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Enhancing Australia's serodiagnostic and surveillance capabilities for sheeppox, goatpox and lumpy skin disease

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Abstract

Capripoxviruses cause sheeppox, goatpox, and lumpy skin disease of cattle, which are the most serious poxvirus diseases of production animals. Although the risk of these diseases entering Australia is considered low, the potential economic impact of an incursion would be considerable. There are currently no validated, high-throughput antibody detection tools available for undertaking post-outbreak surveillance and re-establishing freedom from disease, which is the primary factor that would mitigate the economic impact to the livestock industries in the event of an outbreak. To enhance diagnostic capability and preparedness eight antibody detection ELISA tests, based on recently discovered immunodominant viral antigens, were developed. Of the three assays that showed the most promise, one had a superior ability to differentiate between capripoxvirus infected and uninfected sheep, goats, and cattle, with diagnostic sensitivity and specificity ranging between 98–100%, but it was unable to detect antibodies reliably in vaccinated animals. No cross-reactivity with antibodies against orf or bovine papular stomatitis viruses was detected. Subject to ongoing evaluation to determine assay reproducibility, the ELISA offers the prospect of a reliable and standardised high-throughput serodiagnostic capability to support post-outbreak surveillance and proof of freedom testing, without the requirement for infectious reagents.

Executive summary

Sheeppox, goatpox, and lumpy skin disease are the most serious poxvirus diseases of production animals. Although the risk of these diseases entering Australia is considered low, the potential economic impact of an incursion would be considerable, most likely second only to that anticipated following an introduction of foot-and-mouth disease. A capripoxvirus disease incursion would potentially cause considerable economic losses due to disruption of trade in livestock and livestock products, as well as costs associated with disease control and eradication. All three diseases are categorised as notifiable by the OIE, and sheeppox, goatpox, and lumpy skin disease viruses are listed by the United States Department of Agriculture on the National Select Agent Registry, due to their significance as potential economic bioterrorism agents. Whilst effective vaccines exist, these are based on live-attenuated virus strains. Therefore their use in non-endemic countries, including Australia, in response to an outbreak may not be desirable.

Significantly, there are currently no validated, high-throughput serodiagnostic tools available for undertaking post-outbreak surveillance and re-establishing freedom from disease, which is the primary factor that would mitigate the economic impact to the livestock industries in the event of an outbreak. Importantly the current "gold standard" serum neutralisation test has not been standardised anywhere in the world, it is not suitable for high-throughput antibody detection, and cannot be implemented at AAHL in the absence of live virus, which is not available in Australia (AAHL is not permitted to hold or import live virus).

To enhance diagnostic capability and preparedness eight antibody detection ELISA tests, based on immunodominant viral antigens that had been identified previously using protein microarray technology, were developed. Six were evaluated using sera from experimentally or naturally infected sheep, goats and cattle. Three assays were chosen for more extensive evaluation at AAHL (Australia), the National Centre for Foreign Animal Disease (Canada) and the Capripoxvirus Reference Laboratory at The Pirbright Institute (UK), due to the ability of these antigens to detect viral antibodies in sera from all three host species. Although all three exhibited differential reactivity to sheep, goat, and cattle sera, one antigen, which was produced in mammalian cells, consistently demonstrated broader and stronger reactivity, identifying it as the lead candidate for the ongoing ELISA validation activities. This ELISA performed favourably when sera from sheep, goats, or cattle infected naturally or experimentally with virulent capripoxvirus isolates were tested. Diagnostic sensitivity and specificity ranged between 98–100%, but the assay was unable to detect antibodies reliably in vaccinated sheep, goats, or cattle. Nevertheless, this appears to be a deficiency

encountered with all currently available assays for capripoxvirus serodiagnosis including the serum neutralisation test.

The main outcome of the project has been the development and preliminary validation of a high-throughput antibody detection ELISA for the diseases caused by capripoxviruses (sheeppox, goatpox, and lumpy skin disease). Although based on the same recombinant antigen, validation data have been generated for each host species (sheep, goats, and cattle) meaning, in effect, that three tests have been produced. Reliable methods for large scale preparation and purification of the recombinant antigen have been established. Subject to determining assay reproducibility, the ELISA offers the prospect of a reliable and standardised high-throughput antibody detection capability, without the requirement for infectious reagents, to support post-outbreak surveillance and proof of freedom testing in sheep, goats, and cattle. This would address a major deficiency in AAHL's capability and preparedness for undertaking effective post-outbreak sero-surveillance for capripoxviruses, thereby reducing the time required to re-establish Australia's disease free status and resume trade in livestock and livestock products.

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1 Background

Capripoxviruses cause sheeppox, goatpox, and lumpy skin disease of cattle, which are the most serious poxvirus diseases of production animals. Although the risk of these diseases entering Australia is considered low (Australia has never had an outbreak), the potential economic impact of an incursion would be considerable; in the case of sheeppox, for example, it would likely be second only to that anticipated following an introduction of foot-and-mouth disease (Garner and Lack, 1995). Contributing factors would include the resultant disruption to trade in livestock and livestock products (meat, leather, and wool), the impact of the diseases on animal health and welfare, and costs associated with disease control and eradication. Significantly, however, there are currently no validated, high-throughput serodiagnostic tools available for undertaking post-outbreak surveillance and re-establishing freedom from disease, which is the primary factor that would mitigate the economic impact to the livestock industries in the event of an outbreak.

The Australian Animal Health Laboratory (AAHL) recently led a major international collaborative project (2004–2007, with funding from CSIRO and AWI), with the Canadian National Centre for Foreign Animal Disease (NCFAD), to enhance the diagnostic capability for these diseases. As a result of this work new international collaborations for these long neglected viruses were established, and AAHL now has in place a set of updated tests and reagents that has significantly enhanced its emergency response capability for the primary diagnosis of these diseases (Babiuk *et al.*, 2008a; Babiuk *et al.*, 2009a; Babiuk *et al.*, 2008b; Babiuk *et al.*, 2007; Bowden *et al.*, 2008; Embury-Hyatt *et al.*, 2012; Stubbs *et al.*, 2012). These assays and methods include electron microscopy, histology, immunohistochemistry and quantitative real-time PCR, as well as the capability to undertake virus isolation in cell culture.

In contrast, the OIE-recommended serum neutralisation test has not been standardised anywhere in the world, is not suitable for high-throughput antibody detection, and cannot be implemented at AAHL in the absence of live virus, which is not available in Australia (AAHL is not permitted to hold or import live virus).

To address the deficit in AAHL's serodiagnostic capability for capripoxviruses, development and preliminary evaluation of an ELISA based on recombinant antigens derived from sheeppox virus was undertaken during the AWI-funded project. Although this assay initially appeared suited to detecting antibodies in infected sheep, goats, and cattle (Bowden *et al.*, 2009), additional validation (partially supported by MLA), using cattle sera from outbreaks of lumpy skin disease in Africa, subsequently demonstrated that it does not provide the desired

diagnostic sensitivity and specificity, particularly for cattle. An indirect ELISA, which uses inactivated, sucrose gradient-purified sheeppox virus as coating antigen, was also developed (Babiuk *et al.*, 2009b). However, although suited to screening sera from sheep, goats, and cattle, the viral antigen is difficult and expensive to produce in large quantities. Furthermore, its production requires access to live virus, which is not available in Australia.

To identify alternative candidate antigens for improved test development, CSIRO subsequently funded a collaboration with colleagues at the University of California, Irvine (UC Irvine), who had pioneered a high-throughput cloning and protein microarray chip fabrication pipeline that is ideally suited to profiling immunoreactivity on a large scale (Davies *et al.*, 2005). Fabrication and screening of chips comprising essentially all of the proteins (~450) encoded by sheeppox, goatpox, and lumpy skin disease viruses shed new insights into the host immune response following exposure, and clearly demonstrated the utility of this technology for identification of immunodominant antigens from complex microorganisms in an efficient and straightforward manner (immunodominance being defined as reactivity in >50% of animals in an exposed population). Of ~150 proteins encoded by each viral genome, the greatest number of significantly differentially reactive, immunodominant antigens was recognised by goats (n=31), followed by sheep (n=19), and cattle (n=17). A subset of antigens that were broadly reactive in all three host species was identified, and these were considered to be preferred potential candidates for development of an improved antibody detection ELISA. What follows highlights the significant findings and outcomes of the project without detailing all of the laboratory work undertaken or all of the data that were generated. The main outcome of the project has been the development and preliminary validation of a convenient and cost-effective antibody detection ELISA, based on a recombinant viral antigen, for sheeppox, goatpox, and lumpy skin disease viruses. Subject to determining assay reproducibility, the ELISA offers the prospect of a reliable and standardised high-throughput antibody detection capability, without the requirement for infectious reagents, to support post-outbreak surveillance and proof of freedom testing in sheep, goats and cattle.

