Development of a recombinant ELISA for ovine herpesvirus 2, suitable for use in sheep

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Development of a recombinant ELISA for ovine herpesvirus 2, suitable for use in sheep

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An indirect ELISA, based on this affinity-purified Ov65delB, was optimised for use with sheep and cattle samples and cut-off values were established based on known negative serum samples. Analysis of groups of samples that were either presumed infected (UK sheep) or tested OvHV-2 positive or negative by PCR (cattle MCF diagnostic samples) showed that the assay had 95 % sensitivity and 96 % specificity for sheep serum; and 80 % sensitivity and 95 % specificity for cattle serum. The lower sensitivity with cattle samples appeared to be due to a lack of serological response in some MCF-affected cattle.

This recombinant antigen therefore shows promise as the basis of an inexpensive, simple and reliable test that can be used to detect OvHV-2-specific antibody responses in both MCF-affected animals and in OvHV-2 reservoir hosts.

1. Introduction

Malignant catarhral fever (MCF) is a fatal systemic disease of cattle and other ungulates caused by infection with a group of related viruses now classified as: family Herpesviridae; sub-family Gammaherpesvirinae; genus Macavirus (Davison et al., 2009). The best studied of these viruses are ovine herpesvirus 2 (OvHV-2), which naturally infects sheep worldwide and causes sheep-associated (SA-) MCF, and alcelaphine herpesvirus 1 (AIHV-1), which naturally infects wildebeest and is responsible for wildebeest-associated (WA-) MCF (Russell et al., 2009). MCF viruses infect their natural hosts efficiently and without obvious clinical disease, producing lifelong and generally latent infection. Contact between infected reservoir species and MCF-susceptible host species can lead to infection and subsequent clinical disease that is generally fatal. MCF is characterised by the expansion of virus-infected lymphoid cells, both within the circulation and in most organs, leading to death following infiltration of multiple tissues with infected lymphoid cells (Dewals et al., 2008; Dewals and Vanderplasschen, 2011). This virus expansion in clinical MCF cases makes the detection of infection by PCR of DNA from blood or tissue samples a reliable diagnostic method (Baxter et al., 1997; Bremer et al., 2005; Dungu et al., 2002; Flach et al., 2002; Hussy et al., 2001), and real-time fluorogenic PCR assays for both AIHV-1 and OvHV-2 have been described (Hussy et al., 2001; Russell et al., 2012; Traul et al., 2005). However these approaches may be less reliable for assessing the level of infection in reservoir species, where low virus loads due to latency may make infection difficult to detect in blood cells (Gaudy et al., 2012). In such cases serological tests such as indirect or competitive ELISA (Fraser et al., 2006; Li et al., 2001, 1996; Davison et al., 2009; Russell et al., 2012; Traul et al., 2005).
may be valuable because the antibody response in animals latently infected with MCF viruses may be detectable long after any detectable viraemia has subsided. Serological tests for MCF virus infection may also have broader specificity than PCR and at least one widely cross-reactive MCF virus epitope has been identified by a monoclonal antibody (Li et al., 1995). Indeed, all serological tests for MCF virus infection developed to date used AIHV-1 as the source of antigen, mainly because this virus is readily propagated in culture (Li et al., 2011).

The competitive inhibition (CI)-ELISA for MCF-virus specific antibodies was first reported in 1994 (Li et al., 2001, 1994), based on a monoclonal antibody (15A) that recognised the major glycoprotein complex of the virus. This assay has been used in a range of ruminant species and possession of the 15A epitope appears to be a conserved feature of MCF-causing Macacviruses (Frolich et al., 1998; Li et al., 1996; Probst et al., 2013; Vikoren et al., 2006; Zarnke et al., 2005). This assay is, however, dependent on the 15A mAb and antigen and is available only as a diagnostic assay performed by the Animal Disease Diagnostic Laboratory of Washington State University (WSU-WADDL). No commercial ELISA for MCF is currently available.

Indirect ELISA methods for MCF have been published (Fraser et al., 2006; Russell et al., 2012) but these were designed for use in diagnosis of MCF in cattle and in analysis of MCF vaccine antibody responses. Testing of these assays showed that they could be used successfully in some species (e.g. cattle, deer, wildebeest, bison, buffalo) but did not work in sheep or goats due to the high background in negative samples (GC Russell, unpublished data). This may be related to the crude nature of the antigens used, which were detergent extracts of cultured, attenuated AIHV-1 strains. A recombinant antigen indirect ELISA would be a benefit to the MCF community and to diagnostic laboratories. Ideally such a test should be suitable for use in the reservoir host species as well as MCF-affected animals.

Proteomic analysis of an AIHV-1 ELISA antigen preparation (Bartley et al., 2014) suggested that virus glycoproteins, including gB and gL, and capsid proteins, such as orf65 and orf17.5, could be useful candidates for diagnostic development. Recombinant expression of a range of AIHV-1 and OvHV-2 proteins showed that gB, orf65 and orf17.5 appeared to be antigens recognised by MCF-positive cattle and wildebeest sera (Bartley et al., 2014; Dry et al., 2016). HA-tagged AIHV-1 gB, expressed in mammalian cells, appeared to be suitable for use in ELISA but the recombinant protein lacked the furin cleavage found in gB from AIHV-1 virions and proved difficult to express and purify in quantity (Dry et al., 2016). Similarly, the OvHV-2 Ov8 glycoprotein, expressed in mammalian cells, has been evaluated as a potential diagnostic target for analysis of OvHV-2 infection and showed good agreement with results of the CI-ELISA (Alhajri et al., 2018).

