undoubtedly amongst the most important, facilitating epidemiological research and surveillance [24, 33]. However, no guidelines are recognized for validation of multiplex assays, and recommendations published for the standardization of single assays are typically used. Thus, assays need to be firstly validated individually (as monoplex tests) to establish conditions that are subsequently transferred to the multiplex platform [34].

In this study, both monoplex and pentaplex assays for all viral antigens showed diagnostic sensitivity and specificity above 95 and 93%, respectively, with excellent accuracies (ROC AUC >0.96). Receiver-operating characteristics (ROC) analyses calculate sensitivity and specificity parameters over an uninterrupted range of cut-off points (MFI), subsequently plotting the results. The value for the area under the curve (AUC) of such plots indicate the probability of correctly classifying the sample. A trade-off between diagnostic sensitivity and specificity is inevitable, however selected cut-off points for the assay are established when both parameters are above 90% and the AUC approaches 1, hence complying with the performance guidelines set up by regulatory bodies (AOAC 2002; [25]; *Norma Oficial Mexicana NOM-177-SSA1-2013* 2013 [22]), and efficiently separating between positive

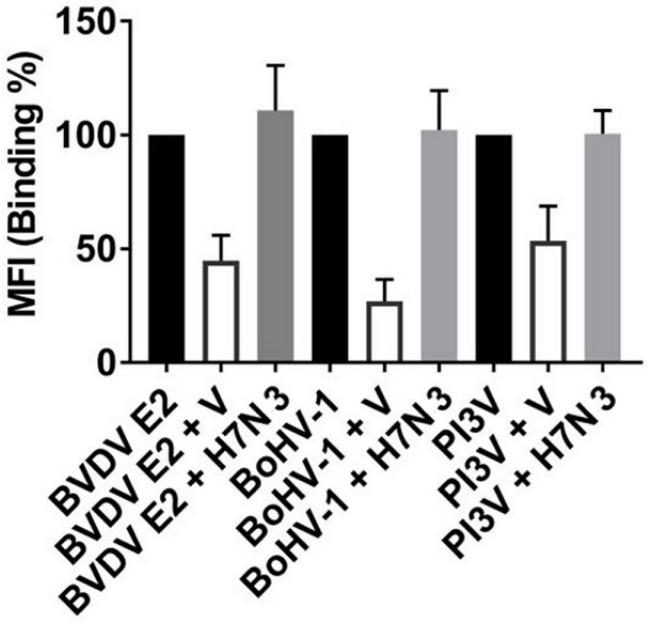

Fig. 3. Binding inhibition caused by the addition of unbound viruses to the assay reaction Bovine viral diarrhoea virus -BVDV-, Bovine Herpes virus-1 -BoHV-1-, Parainfluenza virus type 3 -PI3V-. Total binding to the assay antigen (E2 protein of BVDV; complete BoHV-1, or HN for PI3V) in the absence of unbound virus ( ). The addition of unbound virus caused a reduction in MFI (V;). Binding percentage when a baculovirus-H7N3 ; was added to test a potential antibody binding inhibition caused by the baculovirus proteins.

Table 3. Diagnoses agreement expressed by the corrected Cohen's kappa coefficient, and area under the curve (AUC) for samples analysed simultaneously in monoplex and multiplex Luminex assays for Bovine viral diarrhoea virus (BVDV), Bovine Herpes virus-1 (BoHV-1), Parainfluenza virus type 3 (PI3V), Enzootic Bovine Leukosis virus (EBLV) and Bovine respiratory syncytial virus (BRSV)

Antigen	Monoplex assay AUC	Multiplex assay AUC	Corrected Cohen's kappa coefficient (k´)
gE2 rBVDV	0.937	0.960	0.93
BoHV-1	0.885	0.852	0.92
HN rPI3V	0.846	0.788	0.77
gP51 rEBLV	0.832	0.830	0.86
BRSV	0.943	0.940	0.96

<0 No agreement; 0.0–0.2 slight agreement; 0.41–0.60 moderate agreement; 0.61–0.80 substantial agreement; 0.81–1.00 almost perfect agreement [30, 31].

and negative samples. Further, given the likelihood ratios observed, the probability that samples classified as positive by the assay come from animals that were indeed exposed to the field virus (since tested sera came from non-vaccinated animals) was at least 10 times greater than those with negative results [35].