2 Project objectives

The project objective is to enhance AAHL's capability and preparedness for undertaking effective post-outbreak surveillance for diseases (sheeppox, goatpox, and lumpy skin disease) caused by capripoxviruses, thereby reducing the time required to re-establish Australia's disease-free status and resume trade in livestock and livestock products. Notionally, the project would progress as follows.

1/ Within 6 months: Cloning and expression of novel antigens, with broad reactivity in sheep, goats, and cattle, previously identified as immunodominant following characterisation of the host immune response to infection or vaccination using a capripoxvirus proteome microarray.

2/ Within 12 months: Development and preliminary evaluation of ELISA tests based on well-characterised sera from naturally and experimentally infected or vaccinated sheep, goats, and cattle; preparation of large batches of selected antigens for ongoing test validation.

3/ Within 18 months: Implementation of selected test(s) at AAHL and at collaborating institutes in Canada (National Centre for Foreign Animal Disease (NCFAD)), the UK (The Pirbright Institute) and / or South Africa (Agricultural Research Council - Onderstepoort Veterinary Institute (ARC-OVI)). These international collaborations will facilitate access to well-characterised sera from sheep, goats, and cattle that have been naturally or experimentally infected or vaccinated (The Pirbright Institute and ARC-OVI are both designated OIE Reference Laboratories for sheeppox, goatpox and lumpy skin disease).

4/ Within 24 months: Evaluation of ELISA test performance in association with overseas collaborators, using large panels of cattle sera and, as available, sheep and goat sera. This should enable robust assay performance characteristics (diagnostic sensitivity and specificity) to be determined.

Validation of the ELISA(s) will be conducted to a standard suitable for later implementation in Australia under the Sub-Committee on Animal Health Laboratory Standards (SCAHLS) and internationally under World Organisation for Animal Health (OIE) guidelines. This would facilitate their international acceptance, which would be vital for regaining disease-free status. However, external recognition would be highly dependent on obtaining timely access to sufficiently large numbers of well-characterised sera from infected or vaccinated sheep, goats, and cattle, and may not occur within the timeframe of the project.

3 Methodology

3.1 Sheep, goat, and cattle sera

Sequential serum samples from sheep, goats, and cattle following experimental infection with capripoxvirus isolates of differing host specificity and pathogenicity, as well as sera from naturally infected or vaccinated animals, were provided by the National Centre for Foreign Animal Disease (NCFAD), Winnipeg, Canada, and The Pirbright Institute, UK. Serum samples from New Zealand sheep infected experimentally with orf virus were kindly provided by Professor Andrew Mercer (Virus Research Unit, University of Otago, Dunedin, New

Zealand), while sera from Japanese cattle naturally infected with parapoxviruses were kindly provided by Associate Professor Yasuo Inoshima (Department of Veterinary Medicine, Gifu University, Gifu, Japan).

Sera from Australian animals never exposed to capripoxviruses were obtained anonymously from sheep, goats, and cattle in New South Wales (kindly provided by Dr Peter Kirkland; Elizabeth Macarthur Agricultural Institute, New South Wales, Australia) and from sheep in Victoria (kindly provided by Dr Steve Colegate, Dr Mark Ford, Ms Agnieszka Michalewicz, and Ms Yu Cao; AAHL, Geelong, Australia). Sera from Canadian cattle never exposed to capripoxviruses were provided by the National Centre for Foreign Animal Disease (NCFAD), Winnipeg, Canada.

3.2 Cloning and expression of recombinant proteins

Candidate viral antigens, previously identified as being immunodominant using protein microarray technology, were selected for production as recombinant proteins using well-established microbial or mammalian host expression systems. Due to its general suitability for production of the broadest range of proteins, *Escherichia coli* (*E. coli*) was utilised initially as the expression host. The second production host, usually preferred for more complex integral membrane proteins that possess mammalian-specific modifications that can't be generated using *E. coli*, was the human embryonic kidney (HEK) cell line.

3.2.1 Microbial

Selected open reading frames (ORFs), excluding predicted signal peptides or transmembrane domains, where present, were cloned using the In-Fusion HD EcoDry Cloning Kit (Clontech, USA) according to the manufacturer's guidelines. Briefly, ORFs were amplified by PCR using either Nigerian sheeppox virus or lumpy skin disease virus (Neethling) DNA as template (kindly provided by Dr Shawn Babiuk, NCFAD, Winnipeg, Canada). Following purification, each PCR product was inserted into the expression vectors pET28-MHL and pNIC-CH (Structural Genomics Consortium, Toronto, Canada), which encode an amino- or carboxy-terminal hexahistidine tag, respectively, to facilitate the subsequent purification of proteins using immobilised metal affinity chromatography (IMAC). The resulting plasmids were transformed into *E. coli* DH5α and individual bacterial colonies were tested by PCR to confirm the presence of each construct. Plasmid DNA was subsequently purified from each clone using the QIAprep Spin Miniprep Kit (Qiagen, Germany) and stored at 4 °C prior to generation of expression constructs.

Analytical small-scale recombinant protein expression studies were undertaken following transformation of the plasmid constructs into chemically competent *E. coli* BL21-AI or Rosetta cells. For each pET28-MHL or pNIC-CH clone, 1.5 ml 2YT cultures were induced with 1 mM IPTG and 0.025% (w/v) L-arabinose, and incubated for ~18 h at 18 °C with shaking at 600 rpm. Cells were harvested by centrifugation, freeze-thawed, and then lysed using BugBuster Master Mix (Novagen, USA). The proteins were bound to IMAC resin in 96-well filter plates, washed with binding buffer containing 20 mM imidazole, and the soluble fractions eluted with binding buffer containing 250 mM imidazole. Total and soluble protein fractions were subsequently analysed for capripoxvirus open reading frame expression by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Larger scale expression of candidate antigens was undertaken by inoculating 12.5 ml of overnight starter cultures of *E. coli* strain BL21-AI or Rosetta, transformed with the expression construct of interest, into 1.0 L of TB media in 2.5 L polypropylene shake flasks. Cultures were grown at 37 °C with shaking at 700 rpm until an OD_{600} nm of ~0.6 was reached. Protein expression was then induced by the addition of 1 mM IPTG and 0.025% (w/v) L-arabinose, and the cells grown for ~20 h at 18 °C. Cells were harvested by centrifugation and cell pellets stored at -80 °C prior to purification of the expressed antigens.

3.2.2 Mammalian

Open reading frames corresponding to the ectodomains of viral integral membrane proteins, excluding predicted native signal peptides and transmembrane domains, were chemically synthesized following codon optimization for recombinant expression in mammalian cells, and supplied in the pUC57-Simple vector (GenScript, USA). Each synthetic open reading frame was manufactured to encode an N-terminal secretion signal peptide sequence, as well as a C-terminal FLAG tag (DYKDDDDK) to facilitate protein purification. The codon optimized synthetic inserts were released by digestion with HindIII and EcoRI (Promega, USA), gel purified, and inserted into the mammalian expression vector pEE6.4 (Lonza Biologicals, UK). The expression plasmids were transformed into *E. coli* DH5α, and plasmid DNA from each clone was subsequently purified from 5 ml or 500 ml overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen, Germany) or NucleoBond Xtra Maxi Plus Kit (Macherey-Nagel, Germany), respectively, according to the directions of the manufacturer, quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and stored at 4 °C prior to transfection.

Analytical small-scale recombinant protein expression studies were undertaken following transient transfection of adherent human HEK 293T cells cultured in 2 ml of serum-free

medium (Invitrogen, USA) in 6-well plates. Cells were routinely transfected with 2 µg of plasmid DNA complexed with polyethylenimine (Polysciences, USA) according to the manufacturer's instructions. After 7 days, the cell supernatants were harvested and the cell monolayers lysed in 250 µl of lysis buffer. Biosynthesis and secretion of FLAG-tagged recombinant proteins, in cell lysates and supernatants, was subsequently evaluated by SDS-PAGE and Western blot using a monoclonal antibody to the FLAG tag.