Antigenic virus capsid proteins could be good candidates for recombinant expression due to their lack of complex post-translational modification and consequent potential for high level expression in bacterial systems. However, the low solubility of some capsid proteins, especially when over-expressed in bacteria, remains an issue (Bartley et al., 2014). Of the MCF virus capsid proteins, the orf65 polypeptides from OvHV-2 and AIHV-1 were clearly antigenic but differed in their reactivity, with autologous reactions (such as OvHV-2 sera versus OvHV-2 antigen) being visibly stronger than either heterologous reaction in western blots (Bartley et al., 2014). The same analysis suggested that reactivity to the only other antigenic capsid protein, orf17.5, may be a consequence of vaccination with the culture-adapted attenuated vaccine virus.

The characterised orf65 genes of MCF viruses differ in both size and sequence, with the OvHV-2 polypeptide having 211 residues compared with 252 residues in AIHV-1 orf65, and 266 residues in AIHV-2 orf65, with the bulk of sequence identity found within the first 80 residues of the aligned polypeptide sequences. This region corresponds well with the orf65 domain defined as interacting with other capsid proteins in the gammaherpesvirus Kaposi’s Sarcoma herpesvirus (KSHV; (Dai et al., 2015)) and as necessary and sufficient for capsid assembly in Epstein-Barr Virus (EBV; (Henson et al., 2009)). The remainder of the polypeptide appears to be variable in length, structure and composition between gammaherpesviruses (Dai et al., 2015). Notably, the orf65 polypeptide has also been identified as a diagnostic antigen in KSHV (Spira et al., 2000), with an immunoblot method using the C-terminal 88 residues of the protein expressed in bacteria, while a peptide ELISA was developed using a C-terminal 14-residue peptide (Pau et al., 1998).

In this paper we describe the development of a recombinant ELISA that can detect antibody responses to OvHV-2 infection in both reservoir species and MCF-affected animals with high sensitivity and specificity.

2. Materials and methods

2.1. Cells and media

Escherichia coli JM109 cells (Promega) were used for standard cloning procedures, while BL21/DE3 or Rosetta2/DE3 cells (Merck-Millipore) were used for bacterial expression. Bacterial cultures were grown in LB medium (Miller, 1972) with specific additions as noted below for selection or expression.

A negative coating antigen, to control for background binding and for the presence of residual E. coli proteins in purified Ov65 antigen preparations, was produced from Rosetta2/DE3 cells expressing the non-antigenic OvHV-2 capsid protein orf26 (Ov26) from pCDF-Duet-Ov26 as described previously (Bartley et al., 2014).

2.2. Gene cloning for expression

An E. coli codon-optimised OvHV-2 orf65 sequence was designed, manually edited to adjust GC content and remove homopolymeric tracts and then (i) restriction sites and epitope tag sequences were included at the N- and C-termini (NcoI-His-tag and Strep-tagII-AvrII, respectively) to facilitate cloning, detection and purification; and (ii) internal restriction sites were placed to allow in-frame deletion of segments of the Ov65 coding region. These sites were placed at the junctions of predicted secondary structural elements in the Ov65 polypeptide sequence: BamHI at nt 38 and 245; EcoRI at 251 and 470; and HindIII at 478 and 682 of the 725 bp synthetic gene sequence.

The 725 bp sequence (OHRorf65-OPT-segmented: accession LR899421) was produced by gene synthesis (Eurofins-MWG) and cloned into the bacterial expression vector pCOLA-Duet-1 (Novagen, Merck-Millipore) between its NcoI and AvrII sites. Digestion of plasmid DNA by each of BamHI, EcoRI and HindIII, followed by religation of the plasmids, removed about 210 nt of the orf65 protein coding region at the start, middle or end of the gene, generating pDuet vectors encoding Ov65delB, Ov65delE and Ov65delH, respectively. Further deletions were made by digestion of these plasmids, producing constructs that expressed the N-terminal (Ov65delEH), central (Ov65delBH) and C-terminal (Ov65delBE) domains of the protein. After confirmation of the plasmid structures by DNA sequencing, the orf65 gene variants were transferred to the recommended expression host.

2.3. Protein expression and purification

Expression of the Ov65 p-Duet constructs, all of which retained their respective N- and C-terminal epitope tags, was performed in E. coli BL21/DE3, using small-scale cultures (50 mL LB containing 50 µg/mL kanamycin and 20 % glucose), grown with shaking at 37 °C and induced to express by addition of IPTG to 1 mM when the cells reached mid-to-late log phase (OD600 of 0.4 to 0.6). After 4 h expression phase under the same growth conditions, the cells were harvested and stored as frozen cell pellets.

Gels of crude extracts containing recombinant proteins were produced by resuspending pelleted cells in 1/10 of their original culture volume of phosphate buffered saline (PBS) and then mixing equal
volumes of cell suspension and 2× reducing NuPAGE™ LDS Sample Buffer (Invitrogen). The samples were heated at 100 °C for five minutes and 10 μL aliquots were run on NuPAGE™ 4–12 % Bis-Tris SDS-PAGE gels at 200 V for 50 min.