Assay selectivity, shown by antibody binding to its particular antigen, was warranted firstly by either using the whole virus (BoHV-1 and BRSV) or by selecting immunodominant and highly conserved antigens present at the viral surface (gp51, HN, and gE2 for EBLV, PI3V, and BVDV, respectively). Secondly, selective binding of serum antibodies to the viral antigens was confirmed by a decreased fluorescent signal in monoplex inhibition tests [22], where soluble antigens were added to the assay reaction. Moreover, the addition of baculovirus-H7N3 to the assay did not cause inhibition of the signal, indicating that baculovirus antigens were not recognized by bovine serum antibodies and thus did not interfere with the outcome. This is relevant as the recombinant antigens used herein were produced in a baculovirus expression system.

Conveyance of performance characteristics from monoplex assays to multiplex assays may be challenging as there is the potential for assay cross-reactivity and interference between analytes [34]. However, since all antigens used for assays in this study belonged to different viral families, the possibility of cross-reactivity was scarce. This assertion was further supported by lack of homology

when paired comparisons between the sequences of all antigens were performed (BLAST data not shown, NCBI GenBank). Hence, serum antibodies directed against an antigen are unlikely to recognize a different antigen non-specifically [29, 36].

High repeatability and reproducibility were observed for all the assays validated (monoplex and pentaplex). The intra-assay (r) and inter-assay (R) coefficients of variation were within the acceptable range (i.e. below 20%) [32, 36]. Hence, all validated assays had performance characteristics to be qualified for clinical diagnosis.

Taken together, the above evidence indicates that the monoplex assays validated in this work could be successfully merged to a pentaplex platform, without altering performance parameters. Moreover, to effectively assess the diagnostic efficiency of monoplex and pentaplex formats, samples were tested in both platforms simultaneously, finding a substantial agreement for PI3V and an almost perfect agreement for BoHV-1, EBLV, BRSV and BVDV (Table 3) [31]. Thus, an efficient pentaplex assay for simultaneous antibody quantification against five viral cattle diseases with high diagnostic sensitivity, specificity and selectivity was validated for sero-epidemiological studies.

All assays were highly sensitive and able to discriminate the presence of antibodies from the 1:2 dilution. Nonetheless, the dilution that characterized the detection limit varied slightly for each antigen, with the 1:1024 dilution successfully discriminating positive samples from non-specific binding values for all five antigens tested. Albeit assays could discriminate positive samples in a wide range of dilutions, the MFI was diminished at low dilutions (1:8 or below). This counter-intuitive phenomenon (hook effect) occurs when an excess of antibodies impairs their effectiveness to form immune complexes [37]. With high dilution detection limits, a minimal volume of sample is needed for the determination of antibodies without affecting the diagnostic efficiency of the assay. This attribute is particularly useful when analysing samples combined as pools for epidemiological screening, where antibodies of a positive sample could potentially be diluted [37, 38]. A further advantage of a high sample dilution is decreasing potential interference of unrelated sample components in the assay [36], thereby reducing non-specificity. In this regard, non-specificity of the assay is also diminished by the Luminex xMAP platform since antigens are covalently bound to the surface of the fluorescent beads. This contrasts with most other assay types, where antigens are attached to the plate surface by electrostatic adsorption, leaving multiple active sites that could later bind to non-interest molecules, antibodies or the conjugate to create non-specific signals [23, 39]. Moreover, contrasting with assay platforms where the signal is developed and read in the same well where the reaction takes place, bead signal acquisition in the Luminex xMAP assays occur in a capillary where the antigen-linked beads are individually incited by a laser, reducing the possibility of reading non-specific immune complexes formed during incubation [23].