For larger scale production, transient expression was performed in suspension adapted HEK 293F cells grown in serum-free medium (Invitrogen, USA) in shaker flasks by the addition of 1.0–2.5 mg of plasmid DNA complexed with polyethylenimine (Polysciences, USA) to 1.0–2.5 L of cells, according to the manufacturer's instructions. After 7 days, the cell supernatants were harvested, supplemented with 0.02% (w/v) of sodium azide, filter-sterilised and stored at 4 °C prior to purification.

3.3 Purification of recombinant proteins

3.3.1 His-tagged fusion proteins

Pelleted bacterial cells were resuspended in 10 ml of ice cold lysis buffer (10 mM Tris HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, 2 mM $MgCl_2$, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), Pierce EDTA-free protease inhibitor tablets (1 tablet per 50 ml of lysis buffer), 0.5 mg/ml lysozyme and 5 units/ml benzonase) per gram of wet weight. Cells were lysed at 4 °C by passing three times through an Emulsiflex C5 high-pressure homogenizer (Avestin, Canada). Lysates containing soluble proteins were clarified at 4 °C by centrifugation, passed through a 0.45 µm filter, and loaded on to a 1 ml HisTrap fast flow column (GE Healthcare, Australia) equilibrated in a buffer containing 10 mM Tris HCl, pH 8.0, 300 mM NaCl, 5 mM DTT and 10 mM imidazole. The column was then washed with equilibration buffer containing 30 mM imidazole and bound proteins eluted in buffer containing 250 mM imidazole. Proteins were routinely further purified by passage over a Superdex 200 size exclusion chromatography column (GE Healthcare, Australia) and eluted in 10 mM Tris HCl, pH 8.0, 150 mM NaCl and 0.02% (w/v) sodium azide (TBSA) containing 5 mM DTT. Protein peaks were analysed by SDS-PAGE under reducing conditions, pooled, and routinely concentrated to at least 1.0 mg/ml using Amicon Ultra 10 kDa centrifugal filters (Merck Millipore, Germany). Proteins were then aliquotted, snap frozen in liquid nitrogen, and stored at -80 °C. Protein concentrations were determined prior to storage using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA).

For insoluble proteins, cell lysates were clarified at 4 °C by centrifugation, as above, and the supernatants discarded. The insoluble protein pellets were washed with 50 mM Tris, pH 8.0,

containing 1% (w/v) deoxycholate and 1% (v/v) IGEPAL CA-630, and subsequently solubilised at 4 °C overnight in 50 mM Tris HCl, pH 8.0, containing 8 M urea and 5 mM imidazole. After passing through a 0.45 µm filter, the proteins were loaded on to a 1 ml HisTrap fast flow column (GE Healthcare, Australia) equilibrated in the same solubilisation buffer, washed, and bound proteins eluted in buffer containing 500 mM imidazole. The purity of the eluted proteins was analysed by SDS-PAGE under reducing conditions, and proteins subsequently concentrated to ~1.5 mg/ml using Amicon Ultra 10 kDa centrifugal filters (Merck Millipore, germany). Proteins were aliquotted, snap frozen in liquid nitrogen, and stored at -80 °C.

3.3.2 FLAG-tagged fusion proteins

FLAG-tagged soluble recombinant proteins were recovered from sterile-filtered cell supernatants by affinity chromatography over columns of immobilised anti-FLAG monoclonal antibody covalently coupled to ~50 ml of resin. After adjustment of pH to 8.0 with Tris HCl, media was pumped over the columns at ~2 ml/min. The columns were subsequently washed with 10 column volumes of TBSA, and the bound proteins displaced using a three-step elution. First, 50 ml of FLAG peptide, at 0.25 mg/ml in TBSA, was recirculated through the column for 30 min at room temperature. Then, 100 ml of FLAG peptide (0.025 mg/ml in TBSA) was pumped through the column, followed by 50 ml of TBSA. The column was regenerated with ~150 ml of 0.5 M citrate, pH 3.0, before re-equilibration back to pH 8.0 using TBSA. The pooled eluates were concentrated by stirred-cell ultrafiltration (10 kDa cut-off membrane) and further purified by size exclusion chromatography on a Superdex 200 column (GE Healthcare, Australia) in TBSA. The peak fractions containing the proteins of interest were analysed by SDS-PAGE, pooled and concentrated to 1.0 mg/ml using Amicon Ultra 10 kDa centrifugal filters (Merck Millipore, Germany). They were subsequently aliquotted, snap frozen in liquid nitrogen and stored at -80 °C.

3.4 SDS-PAGE, Western transfer and immunodetection

Protein samples were incubated for 5 min at 100 °C with SDS sample buffer containing 10 mM DTT prior to separation on NuPAGE Novex 4–12% Bis-Tris gels using MES electrophoresis (Life Technologies, USA), as recommended by the manufacturer. Protein bands were visualised by staining with Coomassie Brilliant Blue R-250 (Pierce Biotechnology, USA) and their molecular weights estimated using SeeBlue2 molecular-weight standards (Life Technologies, USA).

For immunodetection, proteins on replicate gels were electrotransferred onto nitrocellulose membranes (Life Technologies, USA). The membranes were blocked for 30 min with

phosphate-buffered saline containing 5% (w/v) skim milk powder and 0.1% (v/v) Tween 20, followed by incubation with an anti-His monoclonal antibody conjugated to horseradish peroxidase (Sigma, USA) to detect His-tagged proteins, or M2 anti-FLAG monoclonal antibody (Sigma, USA), followed by a secondary rabbit anti-mouse IgG-HRP conjugate (Sigma, USA), to detect FLAG proteins. The membranes were washed three times with phosphate-buffered saline containing 0.1% (v/v) Tween 20 before being developed using chemiluminescence substrates according to the manufacturer's recommendations.

3.5 Enzyme linked immunosorbent assay (ELISA)

Ninety-six-well Maxisorp ELISA plates (Nunc, USA) were coated with 60–125 ng of each affinity purified recombinant protein per well in 100 µl of 0.05 M carbonate–bicarbonate buffer (pH 9.6) (Sigma, USA), and incubated overnight at 4 °C. Plates were washed four times with phosphate buffered saline (Life Technologies, USA) containing 0.05% (v/v) Tween 20 (Sigma, USA) (PBST) prior to the addition of 200 µl/well of casein blocking buffer (1X Sigma blocking buffer; Sigma, USA). The plates were incubated for 1 h at 37 °C on a plate shaker and again washed four times with PBST. Test sera, diluted 1:50 in blocking buffer, were serially diluted two-fold across the plates (100 µl/well) or were tested, in duplicate, at a dilution of 1:100. The plates were incubated for 1 h at 37 °C with shaking and washed four times with PBST. Recombinant protein G conjugated to horseradish peroxidase (protein G-HRP; Life Technologies, USA), diluted 1:128 000 in blocking buffer, was subsequently added (100 µl/well). After incubating at 37 °C for 1 h the plates were washed four times with PBST and 100 µl of the substrate 3,3,5,5-tetramethylbenzidine dihydrochloride (TMB; Sigma, USA) was added to each well. An equal volume of 1 M H_2SO_4 was subsequently used to stop the enzyme reaction after a 10 min incubation at room temperature, and the OD of the reaction product was read at 450 nm on an ELISA plate reader (Multiskan EX; Thermo Scientific, USA). To enable comparison of data between different ELISA plates, OD values were routinely normalised against a positive control serum and expressed as percent positivity (mean corrected test OD value ÷ corrected positive control OD value × 100) (Wright *et al.*, 1993). Data analysis was undertaken using GraphPad Prism version 6.03 (GraphPad Software, USA) or MedCalc version 14.12.0 (MedCalc Software, Belgium).

4 Results and discussion

4.1 Strategy for antigen cloning and expression

The newly identified immunodominant viral antigens, which had previously been identified as broadly reactive in sheep, goats, and cattle using protein microarray technology, belonged to various classes of protein. These included core and integral membrane proteins, as well as

proteins of unknown function, and varied in size and complexity. Two main strategies for their production as purified recombinant proteins, fused to affinity tags to facilitate purification, were therefore considered. Both were developed with the intention of producing soluble recombinant proteins, as this improved the probability that they would be modified and folded properly, thereby retaining the ability to bind protein-specific antibodies in sera of infected animals when used as coating antigen in an ELISA test format. The first strategy, generally suited for the initial production of the broadest range of proteins, utilised *Escherichia coli* (*E. coli*) as the expression host. The second, often preferred for more complex integral membrane proteins that possess particular modifications that can't be generated using *E. coli*, utilised human embryonic kidney (HEK) cells.