For solubility analysis, bacterial cells pellets were resuspended in 1/10 original culture volume of 0.8 % CHAPS (3-(3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate hydrate, Sigma) in PBS and mixed for 1 h at room temperature on a rotating platform. The insoluble material was removed by centrifugation for 10 min at 7400× g. The CHAPS-soluble material was harvested and aliquots for PAGE analysis. The protein samples were mixed with the supplied 2× reducing NuPAGE™ LDS sample buffer. Samples containing equivalent original culture volumes of soluble and insoluble material were run on NuPAGE SDS gels as above. Gels were stained using SimplyBlue Safe Stain or Imperial stain (Thermo Fisher) or were prepared for western blotting as described below.

Initial purification of E. coli lysates expressing Ov26 and Ov65 used denaturing metal-affinity batch chromatography. Briefly, pellets from 50 mL of induced bacterial culture were solubilised in 2.5 mL 8 M Urea in phosphate buffer, pH 8.0 (PB), mixed gently for 3 h then spun at 10,000 rpm (7400× g) for 10 min. The soluble fraction was removed and used to resuspend an equal volume of His-Select Nickel affinity gel (Sigma) that had been equilibrated with 8 M Urea in PB. After 15 min incubation at room temperature with mixing, the matrix was pelleted by centrifugation (2000× g for 15 min). The supernatant containing unbound protein was removed carefully and the affinity gel washed serially in 2.5 mL 8 M then 4 M then 2 M Urea in PB. After a final wash with PB, bound His-tagged protein was eluted with the same buffer containing imidazole. Initial step-gradient experiments showed that most of the bound antigen was released by 100 mM imidazole, so antigen elution from His-Select affinity gel batches was done with 5 aliquots of 1 mL of PB containing 100 mM imidazole. Fractions were checked for presence of the cloned antigen by ELISA and western blotting. Positive fractions were pooled and dialysed against PB. Protein content in pooled antigen fractions was assessed by BCA protein assay (Sigma), adjusted to approximately 10 μg/mL and stored at −20 °C in aliquots.

2.4. Western blotting

Proteins run on SDS-PAGE gels were transferred to nitrocellulose membranes at 90 mA for 1 h using a semi-dry electrophoretic transfer cell. Visible protein size markers (SeeBlue Plus2, Thermo) were included in each gel to aid in orientation and alignment of western blots. Membranes were blocked in 4 % non-fat dry milk in PBS in 1 h at room temperature with mixing, washed 3 times for 5 min each in PBS/0.5 M NaCl/0.5 % Tween80 (PNT), before incubating in primary antibody. For detection of His-tagged proteins, anti-His peroxidase antibody conjugate (1:2000) was used (A7058, Sigma), while Ov65 antigenicity was detected using pooled OvHV-2-positive serum. Primary antibody, diluted in PNT, was incubated with each blot for 1 h at room temperature following by washing as above. Cattle serum blots were then incubated with 1:1000 rabbit α-bovine IgG peroxidase secondary antibody conjugate (Sigma, A8917) for 1 h at room temperature with mixing. For detection, all blots were washed 3 x 10 min and then were incubated in SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Scientific). The chemiluminescent signal was promptly analysed using an ImageQuant LAS 4000 CCD camera imaging system.

2.5. Proteomic analysis

2.5.1. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS)

Purified protein samples were digested using an S-Trap micro column (Protifi), following the manufacturer’s instructions. Briefly, the samples were mixed with the supplied 2× SDS lysis buffer, cysteines were reduced with Tris (2-carboxyethyl) phosphine (TCEP) and capped with methyl methanethiosulfonate (MMTS). Proteins were acidified with 12 % Phosphoric acid and bound to the column in S-Trap binding buffer. The column was centrifuged (4000× g for 30 s) and then washed a further 3 times in binding buffer. Proteins were digested overnight at 37 °C on the column in 20 μL of 50 ng/μL Tryptsin (Pierce) in 50 mM triethylammonium bicarbonate (TEAB) buffer. Tryptic peptides were eluted by centrifugation (4000× g for 30 s) in firstly, 40 μL 50 mM TEAB buffer followed by 40 μL 0.2 % formic acid (FA), and finally 40 μL 50 % acetonitrile (ACN) in water and 0.2 % FA. Elutions were pooled and dried under vacuum. Dried peptides were reconstituted in 30 μL of 0.1 % FA.

Liquid chromatography (LC) was then performed on 4 μL from each digest, applied by direct injection to a monolithic reverse phase column (5 cm × 200 μm; Dionex), using an Ultimate 3000 nano-flow HPLC system (Dionex-LC Packings) maintained at 50 °C. Bound peptides were eluted by the application of a 15 min linear gradient of 8–45 % ACN in 0.1 % FA and monitored by UV absorbance in a 3 nL UV detector flow cell. LC was interfaced with a 3-D high-capacity ion trap mass spectrometer (Amazon, Bruker Daltonics) via a low-volume (<50 μL/min) stainless steel nebulizer (Agilent) and electrospray ionization. Digests were analysed for protein content by LC-ESI-MS/MS in ultrascan mode (26,000 amu/s). MS/MS analysis was initiated on a contact closure signal triggered by Chromeleon chromatography software (Dionex).