Multiplexing in its various platforms will likely substitute monoplex assays for massive serological surveillance. As with ELISA, xMAP allows for the in-house development of assays, optimizing the antigens of interest and permitting the customization of antigen panels to suit specific needs. The development of an xMAP assay is no more complicated than that of an ELISA that uses the same antigens, in most cases yielding similar or even higher sensitivity. Several multiplex assays have been developed by other research groups to study bovine diseases. Fontana *et al.* [40] created a multi-antigen assay for the serological detection of bovine tuberculosis using recombinant proteins, reporting a lack of interference in the multiplex reaction and similar signal intensity for all antigens used for both the monoplex and multiplex platforms. Others have developed assays for a single pathogen using multiple antigens with satisfactory results (Yun *et al.* 2007 [17]). Anderson *et al.* [16] devised a multiplex assay similar to the one reported herein, where BRSV, BoHV-1, BVDV and PI3V were studied using viral lysates as capture antigens. These authors found that the performance of the multiplex assay was equivalent to that of the monoplex ELISA for two of their antigens (BoHV-1 and PI3V), while it was lower for the other two (BRSV and BVDV). The reason for the later decline in performance is not clear [16]. Nonetheless, the possibility of protein interference or cross-reactivity when multiple viral lysates are used cannot be ruled out. Although a direct comparison between monoplex ELISAs and the pentaplex assay developed in this work was not made, the contrast between antigen performance in the xMap monoplex versus the pentaplex assay was similar for recombinant proteins and viral lysates.

The viruses chosen for the development of the pentaplex assay in this study (BVDV, BoHV-1, EBLV, BRSV and PI3V), have been targeted in eradication/control programmes in Europe and North America due to increasing disease prevalence [10] and the ensuing negative impact in herd health and productivity [7, 41, 42]. Eradication programmes establish prevalence by regions, and encompass preventive medicine programmes and vaccination strategies, as well as the identification, isolation and elimination of positive animals [7, 41, 42]. Nevertheless, most assessments underestimate the potential impact of secondary complications due to immunosuppression and related synergistic effects with other pathogens. Hence, costs of control and eradication programmes should be regarded as an investment, with expenses of diagnostic testing, removal of infected animals, vaccination and monitoring being factored in against reduced losses in the long term. The inception of any control programme is based on accurate knowledge of prevalences. In this endeavour, the development of efficient diagnostic tools is the corner stone for the implementation of epidemiological surveillance and control.

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Author contributions
A.R. Conceptualization, formal analysis, investigation, methodology, writing – original draft. R.A.M. Conceptualization, methodology, supervision. A.L. Investigation, writing – review & editing. L.R.P. Investigation, writing – original draft. V.R.A. Methodology. C.G.G. Conceptualization, formal analysis, funding acquisition, supervision, writing – review & editing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study was revised and sanctioned by the Internal Committee for the use and care of Animals for experimental research (SICUAE) of the School of Veterinary Medicine, UNAM.

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Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000511.v2.3

James Redfern; Manchester Metropolitan University, UNITED KINGDOM

Date report received: 17 September 2023
Recommendation: Accept

Comments: The work presented is clear and the arguments well formed. Both reviewer comments have been satisfied, and as such as are happy to accept this manuscript for publication.

SciScore report

https://doi.org/10.1099/acmi.0.000511.v2.1

iThenticate report

https://doi.org/10.1099/acmi.0.000511.v2.2

Author response to reviewers to Version 1

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Dr. James Redfern

Editor, *Access Microbiology*

Microbiology Society

Dear Dr. Redfern,

We appreciate the constructive review to our manuscript (ACMI-D-22-00169). We have revised the suggestions made by the referees and made the changes accordingly.

We edited the document to reduce repetition of concepts in the introduction and discussion. We have included a couple of additional references in the introduction to further strengthen the argument on the economic importance of these diseases. Likewise, we have inserted a new paragraph in the introduction to describe the XMap multiplex technology that was used to develop the pentaplex assay, and included a paragraph in the discussion section where we present and contrast work that preceded this paper, as suggested.

Referee 1 mentions a draft document with a couple noted observations that we could not address as we did not receive the file.

There were a few observations on format where we did not agree with the suggested changes (acronyms at the start of the sentence, and the use of further or therefore), and have indicated our reasoning in the reply.

The answers to the reviewers' queries are listed below, and both the manuscript with tracked changes and the new version of the article are enclosed.

We feel that the reviewer comments have improved the manuscript and hope that you will now find it suitable for publication.

Thanking you in advance for your attention.

Yours sincerely,

Carlos G. Gutierrez

Response to reviewers' comments:

Reviewer 1 Comments to Author:

1. Methodological rigour, reproducibility and availability of underlying data

This paper was designed and conducted to test a pentaplex test's diagnostic acuity to detect antibodies simultaneously.

BRSV, PI3V, BoHV-1, BVDV and EBLV in bovine serum.