Since it is not possible to predict ahead of time how well individual novel proteins will express, it was considered prudent to initially screen a greater number of antigens than the minimal optimal subset that had been identified, especially considering that the antigens with the broadest reactivity included integral membrane proteins, which can be difficult to produce in conventional bacterial and mammalian expression systems. Nevertheless, based on a review of the poxvirus literature to identify prior strategies used for production of proteins related to the immunodominant capripoxvirus antigens, 12 proteins were selected for expression – 9 in *E. coli* and 3 in human embryonic kidney (HEK) cells. Considering that capripoxviruses are very closely related antigenically, and based on the outcomes of the prior protein microarray studies, the intention was to produce recombinant antigens with serodiagnostic utility for all three viral species.

4.2 Cloning, analytical expression and characterisation of novel antigens

4.2.1 Microbial

All nine open reading frames (ORFs), excluding predicted signal peptides and transmembrane domains, where present, were amplified by PCR using either sheeppox virus or lumpy skin disease virus genomic DNA as template and inserted into prokaryotic vectors, encoding amino- or carboxy-terminal hexahistidine tags, using ligation-independent cloning. For three of the ORFs (1, 5 and 6 in Table 1), constructs were generated using sheeppox virus or lumpy skin disease virus genomic DNA since subtle differences between these protein coding regions of the viruses were evident, the effect of which on the efficiency of expression could not be predicted. Twenty-four constructs in total were therefore generated, encompassing two or four variants of each of the nine selected open reading frames.

Following propagation and purification of the plasmid DNA, insertion into *E. coli* strain BL21-AI, and small-scale test expression using 1.5 ml *E. coli* cultures and conditions that increased

the probability of producing soluble protein, variants of five of the nine proteins were produced in soluble form as determined by SDS-PAGE and staining with Coomassie. Subsequently, small-scale expression of all 24 constructs using the Rosetta strain of *E. coli* resulted in the production of variants of seven of the nine antigens, including two of the four that had previously been identified as being broadly reactive using protein microarray technology. However, one of these two additional recombinant proteins was predominantly insoluble (Table 1).

Table 1. Analytical expression of novel antigens in *E. coli*

ORF	Construct [a]	Antigen ID	BL21-AI			Rosetta		
			Expressed	Yield [b]	Soluble	Expressed	Yield [b]	Soluble
1	pET28-MHL_1L	Cp 1	N			N		
	pNIC-CH_1L	Cp 2	N			Y	+++	N
	pET28-MHL_1S	Cp 3	N			Y	+++	N
	pNIC-CH_1S	Cp 4	N			Y	+++	N
2	pET28-MHL_2L	Cp 5	N			N		
	pNIC-CH_2L	Cp 6	N			N		
3	pET28-MHL_3L	Cp 7	N			N		
	pNIC-CH_3L	Cp 8	N			N		
4	pET28-MHL_4S	Cp 9	Y	+	Y	N		
	pNIC-CH_4S	Cp 10	Y	+	Y	Y	+	Y
5	pET28-MHL_5L	Cp 11	Y	+	Y	Y	+	Y
	pNIC-CH_5L	Cp 12	Y	+	Y	Y	+	Y
	pET28-MHL_5S	Cp 13	Y	+	Y	Y	+	Y
	pNIC-CH_5S	Cp 14	Y	++	Y	Y	++	Y
6	pET28-MHL_6L	Cp 15	Y	+++	Y	Y	+++	Y
	pNIC-CH_6L	Cp 16	Y	++	Y	Y	++	Y
	pET28-MHL_6S	Cp 17	Y	++	Y	Y	++	Y
	pNIC-CH_6S	Cp 18	Y	+	Y	Y	+	Y
7	pET28-MHL_7S	Cp 19	Y	+	Y	Y	+	Y
	pNIC-CH_7S	Cp 20	Y	+	Y	Y	+	Y
8	pET28-MHL_8L	Cp 21	Y	+	Y	Y	+	Y
	pNIC-CH_8L	Cp 22	Y	++	Y	Y	++	Y
9	pET28-MHL_9S	Cp 23	N			Y	++	Y
	pNIC-CH_9S	Cp 24	N			Y	++	Y

[a] The number and letter suffixes denote the open reading frame and nature of genomic template (L: lumpy skin disease virus (Neethling); S: Nigerian sheeppox virus).
[b] Relative estimate of amount of soluble protein produced. "+" indicates low; "++" indicates medium; "+++" indicates high.

4.2.2 Mammalian

For the remaining three ORFs synthetic DNA templates, sequence-optimised for expression in mammalian (HEK) cells and encoding the relevant regions of three viral integral membrane

proteins, were manufactured by a commercial provider. Following transfer of the protein coding region into an expression vector that encoded a carboxy-terminal FLAG tag, the plasmid DNA was propagated in *E. coli*, purified, and transfected into HEK cells complexed with polyethylenimine. After seven days it appeared that all three synthetic gene constructs were being produced and secreted as soluble recombinant proteins. However, the yields, as determined by immunoblotting with an antibody to the marker tag attached to the C-terminus of each protein, were lower than anticipated (data not shown). Following minor modifications to the transfection protocol, expression of these proteins was repeated and Western blotting of the harvested supernatants confirmed that two of the three were being secreted in soluble form (Table 2).

Table 2. Analytical expression of novel antigens in HEK cells

ORF	Construct [a]	Antigen ID	HEK 293T		
			Expressed	Yield [b]	Secreted
10	pEE6.4_10S	Cp 25	Y	+	Y
11	pEE6.4_11L	Cp 26	N		
12	pEE6.4_12S	Cp 27	Y	+	Y

[a] The number and letter suffixes denote the open reading frame and identity of genomic template (S: Nigerian sheeppox virus; L: lumpy skin disease virus (Neethling)).
[b] Relative estimate of amount of secreted protein produced. "+" indicates low; "++" indicates medium; "+++" indicates high.

4.3 Scale-up expression, purification and reactivity of selected antigens

Larger scale expression of variants of all seven *E. coli*-expressed antigens, using the optimal strain of *E. coli* as previously determined for each, was subsequently undertaken to obtain sufficient quantities of the recombinant proteins for development and evaluation of the prototype ELISAs. Following induction of 1.0 L cultures of *E. coli* transformants in TB medium, bacterial cells were pelleted, lysed mechanically, and purified using standard immobilised nickel affinity chromatography and size exclusion chromatography purification protocols. Expression products were routinely analysed by SDS-PAGE and Western blotting, concentrated to at least 1.0 mg/ml, and snap frozen in 100 µl aliquots prior to storage at -80 °C. Notably, although they expressed well, variants of one of the two proteins that had previously been identified as broadly reactive in sheep, goats, and cattle were not successfully purified since they would not bind efficiently to the nickel column matrix. These proteins, Cp 11 and 14, were not developed further. Variants of six of the nine antigens selected for expression in *E. coli* were, however, obtained in sufficient quantities and purity to evaluate their immunoreactivity when used as coating antigen in an indirect ELISA test format. Data for two of the antigens were suboptimal, whereas data for the other four were

very promising (Table 3). Testing confirmed that four of the purified antigens exhibited differential reactivity to various combinations of cattle, goat, and sheep sera. Specifically, one antigen (Cp 2) exhibited differential reactivity to sheep, goat, and cattle sera, two (Cp 10 and 15) exhibited differential reactivity to cattle and goat sera, and one (Cp 21) exhibited differential reactivity to cattle and sheep sera. All four showed differential reactivity to cattle sera, all but Cp 21 showed differential reactivity to goat sera, while Cp 2 and 21 showed differential reactivity to sheep sera.