2.5.2. Proteomic analysis of MS/MS data

Deconvoluted MS/MS data in mgf (Mascot Generic Format) format were imported into ProteinScapeTM V2.1 (Bruker) proteomics data analysis software for searching against a custom database of the Ov65 and Ov26 tagged sequences combined with all of the E. coli entries in NCBI (November 2020) utilising the MascotTM V2.5.1 (Matrix Science) search algorithm. Mascot search parameters were set in accordance with published guidelines (Taylor and Goodlett, 2005) and to this end, fixed and variable modifications selected were carbamidomethyl (C), and oxidation (M), respectively, while peptide (MS) and fragmentation (MS/MS) tolerance values were set at 1.5 and 0.5 Da, respectively. Individual protein candidates identified were inspected manually and considered significant only if: (a) a minimum of two peptides were matched for each protein; and (b) each peptide contained an unbroken “b” or “y” ion series comprising a minimum of four amino acid residues.

2.6. ELISA

For analysis of antibody binding to OvHV-2 antigens, purified fractions of each antigen (at about 10 μg/mL) were diluted in 0.1 M Carbonate buffer pH 9.6. Pairs of adjacent rows of 96-well microtitre plates (Microlon-High Binding, Greiner Bio-one) were coated with 50 μL of positive and negative antigen respectively and left overnight at 4 °C to adsorb. Wells containing bound antigen were washed with PBS/0.05 % Tween20 (PBS-Tween), before blocking with 4 % Infasoy (Soya-based infant formula)/PBS or 4 % filtered non-fat dried milk/PBS for 1 h. The plates were then washed as above and serum or plasma samples, diluted 1:200 using 2 % diluent buffer (either 2 % Infasoy in PBS-Tween or 2 % filtered non-fat dried milk in PBS-Tween), were applied to the positive and negative antigen wells in duplicate. After 1.5 h, the plates were washed again and bound antibodies were detected using rabbit α bovine IgG-HRP conjugate (AS295, Sigma) for bovine samples or rabbit α sheep HRP conjugate (P0163, Dako) for sheep samples, diluted 1:1000 in PBS-Tween, incubated for 1 h. The plates were then washed, and Sureblue TMB-peroxidase substrate (KPL) applied for five minutes. The reaction was stopped by the addition of 0.1 M HCl and the plates were read at 450 nm in a plate reader (Dynex MRX II).

ELISA values for each serum sample were calculated as the difference between the average absorbance values at 450 nm (OD450) for the positive antigen (e.g. Ov65delB) minus the average OD values for negative antigen (Ov26).
2.7. Sample sources

Sheep sera used included (i) a set of 22 samples from adult sheep in a single OvHV-2 negative flock generated and kept at University of Zurich (Muller-Doblies et al., 2001); and (ii) a set of 239 sheep samples from animals over three months of age, derived from ongoing research projects at Moredun Research Institute. Cattle samples were all from animals over three months of age and included (i) a set of 80 samples from a herd with no history of MCF (Fraser et al., 2006); and (ii) blood plasma from 186 diagnostic submissions to the Moredun Research Institute Virus Surveillance Unit (MRI VSU) for MCF PCR testing, dating from 2009 and 2010. Consent for use in anonymous surveillance or test validation was included in the VSU sample submission.

All samples analysed as part of this work were taken either by veterinary surgeons and submitted as diagnostic samples for MCF diagnostic testing, or by authorised and trained personnel working in compliance with the UK ‘Animals (Scientific Procedures) Act 1986’. In each case the anonymous re-use of such samples for diagnostic test development was appropriate and in line with the principles of 3Rs in research.

2.8. Analysis of data

Initial analysis of ELISA data, including calculation of sample to positive (S/P) values, means and sample standard deviation (SD), and the plotting of all graphs and derivation of ROC curves, was done in Microsoft Excel. Further ROC curve analysis was done using the EPIT-OOLS suite of statistical calculators (https://epitools.ausvet.com.au/roccurves). This calculated summary statistics for each group of animals, plus sensitivity and specificity of each assay (with 95 % confidence limits) for a range of cut-offs, allowing a cut-off for each assay to be selected, which optimised both sensitivity and specificity.

The Welch Two Sample T-test was done within RStudio (Version 1.3.959; https://rstudio.com/).

3. Results

3.1. Expression and solubility of Ov65 derivatives

The E. coli codon-optimised version of the Ov65 gene was cloned into the pCOLA-Duet-1 vector between Ncol and AvrII sites, replacing both MCS regions and their associated epitope tags. This plasmid – pCOLA-Duet-Ov65 - was then subjected to serial deletions using BamHI, EcoRI and HindIII. The resulting plasmids were checked by restriction enzyme digestion and sequence analysis to confirm that the structures of all plasmid constructs were exactly as expected.