It is my judgment, first of all, that it is very well written and presented, with a lot of coherence and a remarkable structure. Secondly, I think it is a very well-designed and executed work with high rigor in the laboratory, using appropriate methods. I had doubts about the sample size, as it is not specified why the number of samples used is a desirable detail to have a better idea of the power of the results. Fortunately, the results were outstanding, which helped the authors not notice this absence. The statistical tests, moreover, are appropriate for this type of study.

Unfortunately, the absence of animals entirely negative for the five diseases did not allow us to see the actual performance of this diagnostic test in serum from adult cattle naturally exposed to multiple infectious agents that could cause cross-reactions and possible false positives; however, the use of fetal bovine serum is the right thing to do when utterly seronegative adult cattle are not available.

We agree with the reviewers observation, the estimation of the sample size needed for validation was based on the recommendations published by the OIE (2018), with an expected 95% diagnostic Se, and a 5% error. We have inserted this specification in the paper.

2. Presentation of results

The form in which the results are presented is appropriate for the most part. A couple of details are noted as observations in the draft document (attached). The tables and figures are well constructed, and the titles are appropriate.

3. How the style and organization of the paper communicates and represents key findings

As I noted, the article's structure is adequate because it has absolute coherence, from the objective to the conclusion, including the methodology, results, and discussion. The authors do not get lost in the search for answers to their questions. The style is clear and facilitates the fluent reading of the work, leaving space to look carefully at the details, especially the methodological ones.

4. Literature analysis or discussion

The literature review used for the different sections of the paper is adequate. Perhaps, I suggest including a couple more references on the economic effects of diseases, trying to give more weight to the relevance of the study and its results.

We have included a couple of additional references in the introduction to further strengthen the argument as suggested.

The introduction presents the necessary contents to learn about the subject of the study. The discussion has a correct thread, in addition to analyzing and contextualizing the results.

Perhaps the only thing I would add is a paragraph on the possible limitations in the development of the study, which in some way affect its scope.

We added a paragraph that addresses comments of both reviewers regarding assay characteristics and discusses findings by other groups.

5. Any other relevant comments

This type of assay will help cattle producers in their goal to control or eradicate these diseases, even to reduce their economic impact.

Please rate the manuscript for methodological rigour

Reviewer 2: Poor

Please rate the quality of the presentation and structure of the manuscript

Reviewer 2: Good

To what extent are the conclusions supported by the data?

Reviewer 2: Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

Reviewer 2: No:

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Reviewer 2: Yes:

Reviewer 2 Comments to Author: This manuscript describes the development and validation of a Luminex assay for the detection of five endemic diseases of cattle. Whilst it is generally well written, there are a number of gaps in the manuscript that need to be revised before being considered acceptable for publication which include:

1. See list for minor amendments.

The list of minor amendments has been attended.

2. The authors need to describe the Luminex assay in more detail e.g. intro and methodology.

3. Consider condensing the introduction on viral diseases and repetitiveness throughout the document - the focus should be about the technology, test developed and application.

*We have inserted a new paragraph in the introduction to describe the XMap multiplex technology that was used to develop the pentaplex assay. Details on the workings of the XMap technology are included in lines 194 to 204*of the new version. *Details specific to the immunoassays described herein are included on lines 209 to 226.*

We edited the manuscript to eliminate concepts that were repetitive.

4. There are no acknowledgments of other assays developed in this area or compared/contrast or benefits of this platform compared to other multiplex platforms.

We have added a paragraph in the discussion section where we present and contrast work that preceded this paper, as suggested.

5. Present the order of antigen results in the same order each time, currently different order in each figure/table.

The order of the antigens was standardised in the figures and tables.

6. There are a number of inconsistencies in the data presented in tables, figures and/or text which do not always correlate e.g. Figure 1 BVDV monoplex AUC 0.981 whereas table 3 AUC 0.885. Therefore, this needs to be amended or clarified before being considered suitable for publishing.

The results presented on Figure 1 and Table 3 differ because they arise from different assays, performed at different times with different samples. Further, even when monoplex and multiplex assays where ran with the same samples at the same time (Table 3), not significant (numerical) difference in the AUC can be observed.

Font - different fonts have been used throughout the manuscript, please change to single font recommended by journal.

The font was changed to Arial 11 throughout the manuscript.

Title - To aid the reader please consider amending the word undiagnosed as misleading as many countries have eradicated or have eradication programmes in place for many of the diseases that you are describing.

The title was amended replacing the word "underdiagnosed" with term "common viral"

Line 31-33 Please rephrase sentence and add in punctuation.