Table 3. Characterisation and immunoreactivity of recombinant capripoxvirus proteins

ORF	Construct [a]	Antigen ID	Expression Host	Soluble	Purified	Yield (mg/L)	ELISA Reactivity [b]
1	pNIC-CH_1L	Cp 2	Rosetta	N	Y	15.0	SGC
4	pNIC-CH_4S	Cp 10	Rosetta	Y	Y	0.4	GC
5	pET28-MHL_5L	Cp 11	Rosetta	Y	N	-	ND
	pNIC-CH_5S	Cp 14	BL21-AI	Y	N	-	ND
	pNIC-CH_5S	Cp 14	Rosetta	Y	N	-	ND
6	pET28-MHL_6L	Cp 15	BL21-AI	Y	Y	10.5	GC
7	pNIC-CH_7S	Cp 20	Rosetta	Y	Y	0.8	-
8	pET28-MHL_8L	Cp 21	BL21-AI	Y	Y	2.0	SC
9	pNIC-CH_9S	Cp 24	Rosetta	Y	Y	11.2	-
10	pEE6.4_10S	Cp 25	HEK 293F	Y	Y	2.0	SGC
12	pEE6.4_12S	Cp 27	HEK 293F	Y	Y	2.0	SGC

[a] The number and letter suffixes denote the open reading frame and origin of genomic template (L: lumpy skin disease virus (Neethling); S: Nigerian sheeppox virus).
[b] Immunoreactivity of purified recombinant proteins, to sera from capripoxvirus-infected animals, was determined by indirect ELISA. "S", "G" and "C" indicate reactivity to sheep, goat and cattle sera, respectively; "-" indicates poor reactivity; "ND" indicates not determined.

Larger scale production of the two mammalian-expressed antigens, Cp 25 and 27, which were secreted as soluble glycoproteins, was routinely undertaken in 1.0 or 2.5 L culture volumes. Each was successfully purified using an antibody to the marker tag, followed by size exclusion chromatography. The expressed proteins were routinely analysed by SDS-PAGE and Western blotting, and the production between batches shown to be reproducible. Each was concentrated to 1.0 mg/ml, snap frozen in 100 µl aliquots and stored at -80 °C. Testing by ELISA demonstrated that both antigens exhibited very good differential reactivity to sheep, goat, and cattle sera of known antibody status (Table 3).

Of the eight ELISA tests that underwent preliminary assessment, six were therefore selected for ongoing evaluation and validation based on their superior ability to differentiate between capripoxvirus neutralising antibody positive and negative sera. At least several of these antigens offered the prospect of a high-throughput serodiagnostic capability for sheeppox, goatpox, and lumpy skin disease, without the requirement for infectious reagents. However,

the three recombinant integral membrane proteins Cp 2, Cp 25 and Cp 27, which exhibited differential reactivity to sera from all three host species, were of particular interest due to their potential to be used in a universal test format for sheep, goats, and cattle.

4.4 Preliminary evaluation of Cp 2, Cp 25 and Cp 27 antibody detection ELISAs

Of the eight ELISAs that had previously undergone preliminary assessment at AAHL, six were transferred to the National Centre for Foreign Animal Disease in Winnipeg, Canada, for more comprehensive evaluation (26 October – 1 December 2013) using sheep, goat, and cattle sera, of known viral neutralising antibody status, which had not been subjected to gamma irradiation. The three recombinant integral membrane proteins, which had previously shown differential reactivity to sera from all three host species, continued to show superior performance characteristics and were therefore considered to be the preferred candidate antigens. The two mammalian-expressed proteins exhibited lower background reactivity than the bacterial-expressed antigen, enabling sera to be tested at a lower (more concentrated) dilution. Furthermore, one of the mammalian-expressed antigens, Cp 25, appeared to be more broadly reactive and therefore the probable antigen of choice (Figure 1).

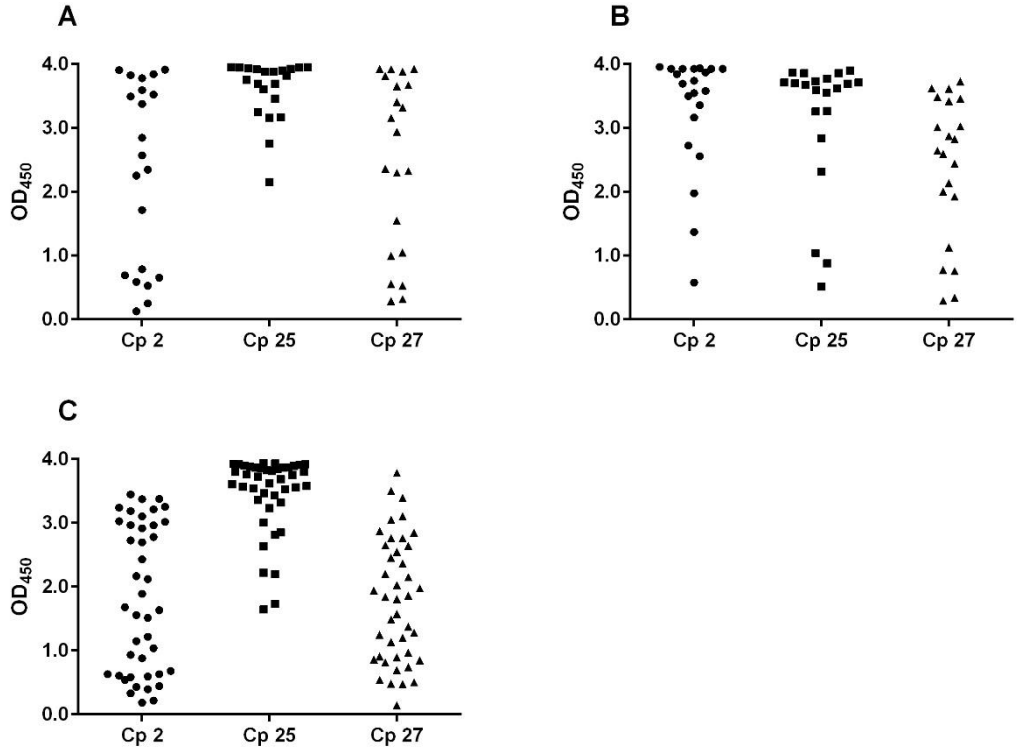

Figure 1. Comparative immunoreactivity of Cp 2, Cp 25 and Cp 27. Immunoreactivity of the purified recombinant proteins was determined by indirect ELISA. The same panels of neutralising antibody positive sheep (n=21) (A), goat (n=21) (B) and cattle (n=42) (C) sera were evaluated against each

recombinant protein using the optimal coating concentrations for each antigen and a 1:100 dilution of test sera. Corrected OD values, with background (from no serum control wells) subtracted, are shown.

All three assays were subsequently transferred to the Capripoxvirus Reference Laboratory at The Pirbright Institute in the UK, where further testing was undertaken (28 February – 7 April 2014) using additional well-characterised sheep, goat, and cattle sera from naturally and experimentally infected animals. Although all three ELISAs were clearly able to detect antibodies in neutralising antibody positive sera from sheep, goats, and cattle, the performance of the assay based on the mammalian-expressed antigen, Cp 25, was again shown to be superior to the other two assays. This antigen demonstrated broader and stronger reactivity using sera from all three host species, confirming its status as the preferred antigen for the ongoing ELISA validation activities (data not shown).

4.5 Validation of the Cp 25 ELISA using sheep, goat, and cattle sera

4.5.1 Repeatability

Positive and negative control sera from experimentally infected sheep, goats, or cattle were routinely included on every plate to evaluate assay repeatability, as well as to enable normalisation of data within and between runs. For sheep and goat sera the control samples were tested in duplicate whereas, for cattle, the control sera were tested in quadruplicate. Estimates of upper and lower control limits for the strong positive serum dilutions are shown in Figure 2. Six to nine plates were routinely tested per run. Across approximately 40 plates for each species, the average coefficient of variation of the strong positive control serum was 6.1% (mean OD 1.71 ± 0.10 SD), 13.0% (mean OD 1.35 ± 0.18 SD), and 7.8% (mean OD 2.40 ± 0.19 SD), for sheep, goats, and cattle, respectively.

4.5.2 Analytical sensitivity

Based on the preliminary immunoreactivity data of Cp 25, and checkerboard titrations, 60 ng of antigen per well was used in an indirect ELISA for analysis of test sera. Titration of samples, that had been collected from sheep, goats, or cattle infected experimentally or naturally with capripoxviruses that differed in geographic origin and virulence, was routinely undertaken by serial two-fold dilution across the plate, commencing at 1:50. The ELISA was clearly able to detect antibodies in sera from all three host species (Figure 3). By arbitrarily defining the antibody titre as the reciprocal of the final dilution of serum in which the OD_{450} exceeded background, antibodies were readily detectable in sheep, goat, and cattle sera at titres exceeding 6 400, with the peak in detectable antibody occurring at 51 200 in sheep sera and at 25 600 in goat or cattle sera.