Previous studies (Bartley et al., 2014) showed that the Ov65 protein expressed in the pDuet system was largely insoluble under non-denaturing buffer conditions, therefore initial experiments were performed to determine whether a truncated version of Ov65 could be produced that was more soluble than the full-length protein but retained antigenicity. The influence of deletion of the N-terminal, central and C-terminal thirds of the Ov65 polypeptide in Ov65delB, Ov65delE and Ov65delH, respectively, were analysed from small-scale culture experiments. The soluble and insoluble induced cell lysates from pDuet-Ov65 and its deletion derivatives were analysed by western blotting with pooled OvHV-2-positive cattle sera (Fig. 1). This showed that the Ov65 protein could be detected specifically by such MCF-positive sera in both pellets and supernatants of bacterial cell lysates but most of the protein was found in the insoluble fraction. In contrast, the N-terminally truncated Ov65delB protein was more abundant in the soluble fraction than insoluble, while the centrally deleted Ov65delE was mainly insoluble. The C-terminal deletion variant Ov65delH was detected poorly and was only visible as a faint band in the soluble fraction.

Further deletion to produce plasmids expressing the C-terminal, N-terminal and central thirds of Ov65 - Ov65delBE, Ov65delEH and Ov65delHB, respectively - showed poor antigenicity of the smaller fragments compared with Ov65delB and Ov65delE (data not shown), confirming that the Ov65delB deletion gave the best combination of antigenicity and solubility. The construct expressing this truncated gene (pCOLA-Duet-Ov65delB) was therefore used for expression and purification of the Ov65delB polypeptide using metal affinity chromatography (MAC) under non-denaturing conditions. The MAC-enriched Ov65delB and Ov26 extracts were analysed by SDS-PAGE and mass spectrometry to confirm the identities of proteins present before use in ELISA optimisation and testing.

Gel electrophoresis and staining of the MAC-enriched Ov65delB and Ov26 antigen fractions (Fig. 2) showed multiple visible bands, of which only one band in each fraction was detected by western blotting with anti-His peroxidase antibody conjugate. For Ov65delB, a band could be identified on the stained gel in a position corresponding to the His-tagged band (Fig. 2) and this was excised for analysis by mass spectroscopy to confirm the identities of proteins present before use in ELISA optimisation and testing.

Fig. 1. Chemiluminescent Western blot image of Ov65 and deletion derivatives, expressed in E. coli and assessed for solubility and antigenicity by western blotting with pooled OvHV-2 specific serum from cattle suffering from clinical MCF. Lanes are marked as follows: 65, intact epitope-tagged Ov65; ΔB, Ov65delB deletion derivative, lacking the N-terminal third of the Ov65 polypeptide; ΔE, Ov65delE deletion derivative, lacking the central third of the Ov65 polypeptide; ΔH, Ov65delH deletion derivative, lacking the C-terminal third of the Ov65 polypeptide; C, control tagged Ov65 antigen purified by denaturing His-affinity chromatography. The numbers on the left of the blot indicate the approximate positions and molecular weights (kDa) of SeeBlue Plus2 (Thermo) pre-stained protein standards.

Fig. 2. Stained protein gels and chemiluminescent western blot images of E. coli extracts enriched by metal affinity chromatography. Panel A: Ov65delB extract; Panel B: Ov26 extract. Lane labels are as follows: M, SeeBlue Plus2 pre-stained markers; P, concentrated, pooled positive fractions; 1, 2, 3, 4, fractions eluted from His-Select Nickel affinity gel.
spectrometry. This band showed good evidence of the presence of the expected recombinant protein, by both tryptic peptide mass finger-
printing and MS/MS fragmentation/sequence analysis. The unusual sequence composition of the Ov65delB polypeptide, however, made its
detection difficult due to the lack of trypsin-cleavable residues in the
central region and therefore only N- and C-terminal peptides could be
identified (data not shown). Bands excised from the relevant area of
Ov26 lanes did not yield clearly identifiable Ov26 peptides.

Concentrated MAC-enriched Ov65delB and Ov26 fractions were
subjected to solution trypsinolysis and LC-MS-MS analysis, searching
against a database containing all E. coli proteins and the predicted
Ov65delB and Ov26 tagged sequences. This analysis showed than in
addition to the overexpressed O65delB and Ov26 polypeptides, there
was evidence of a number of E. coli proteins that appeared to co-purify
with the recombinant polypeptides (Supplementary Table 1).

3.2. ELISA

A series of chequerboard assays were used initially to optimise the
dilutions of antigens and test sera and the composition of blocking buffer
used. For the Ov65delB positive antigen and the Ov26 negative antigen,
dilutions resulting in about 50 ng total protein per well were found to be
optimal. A 4 % non-fat-milk-based blocking buffer gave lowest back-
ground for cattle samples, while blocking buffer containing 4 % Infasyso
was best for sheep samples. For both species a 1:200 dilution of test
serum/plasma was considered optimal.

In a standard assay, each sample was tested in duplicate with each of
the Ov65delB positive antigen and Ov26 negative antigen and the mean
difference in OD450 calculated as the ELISA value. To control for vari-
ation in the assay between plates, the same positive control serum pool
sample was included in all plates. This was then used to calculate the
normalised sample to positive (S/P) ratio for each sample, which could
be compared between plates.

To establish a baseline for diagnostic testing, sets of negative samples
were identified for sheep and cattle sera for inclusion in the analysis.
These were a set of 22 adult sheep samples from an Ovh2V2-free flock
maintained by University of Zurich (Muller-Doblies et al., 2001) and a
set of 80 cattle samples from a herd with no previous record of MCF and
which had been used in previous MCF ELISA development (Fraser et al.,
2006).