The sentence has been revised.

Line 34 - lower case h for Herpes

Line 35 - lower case b & l

The above editions have been made.

Line 35 - the use of 'underdiagnosed' is too strong and as described above many countries have eradicate or have eradication/ surveillance programmes in place for these diseases.

We qualified the term underdiagnosed to make it less strong.

Line 38 - omit the word 'serum'

Line 39 - Add in the 'in serum' after EBLV

Line 41 - lysates may be more appropriate

Line 42 - change sera to serum

Line 43 - change towards to 'for'

Line 44 - consider including Luminex in this sentence

Line 46 - ensure the word 'diagnostic' sensitivity and specificity is used throughout the document

Line 49 - change diagnose to diagnostic

Line 51 - omit serum

Line 52 - add 'in serum'

All the above editions have been made.

Line 54 - add in the potential application of this assay e.g. how it would be used or its benefits.

We have included a potential use of the assay in the sentence.

Line 67 - lowercase h for herpes

Line 68 - lowercase b & l

Line 69 - add in 'often' underdiagnosed

Line 70-71 - merge sentences to help with flow

All the above editions have been made.

Line 75 - PI3V.

As it is the start of the sentence, we left the full name and added the acronym for clarity.

Line 77 - in cattle

Line 79 - lowercase h

84 - add in comma

Line 91 - lowercase EBL

All the above editions have been made.

Line 101 - change further to therefore.

Further is the appropriate junction since this sentence provides additional information to that presented before and is not the consequence of what was previously stated.

Line 103 - state impact and why.

The sentence has been revised to include possible impact.

Line 107 - would also confirm vaccination success.

Acknowledgement of this statement was included at the end of the paragraph.

Line 111- or treatment regimens?

The viable options for control of viral diseases in cattle are segregation and immunisation. Available treatments address only symptoms and thus are not sustainable. However, we recognise that the serological surveillance could provide feedback on the success of immunisation programmes.

Line 114 - omit causing endemic viral diseases

Omitted as suggested.

Line 115 - where? Or do you mean in general?

We specified the use of the assay for cattle production units.

Line 119 - NADL strain?

This refers to the identification of the viral strain identification (https://www.lgcstandards.com/ES/es/BVDV-Strain-NADL/p/ATCC-VR-1422). NADL, stands forNational Animal Disease Laboratory.

Line 120 - change to BHV1

Line 122 - MDBK change to 'Madin-Darby bovine kidney' cells

Line 123 - MEM change to 'Minimum essential media eagle'

All the above editions have been made.

Line 125 - Was trypsin used to remove cells from flasks?

No trypsin was used in the assay. Cells were subjected totwo freeze-thaw cycles and later centrifuged at 4,000g for 10 min to remove cellular debris.

Line 131 - there is not much detail on how the monoplex assays were developed or do you have a reference that you can use?

The conditions for the Luminex monoplex assays (antigen concentration, sample and secondary antibody dilutions) were established in individual ELISA assays using the same viral antigens. These methods are mentioned on lines 186 to 193 of the new manuscript.

Line 133 – BVD

We did not find any guidelines as to whether acronyms could be used at the start of a sentence. Usually this is not the case, but we will follow the recommendation of the editorial office.

Line 180 - change reaction to 'interaction'

Changed as suggested.

Line 185 - The papers you have reference do not refer to the chemistry or development of Luminex they are just applications of Luminex (like your study). Identify suitable references to illustrate the Luminex chemistry etc.

Thanks for the observation. We have substituted the references.

Line 192 - remove gap after uL plus capital L for litres throughout manuscript.

We have capitalized the L throughout the document as suggested.

Line 195-197 - does not state conditions e.g. temp, shaking

These conditions are stated on lines 220-221.

Line 199 - No details of blocking buffer composition? Did you sonicate the beads to mix?

Details of the buffer composition were added to the text. Beads are not sonicated at this stage as sonication could uncouple the antigens.

Line 202 - add conditions above

As assay conditions were the same throughout, the inclusion of temperature, time, etc. on every step can result cumbersome and repetitive. We believe that for the sake of readability, the assay conditions are best placed at the end of the paragraph, where they stand.

Line 214 - add value and full stop.

A minimum positive likelihood ratio of 10 was set since it indicates that a qualitatively low to intermediate probability of disease in a pre-test clinical assessment would translate into an intermediate to high post-test probability of disease(Hayden and Brown, 1999).