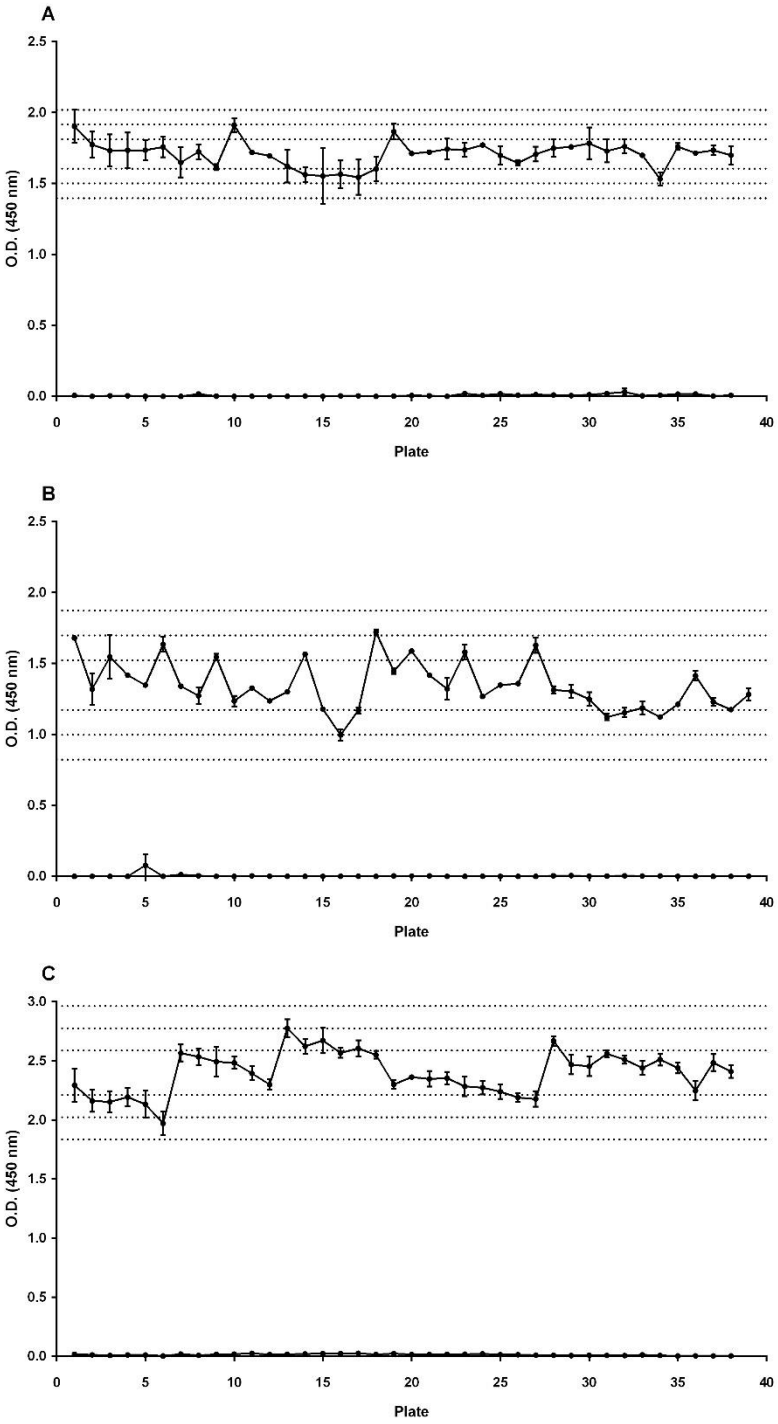

Figure 2. Cp 25 ELISA repeatability and internal quality control. Estimates of the variation observed in high positive and negative control sheep (A), goat (B), or cattle (C) serum samples that were run on every plate for data normalisation within and between runs. Data for each plate are shown as the mean of two replicates ±1 SD for sheep and goats or as the mean of four replicates ±1 SD for cattle. Estimates of assay upper and lower control limits are shown as dotted lines indicating one, two and three standard deviations above or below the mean of each positive control serum.

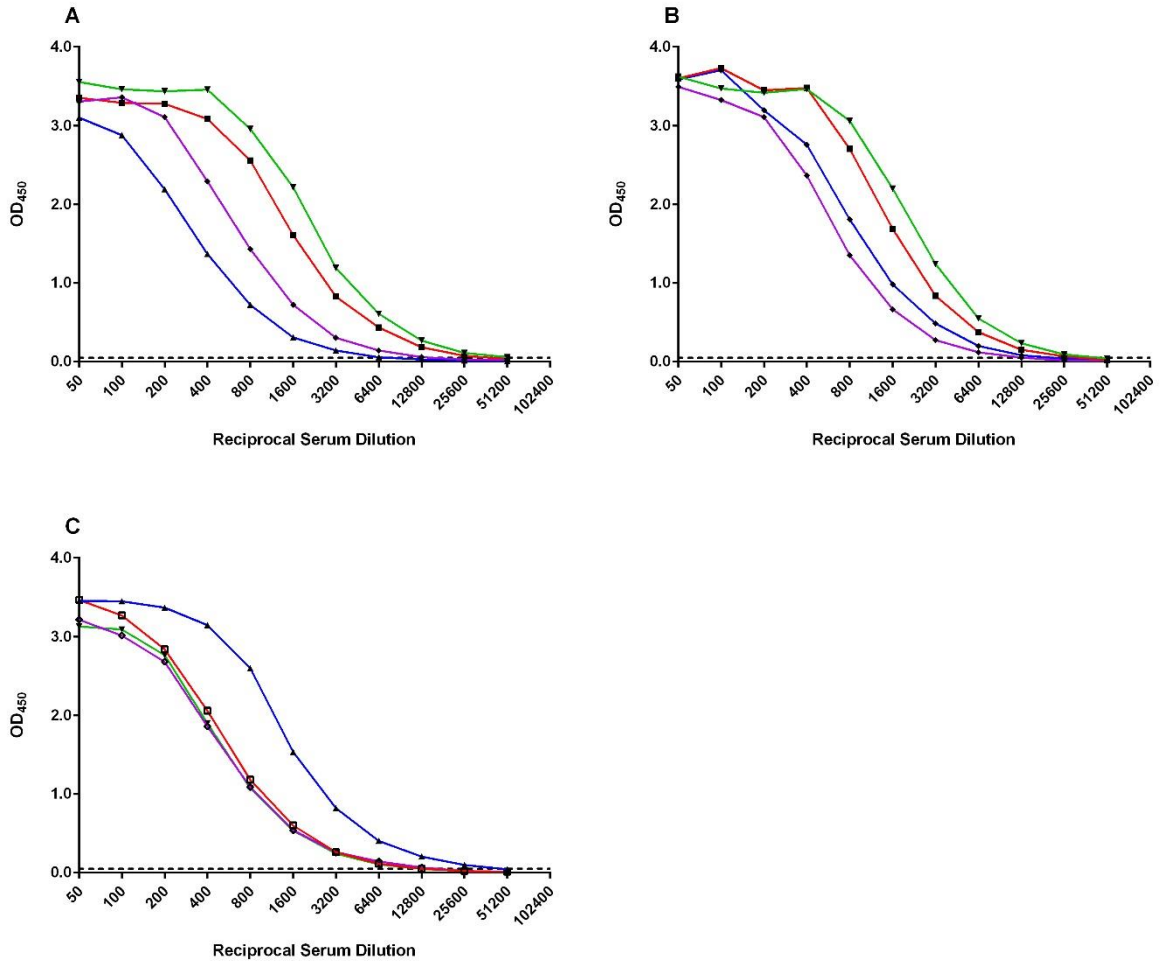

Figure 3. Titration of sera collected from sheep (n=4) (A), goats (n=4) (B), or cattle (n=4) (C) infected with isolates of sheeppox, goatpox, or lumpy skin disease viruses, respectively. Sera were serially diluted two-fold across each plate. Corrected OD values, with background (from no serum control wells) subtracted, are shown. Cut-off values for determination of serum antibody end point titres are depicted as dashed lines.