Analysis of the sheep negative sample set gave S/P values ranging from
−0.017 to 0.352 (Supplementary data Table 2; mean 0.030, SD
0.073).

Serum samples gathered from 239 healthy sheep in the Morendun
Research Institute flock were then used to test the Ov65delB ELISA in
the Ovh2V2 reservoir species, where a high rate of prevalence was expected
because Ovh2V2 infection of sheep is endemic in the UK. Within the 239
samples, S/P values ranged from -0.17 to 1.8 (mean 0.486, SD 0.325).
The respective ELISA and S/P values are provided in Supplementary
Table 3.

The sheep data were then analysed by construction of a receiver
operating characteristic (ROC) curve, based on the assumptions that the
negative flock represented true negatives and that the MRI flock samples
represented true positives (Supplementary data Fig. 1). The area under
the ROC curve (AUC) for the sheep dataset was 0.955 (95 % confidence
interval (CI) = 0.917 – 0.993), suggesting that the test is highly accurate
for sheep samples. For this assay, plotting sensitivity and specificity
across a range of cut-off values showed that a diagnostic cut-off of 0.05
maximised sensitivity of 0.950 (95 % confidence limits (CL) =
0.914 – 0.971) and specificity of 0.955 (95 % CI = 0.782 – 0.992).

Testing of the cattle MCF-negative herd samples (Fraser et al., 2006)
using the Ov65delB antigen under optimised conditions gave S/P values
ranging from −0.033 to 0.092 (mean 0.008; SD 0.019) (Supplementary
data Table 4).

The optimised cattle assay was then used to test 176 cattle plasma
samples from uncoagulated blood that had been submitted as suspected
MCF cases to the MRI VSU for testing by PCR. The ELISA and S/P values
for these samples are given in Supplementary data Table 5.

ROC curve analysis of these data (Supplementary data Fig. 2) was
done with the assumption that the negative herd and PCR-negative
samples were uninfected, and the PCR-positive samples were infected.
The area under the ROC curve (AUC) for the cattle dataset was 0.896 (95
% CI = 0.845 – 0.948), suggesting that the test in cattle samples was at
the boundary of moderately to highly accurate tests (0.9; Greiner et al.,
2000). For this assay, plotting sensitivity and specificity for different
cut-offs showed that a diagnostic cut-off of 0.03 could maximise sensi-
tivity (0.86; 95 % CI 0.77 – 0.91) and specificity (0.86; 95 % CI
0.80 – 0.91). However, use of a higher cut-off of 0.05, as defined for
sheep samples, increased specificity to 0.95 (95 % CI 0.907 – 0.98) at the
expense of test sensitivity (0.80; 95 % CI 0.71 – 0.87) (Fig. 3).

Comparing the S/P values with PCR results showed that 5 of 86 cattle
samples that were OvHV-2 negative by PCR had S/P values greater than
0.05, while 20 of 90 PCR-positive samples had S/P values below 0.05
(Fig. 4). Analysis of the S/P values of PCR-positive and PCR-negative
samples by a two-sample T-test suggested that the range of S/P values
among PCR positive and negative samples were significantly different
from each other (p<10^-16; Fig. 4).

4. Discussion

Established serological tests for MCF in either MCF-susceptible or
reservoir species, irrespective of the virus responsible, have to date used
reagents derived from strains of alcelaphine herpesvirus 1. These assays
include virus neutralisation tests, fluorescent antibody tests, and both
indirect and competitive ELISA formats (OIE, 2018; Fraser et al., 2006;
Haig et al., 2008; Li et al., 2001; Russell et al., 2012; Taus et al., 2015).
Historically, this relates to the inability of most MCF viruses to be
propagated in culture but recent work has shown that MCF virus anti-
gen can be expressed as recombinant proteins in either mammalian or
bacterial systems (Bartley et al., 2014; CUNHA et al., 2015; Dry et al.,
2016), and exploited in development of assays (Alhajri et al., 2018;
CUNHA et al., 2015; Taus et al., 2015).

However, a simple ELISA for MCF virus-specific antibodies has
remained elusive and no commercial assay is currently available. For
example, the competitive inhibition ELISA developed at Washington
State University (Li et al., 2001) has broad specificity (all MCF viruses
appear to share the epitope detected in the assay) and has been used in a
variety of species including both reservoir and MCF-affected cases
(Frolich et al., 1998; Li et al., 1996; Probst et al., 2011; Vikoren et al.,
2006; Zarnke et al., 2002). Unfortunately, this assay, which uses AIHV-1
antigen and an MCF-virus-specific monoclonal antibody, is only now
available as a test run by the WSU-WADDL. More recently, a novel
antibody ELISA for OvHV-2 infection has been described (Alhajri et al.,
2018). This assay was based on the ectodomain of the Ov8 gene product
expressed as a recombinant protein in mammalian cells. This assay had
high sensitivity and specificity for sheep samples when compared with
CI-ELISA but had lower sensitivity for samples from PCR-tested MCF
cases in Bison (Alhajri et al., 2018). Thus the Ov8-based assay showed
similar performance to the Ov65delB ELISA described here.