As different sensitivities and specificities are considered in ROC analyses a different LR will be obtained for every pair of Se-Sp. A likelihood ratio value of 10 was set as an additional minimum desirable criterion for all the assays. The actual LR+ values obtained in thepentaplex assay are displayed in Table 2. The reference quoted above was included in the manuscript.

Line 223 - I do not understand what is meant by 'Selectivity was evaluated'? Please rephrase.

Selectivity is the "ability to discriminate between the analyte and interfering components" (Vessman et al., 2001). In this case, it was important to determine that antibodies recognise their target antigens, and this was achieved by adding soluble antigens (free virus) to the reaction.

The sentence was amended for clarity as suggested.

Line 224 - Neat rather than 0?

amended

Line 228 - You have referenced OIE for your own method that you have developed as part of this study?

By adding the OIE reference, we meant that OIE guidelines were followed. However, as this is unclear, we deleted the reference.

Line 237 - 1:2? You mentioned neat earlier? Please align.

The previous section dilution refers to a different assay from that presented in section pertaining line 237. The later section refers to the range of dilutions tested for the serum samples, whereas the former section (that includes the "neat" dilution indicated by the referee), refers to the addition of viral antigens in the inhibition assay. Since assays are different, there is no misalignment on the methods.

Line 248 - change further to 'therefore'

The sentence preceding ´further´ talks about specific inhibition of binding by soluble antigens. In contrast, the second sentence refers to evidence of nonspecific non-specificity of the baculovirus and this does nor derive from the results presented in the previous sentence. Hence the use of ´therefore´ is not adequate.

Line 394 - change to therefore

As before the evidence presented in the second sentence cannot be inferred from the prior sentence. Therefore, the use of "therefore" is not appropriate.

Line 467 - EBL should be abbreviated

In an effort to reduce repetition of concepts in the introduction and discussion, the paragraph was edited, and the sentence deleted.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000511.v1.5

James Redfern; Manchester Metropolitan University, UNITED KINGDOM

Date report received: 01 March 2023
Recommendation: Major Revision

Comments: To ensure the article meets the Open Data Policy of Access Microbiology, please ensure all data that went into the manuscript is uploaded and made available. Please deposit the data underlying the work in the Society's data repository Figshare account here: https://microbiology.figshare.com/submit. Please also cite this data in the Data Summary of the main manuscript and list it as a unique reference in the References section. When you resubmit your article, the Editorial staff will post this data publicly on Figshare and add the DOI to the Data Summary section where you have cited it. This data will be viewable on the Figshare website with a link to the preprint and vice versa, allowing for greater discovery of your work, and the unique DOI of the data means it can be cited independently.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000511.v1.3

Anonymous.

Date report received: 28 February 2023
Recommendation: Major Revision

Comments: This manuscript describes the development and validation of a Luminex assay for the detection of five endemic diseases of cattle. Whilst it is generally well written, there are a number of gaps in the manuscript that need to be revised before being considered acceptable for publication which include: 1. See list for minor amendments. 2. The authors need to describe the Luminex assay in more detail e.g. intro and methodology. 3. Consider condensing the introduction on viral diseases and repetitiveness throughout the document - the focus should be about the technology, test developed and application. 4. There are no acknowledgments of other assays developed in this area or compared/contrast or benefits of this platform compared to other multiplex platforms. 5. Present the order of antigen results in the same order each time, currently different order in each figure/table. 6. There are a number of inconsistencies in the data presented in tables, figures and/or text which do not always correlate e.g. Figure 1 BVDV monoplex AUC 0.981 whereas table 3 AUC 0.885. Therefore, this needs to be amended or clarified before being considered suitable for publishing. Font - different fonts have been used throughout the manuscript, please change to single font recommended by journal. Title - To aid the reader please consider amending the word undiagnosed as misleading as many countries have eradicated or have eradication programmes in place for many of the diseases that you are describing. Line 31-33 Please rephrase sentence and add in punctuation. Line 34 - lower case h for Herpes Line 35 - lower case b & l Line 35 - the use of 'underdiagnosed' is too strong and as described above many countries have eradicate or have eradication/surveillance