The kinetics of antibody production in sheep, goats, and cattle were also assessed by testing, at a dilution of 1:100, sequential sera that had been collected at various time points following experimental challenge (Figure 4). Antibodies were first detected in sheep by 8 days post inoculation (DPI) and in goats by DPI 12, with all sheep and goats showing seroconversion by DPI 12 and 14, respectively. For cattle, antibodies were not detectable until after DPI 10. However, all animals tested had seroconverted by DPI 13, which is comparable to what was observed for sheep and goats.

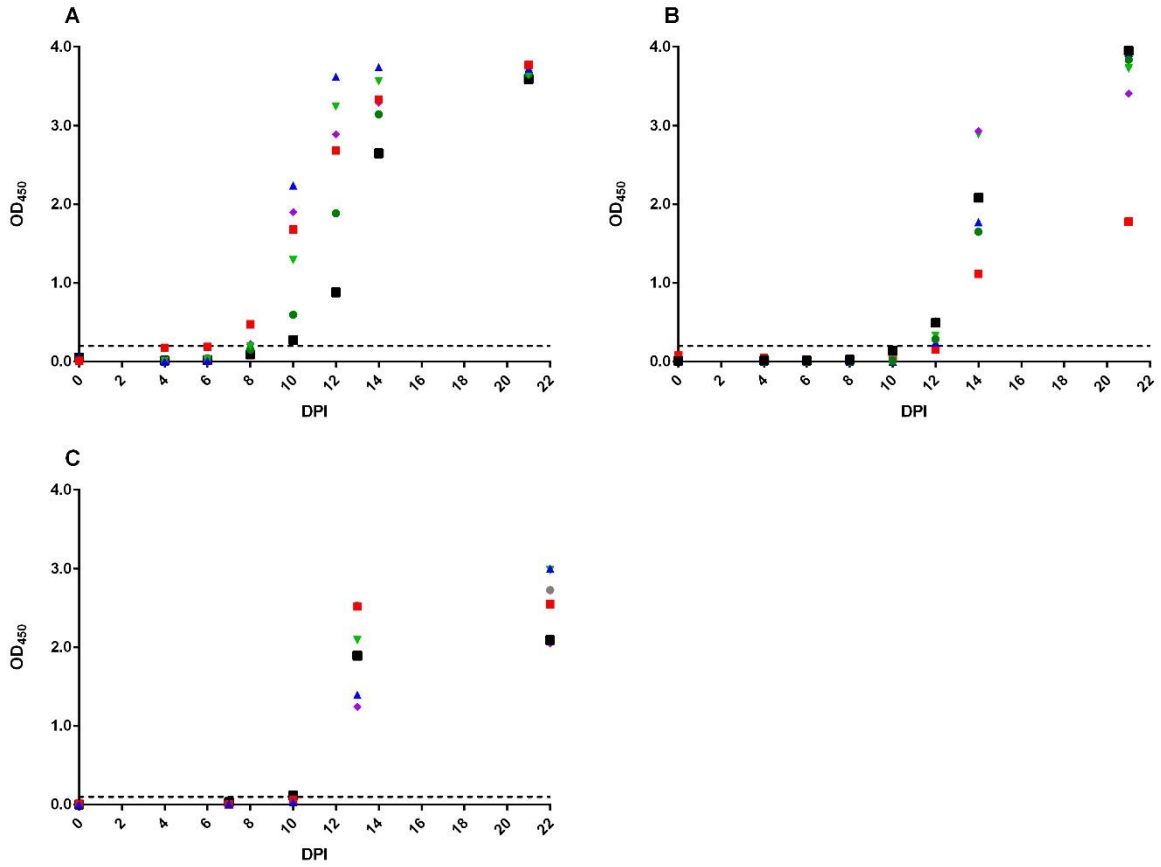

Figure 4. Immunoreactivity of sera collected from sheep, goats, or cattle infected with virulent capripoxvirus isolates. Sequential sera collected at various days post inoculation (DPI) were tested at a dilution of 1:100. (A) Sera from sheep (n=6) inoculated with Nigerian sheeppox virus. (B) Sera from goats (n=6) inoculated with Yemen goatpox virus. (C) Sera from cattle (n=6) inoculated with lumpy skin disease virus (Neethling). Corrected OD values, with background (from no serum control wells) subtracted, are shown. Cut-off values for determination of positive test samples are depicted as dashed lines.

4.5.3 Analytical specificity

To assess analytical specificity of the Cp 25 ELISA, sera collected from 31 sheep that had been infected experimentally with orf virus, a parapoxvirus which is known to cause cross-reactivity in the agar gel immunodiffusion (Kitching *et al.*, 1986) and indirect immunofluorescence antibody (Tuppurainen, 2014a, b) tests, were evaluated. At a serum dilution of 1:400, OD values using an orf virus indirect ELISA, which used an infected cell lysate as coating antigen, ranged from 0.06–1.8, whilst the corresponding OD values using the Cp 25 ELISA, at a serum dilution of 1:100, were uniformly low (0.010 ± 0.015) (Figure 5).

The lack of correlation between the two sets of data demonstrated an absence of cross-reactivity between the recombinant capripoxvirus protein Cp 25 and orf virus antigens. Additional sera from Canadian and Australian sheep and goats that had been infected naturally or experimentally with orf virus also failed to react using the Cp 25 ELISA (data not shown), confirming the utility of this antigen for specific detection of antibodies to sheeppox and goatpox viruses.

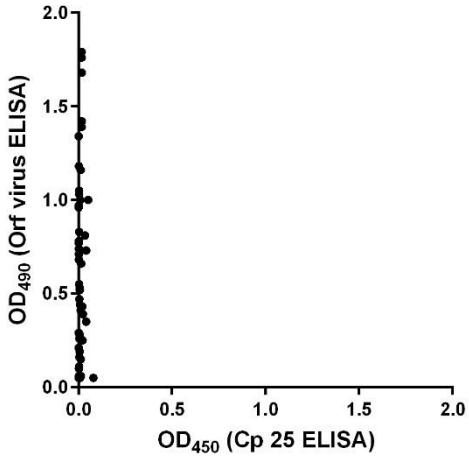

Figure 5. Evaluation of the Cp 25 ELISA analytical specificity using sheep sera. Sequential sera from 31 sheep infected experimentally with orf virus were tested at a dilution of 1:100. A lack of correlation between the corrected OD values using the capripoxvirus ELISA (x-axis) and an orf virus ELISA (y-axis) confirmed the absence of cross-reactivity between the capripoxvirus recombinant protein Cp 25 and orf virus antigens.

In addition, sera collected from cattle in Japan that had been naturally exposed to parapoxviruses (orf or bovine papular stomatitis viruses), as determined by agar gel immunodiffusion (Ito *et al.*, 2011), were also tested. As shown in Figure 6 the OD values for both the agar gel immuodiffusion negative (0.030 ± 0.012) and positive (0.036 ± 0.013) samples, when tested at a dilution of 1:100, were uniformly low, demonstrating the absence of cross-reactivity between the recombinant capripoxvirus protein Cp 25 and parapoxvirus antigens in cattle.

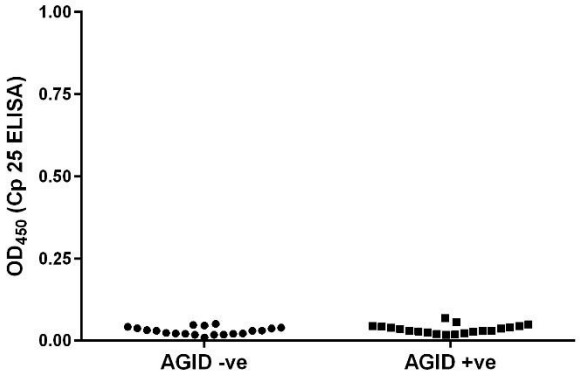

Figure 6. Evaluation of the Cp 25 ELISA analytical specificity using cattle sera. Sera, collected from cattle in Japan that had been naturally exposed to parapoxviruses, were tested at a dilution of 1:100. The absence of detectable antibodies to Cp 25 in both the agar gel immuodiffusion (AGID) negative (n=20) and positive (n=20) samples confirmed the lack of antigenic cross-reactivity to parapoxviruses in cattle sera.