Indirect ELISAs described by the Morendun Research Institute (Fraser et al., 2006; Russell et al., 2012) have been used mainly in the context of
vaccine trials for WA-MCF (Cook et al., 2019; Haig et al., 2008;
Lankester et al., 2016a, b; Orono et al., 2019; Russell et al., 2012). These
assays work well to detect MCF virus seroconversion in cattle, wildbeest,
bison, deer and some other species, but have not proved useful for
testing sheep or goat sera due to high background (G. Russell, unpub-
lished data). This work was therefore aimed at developing an assay for
the major form of MCF worldwide, with the potential for use in both
MCF-susceptible and MCF host species, and which used an antigen that
would be simple to produce and to purify. The OvHV-2 orf65 protein
was selected based on its clear antigenicity (Bartley et al., 2014) and its
lack of glycosylation, making it a good candidate for bacterial
expression. The poor solubility of this capsid protein was addressed by serial deletion of a codon-optimised version of the native Ov65 gene and selecting a derivative that combined antigenicity with improved solubility, both in the bacterial overexpression system and during purification.

This derivative, Ov65delB, was produced and enriched simply and with high yield (estimated at over 1 mg from 250 mL of induced culture), was used in small quantities (~50 ng/well in the current configuration) and showed good specificity and sensitivity for both sheep and cattle samples. It therefore appears to be an excellent candidate for further development as a test of infection by OvHV-2 and for sheep-associated MCF. Rapid metal affinity chromatography led to an enrichment of the two antigens used here but there was clearly a background of co-purifying E. coli proteins in the eluents (supplementary table 1). These included recognised His-tag purification contaminants such as peptidylprolyl isomerase (SlyD), cAMP-activated global transcriptional regulator (CRP), and ribosomal proteins (Andersen et al., 2013; Bolanos-Garcia and Davies, 2006). The use of a negative control extract based on a tagged version of the non-antigenic Ov26 protein (Bartley et al., 2014) was aimed at removing background due to contamination with co-purifying E. coli proteins. Western blotting and proteomic analysis confirmed that the Ov26 antigen preparation contained both the target protein and multiple E. coli proteins, including one identified in the enriched Ov65delB preparation. This suggests that the Ov26 MAC eluent is a suitable negative antigen preparation for this ELISA. Additional chromatography steps might improve the purity of the antigens required. Indeed, the codon-optimised Ov65 clone produced here carried both N-terminal His-tag and C-terminal Strep-TagII affinity tags, providing scope for a two-step affinity purification. Such purification might remove the requirement for a negative antigen background control.

The difference in performance of the test in sheep (95% specificity and 96% sensitivity) versus cattle (95% specificity and 80% sensitivity) at a cut-off S/P value of 0.05 may reflect the relative value of PCR and serological testing in the detection of clinical MCF in cattle. A study (Muller-Doblies et al., 1998) that used histopathology as the gold-standard for SA-MCF diagnosis, compared serology (CI-ELISA, (Li et al., 1994)) and semi-nested PCR (Baxter et al., 1993) of samples from MCF-affected and clinically healthy cattle. This found that PCR had sensitivity and specificity greater than 94% while serology had lower sensitivity of at least 56% and specificity greater than 91%, with good correlation between the different methods for diagnosis of SA-MCF. More recently, detection of WA-MCF in Kenya by clinical observation, nested PCR (Li et al., 2000) and indirect ELISA of cattle samples was compared (Orono et al., 2019). In this comparison, the indirect ELISA (Russell et al., 2012) had sensitivity of 64% and specificity of 90% when

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**Fig. 3.** Histogram of Ov65delB ELISA S/P values from sheep samples. The optimal negative cut-off of 0.05 for the assay is indicated by the horizontal line.

**Fig. 4.** Histogram of Ov65delB ELISA S/P values for MCF PCR-tested cattle samples. The suggested cut-off value of 0.05 is marked by a horizontal line. Comparison of the PCR-negative and PCR-positive sample S/P values by T-test shows that the two groups are significantly different (** p<10^{-12}).**
PCR was used as the gold standard. These studies confirm the view of the OIE terrestrial manual chapter on MCF ([OIE], 2018) that antibody can be detected in 70–80% of WA-MCF affected cattle. Some MCF-affected animals may not seroconvert due to the rapid progression of clinical disease ([OIE], 2018). This disparity between infection and seroconversion has also been observed in experimental infections with AIHV-1 (Russell et al., 2012) and in an OvHV-2 outbreak in bison where 98% of affected animals were PCR positive but only 73% were serologically positive (Li et al., 2006). These figures for seroconversion in MCF-affected livestock agree well with the sensitivity of the Ov65 ELISA in cattle, suggesting that the assay detects most sero-positive samples. Our observations here, that S/P values among PCR-positive samples formed a continuous progression from negative to positive (Fig. 4) may indicate that serological responses in MCF-affected cattle are not binary but reflect a range of MCF-specific immune responses. This would explain the reduced sensitivity of all serological assays for detecting MCF cases.