programmes in place for these diseases. Line 38 - omit the word 'serum' Line 39 - Add in the 'in serum' after EBLV Line 41 - lysates may be more appropriate Line 42 - change sera to serum Line 43 - change towards to 'for' Line 44 - consider including Luminex in this sentence Line 46 - ensure the word 'diagnostic' sensitivity and specificity is used throughout the document Line 49 - change diagnose to diagnostic Line 51 - omit serum Line 52 - add 'in serum' Line 54 - add in the potential application of this assay e.g. how it would be used or its benefits Line 67 - lowercase h for herpes Line 68 - lowercase b & l Line 69 - add in 'often' underdiagnosed Line 70-71 - merge sentences to help with flow Line 75 - PI3V Line 77 - in cattle Line 79 - lowercase h 84 - add in comma Line 91 - lowercase EBL Line 101 - change further to therefore Line 103 - state impact and why Line 107 - would also confirm vaccination success Line 111- or treatment regimens? Line 114 - omit causing endemic viral diseases Line 115 - where? Or do you mean in general? Line 119 - NADL strain? Line 120 - change to BHV1 Line 122 - MDBK change to 'Madin-Darby bovine kidney' cells Line 123 - MEM change to 'Minimum essential media eagle' Line 125 - Was trypsin used to remove cells from flasks? Line 131 - there is not much detail on how the monoplex assays were developed or do you have a reference that you can use? Line 133 - BVD Line 180 - change reaction to 'interaction' Line 185 - The papers you have reference do not refer to the chemistry or development of Luminex they are just applications of Luminex (like your study). Identify suitable references to illustrate the Luminex chemistry etc. Line 192 - remove gap after uL plus capital L for litres throughout manuscript Line 195-197 - does not state conditions e.g. temp, shaking Line 199 - No details of blocking buffer composition? Did you sonicate the beads to mix? Line 202 - add conditions above Line 214 - add value and full stop Line 223 - I do not understand what is meant by 'Selectivity was evaluated'? Please rephrase Line 224 - Neat rather than 0? Line 228 - You have referenced OIE for your own method that you have developed as part of this study? Line 237 - 1:2? You mentioned neat earlier? Please align Line 248 - change further to 'therefore' Line 394 - change to therefore Line 467 - EBL should be abbreviated

Please rate the manuscript for methodological rigour
Poor

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

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Recommendation: Accept

Comments: 1. Methodological rigour, reproducibility and availability of underlying data This paper was designed and conducted to test a pentaplex test's diagnostic acuity to detect antibodies simultaneously. BRSV, PI3V, BoHV-1, BVDV and EBLV in bovine serum. It is my judgment, first of all, that it is very well written and presented, with a lot of coherence and a remarkable structure. Secondly, I think it is a very well-designed and executed work with high rigor in the laboratory, using appropriate methods. I had doubts about the sample size, as it is not specified why the number of samples used is a desirable detail to have a better idea of the power of the results. Fortunately, the results were outstanding, which helped the authors not notice this absence. The statistical tests, moreover, are appropriate for this type of study. Unfortunately, the absence of animals entirely negative for the five diseases did not allow us to see the actual performance of this diagnostic test in serum from adult cattle naturally exposed to multiple

infectious agents that could cause cross-reactions and possible false positives; however, the use of fetal bovine serum is the right thing to do when utterly seronegative adult cattle are not available. 2. Presentation of results The form in which the results are presented is appropriate for the most part. A couple of details are noted as observations in the draft document (attached). The tables and figures are well constructed, and the titles are appropriate. 3. How the style and organization of the paper communicates and represents key findings As I noted, the article's structure is adequate because it has absolute coherence, from the objective to the conclusion, including the methodology, results, and discussion. The authors do not get lost in the search for answers to their questions. The style is clear and facilitates the fluent reading of the work, leaving space to look carefully at the details, especially the methodological ones. 4. Literature analysis or discussion The literature review used for the different sections of the paper is adequate. Perhaps, I suggest including a couple more references on the economic effects of diseases, trying to give more weight to the relevance of the study and its results. The introduction presents the necessary contents to learn about the subject of the study. The discussion has a correct thread, in addition to analyzing and contextualizing the results. Perhaps the only thing I would add is a paragraph on the possible limitations in the development of the study, which in some way affect its scope. 5. Any other relevant comments This type of assay will help cattle producers in their goal to control or eradicate these diseases, even to reduce their economic impact.

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Very good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000511.v1.1

iThenticate report

https://doi.org/10.1099/acmi.0.000511.v1.2