The ELISA described here uses the C-terminal ~140 residues of the OvHV-2 minor capsid protein orf65 as the target antigen. This minor capsid protein is conserved among gammaherpesviruses, but only the N-terminal domain of up to 80 residues is required for correct capsid assembly, while the remainder of the polypeptide is dispensable (Dai et al., 2015). In accord with this observation, the first 70 residues of Ov65 constitutes the only region of similarity with other MCF virus minor capsid proteins, including the orf65 gene from AIHV-1, which has 71% identity in this region. The deletion of this conserved domain from the soluble antigenic Ov65delB derivative is therefore in keeping with the low level of cross-reactivity between AIHV-1 and OvHV-2 specific sera and the recombinant orf65 proteins (Bartley et al., 2014). Additionally, the observation that the N-terminal domain of orf65 is associated with capsid assembly suggests this region may also be involved in aggregation and lack of solubility of the native Ov65 protein expressed in bacteria.

The lack of cross-reactivity between orf65 homologues also suggests that the Ov65delB antigen is likely to be of less value in the diagnosis of WA-MCF or in the serology of AIHV-1 infection. We have, however, demonstrated in preliminary studies that the AIHV-1 orf65 protein may be used as an ELISA antigen that can detect both naturally infected and vaccinated or experimentally challenged cattle and wildebeest ([Bartley et al., 2014]; GC Russell; unpublished data). We are currently developing an orf65-based assay for detection of AIHV-1 antibodies, which may be employed in situations where WA-MCF is a potential hazard (such as in sub-Saharan Africa or in wildlife collections).

The value of serology as a tool for detection of OvHV-2 infection and for MCF may be limited because not all MCF-affected cattle have a detectable serological response (Muller-Doblies et al., 1998; Orono et al., 2019; Russell et al., 2012). In contrast, the Ov65 ELISA, used in sheep from the Morefun flock, had both sensitivity and specificity of at least 95%, suggesting that it would be a good assay for the detection of OvHV-2 infection of sheep. It may also have value in zoological collections where rare and potentially endangered exotic ungulates may be at risk of OvHV-2 infection via direct or indirect contact with domesticated or wild ovine/caprine species (Cooley et al., 2008; Frontoso et al., 2016; Hanichen et al., 1998; Li et al., 1999; Meteyer et al., 1998; Probst et al., 2011; Yesilbag et al., 2011). Serological analysis of potential reservoir/susceptible hosts would allow the risks from MCF to be understood and managed by appropriate biosecurity.

This new assay, therefore, constitutes an inexpensive, simple and reliable test that can be used to detect OvHV-2-specific antibody responses in multiple species and that may be employed in situations where resources for PCR testing are not available or the samples available are not amenable to PCR.

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CRediT authorship contribution statement

George C. Russell: Conceptualization, Formal analysis, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. Ann Percival: Investigation, Formal analysis, Data curation, Methodology, Supervision, Writing - original draft. Dawn M. Grant: Investigation, Methodology, Validation, Writing - review & editing. Kathryn Bartley: Investigation, Methodology, Resources, Writing - review & editing. Dylan Turnbull: Formal analysis, Investigation, Methodology, Validation, Writing - review & editing. Kevin McLean: Formal analysis, Data curation, Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing. Julia Liенhard: Formal analysis, Data curation, Investigation, Methodology, Visualization, Writing - original draft. Claudia Bachofen: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

(OIE), W.O.F.A.H., 2018. Malignant Catarrhal Fever, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris.

Alhajri, S.M., Cunha, C.W., Knowles, D.P., Li, H., Taus, N.S., 2018. Evaluation of glycoprotein Ov8 as a potential antigen for an OvHV2-specific diagnostic assay. PLoS One 13, e0200130.

Andersen, K.R., Leksa, N.C., Schwartz, T.U., 2013. Optimized E. coli expression strain LOBSTRA eliminates common contaminants from His-tag purification. Protein 81, 1857–1861.

Bartley, K., Deane, D., Percival, A., Dry, I.R., Grant, D.M., Inglis, N.F., McLean, K., Manson, E.D.T., Imrie, L.H.J., Haig, D.M., Lankester, F., Russell, G.C., 2014. Identification of immuno-reactive capsid proteins of malignant catarhal fever viruses. Vet. Microbiol. 173, 17–26.

Baxter, S.I.F., Wiyono, A., Pow, I., Reid, H.W., 1997. Identification of ovine herpesvirus-2 in sheep. Arch. Virol. 142, 823–831.

Bremer, C.W., Stuart, H., Dobora, F.A., Dungu, B., Romito, M., Viljoen, G.J., 2005. Discrimination between sheep-associated and wildebeest-associated malignant catarrhal fever virus by means of a single-tube duplex nested PCR. Understeepoet J. Vet. Res. 72, 285–291.
Cook, E., Russell, G., Grant, D., Mutisya, C., Omodo, L., Dobson, E., Lankester, P., Nene, V., 2019. A randomised vaccine field trial in Kenya demonstrates protection against wildlife-associated malignant catarrhal fever in cattle. Vaccine 37, 5946–5953.

Cooley, A.J., Taus, N.S., Li, H., 2009. Development of a management program for a field vaccine trial in Tanzania demonstrates partial protection against malignant catarrhal fever in cattle. Vaccine 34, 831–838.

Li, H., Shen, D.T., Knowles, D.P., Gorham, J.R., Crawford, T.B., 1994. Competitive-inhibition enzyme-linked immunosorbent-assay for antibody in sheep and other...