4.5.4 Diagnostic sensitivity and specificity

The Cp 25 ELISA was subsequently evaluated using additional experimental and field sera from sheep, goats, and cattle to determine the diagnostic performance characteristics. The testing was conducted at AAHL, as well as at The Pirbright Institute in England (28 February – 7 April 2014) and the National Centre for Foreign Animal Disease in Winnipeg, Canada (7 June – 7 July 2014), both of which hold neutralising antibody-positive sheep, goat, and cattle sera that have not been subjected to gamma irradiation. All sera were tested, in duplicate, at a dilution of 1:100 and, to enable comparison of data between different ELISA plates, the OD data were normalised against positive control sera and expressed as percent positivity values (mean corrected test OD value ÷ corrected positive control OD value × 100).

Including sequential sera from animals that had been vaccinated, or infected experimentally with virulent virus isolates, as well as sera from natural outbreaks, >2150 samples (>700 sheep, >450 goat and >1000 cattle sera) were used to evaluate the Cp 25 ELISA. However, since repeated observations from the same animals are not considered acceptable for establishing diagnostic sensitivity and specificity (Jacobson and Wright, 2014), the analyses for this purpose were restricted to single samples from individual animals so that the statistical requirement of independent observations was met. The ELISA performed favourably when sera from sheep, goats and cattle were tested, with diagnostic sensitivity and specificity ranging between 98–100% (Figure 7).

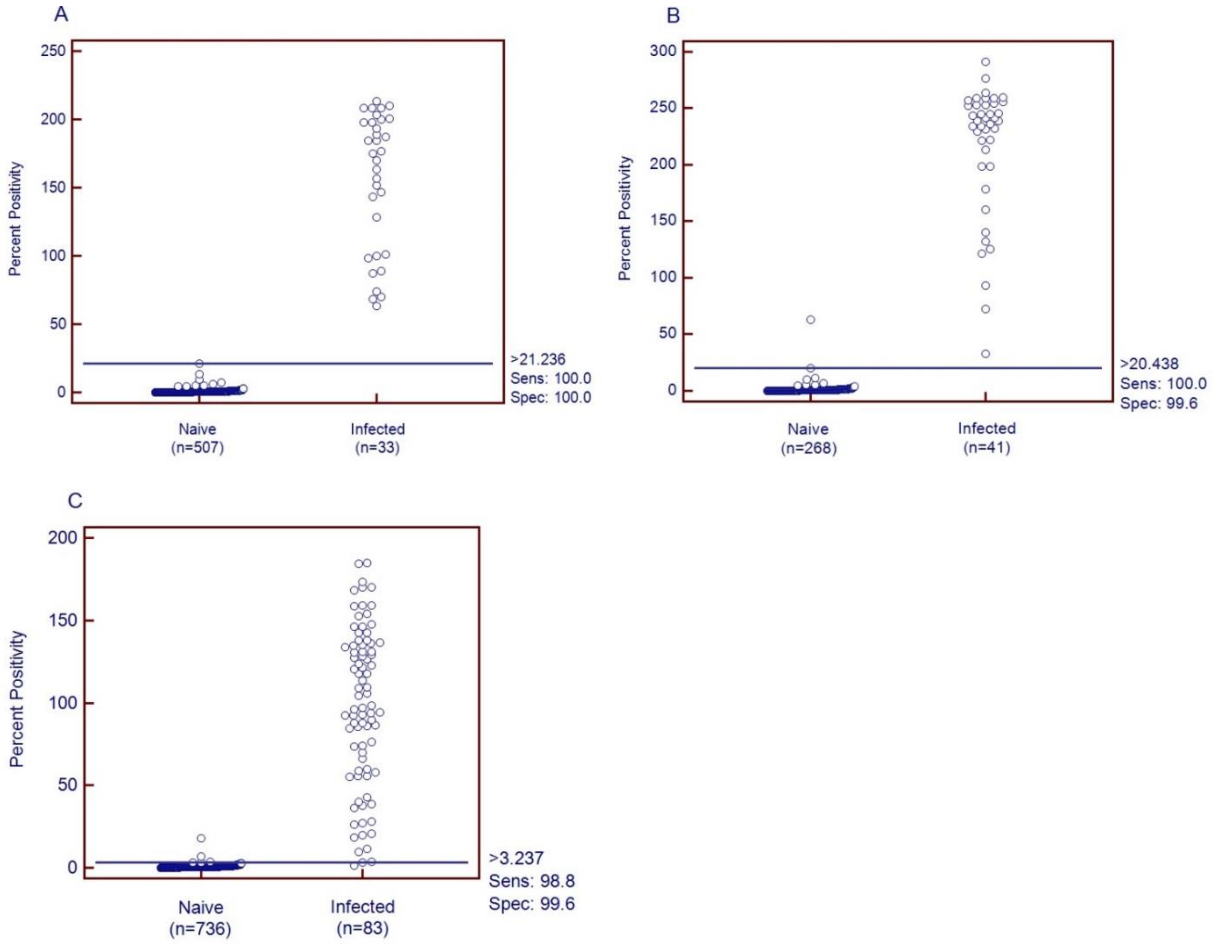

Figure 7. Differentiation of capripoxvirus-naive and infected sheep (A), goat (B), and cattle (C) populations using the Cp 25 ELISA. For each species, the respective cut-off values were determined by receiver operating characteristic (ROC) curve analysis, and are shown as horizontal lines in each panel (21.2, 20.4 and 3.2 for sheep, goats and cattle, respectively).

To determine estimates of diagnostic specificity, sera from Australian and Canadian naive animals were tested. However, since capripoxviruses are exotic to Australia and cannot be imported into the country, these data were compared to the percent positivity values obtained by testing neutralising antibody positive sera from naturally or experimentally infected sheep, goat, and cattle sera held at the collaborating UK and Canadian facilities to determine diagnostic sensitivity. For each host species, the diagnostic performance characteristics (excluding sera from vaccinated animals) were determined to be as follows.

1/ Sheep (number of uninfected animals: 507; number of infected animals: 33)

Diagnostic sensitivity: 100% (95% confidence interval, 89.4–100.0%)

Diagnostic specificity: 100% (95% confidence interval, 99.3–100.0%)

2/ Goats (number of uninfected animals: 268; number of infected animals: 41)

Diagnostic sensitivity: 100% (95% confidence interval, 91.4–100.0%)

Diagnostic specificity: 99.6% (95% confidence interval, 97.9–100.0%)

3/ Cattle (number of uninfected animals: 736; number of infected animals: 83)

Diagnostic sensitivity: 98.8% (95% confidence interval, 93.5–100%)

Diagnostic specificity: 99.7% (95% confidence interval, 98.8–99.9%)

Of note, the ELISA was unable to detect antibodies reliably in sheep, goats, or cattle following vaccination with live-attenuated capripoxvirus vaccine strains (data not shown). However, this appears to be a deficiency encountered with all currently available assays for capripoxvirus serodiagnosis including the serum neutralisation test.

5 Conclusions and recommendations

To enhance diagnostic capability and preparedness eight antibody detection ELISA tests, based on immunodominant viral antigens that had been identified previously using protein microarray technology, were developed during this project. Six were evaluated using sera from experimentally or naturally infected sheep, goats, and cattle. Three assays were chosen for more extensive evaluation at AAHL (Australia), the National Centre for Foreign Animal Disease (Canada), and the Capripoxvirus Reference Laboratory at The Pirbright Institute (UK), based on the ability of the recombinant antigens to detect viral antibodies in sera from all three host species. Although all three exhibited differential reactivity to sheep, goat, and cattle sera, one antigen, Cp 25, consistently demonstrated broader and stronger reactivity, identifying it as the lead candidate for the ELISA validation activities. This ELISA performed favourably when sera from sheep, goats, or cattle infected naturally or experimentally with virulent capripoxvirus isolates were tested, with diagnostic sensitivity and specificity ranging between 98–100%, but it was unable to detect antibodies reliably in vaccinated sheep, goats, or cattle. Nevertheless, this appears to be a deficiency encountered with all currently available assays for capripoxvirus serodiagnosis including the serum neutralisation test.

The key outcome of the project has been the development and preliminary validation of a high-throughput antibody detection ELISA for the diseases caused by capripoxviruses (sheeppox, goatpox, and lumpy skin disease). Although based on the same recombinant antigen, validation data have been generated for each host species (sheep, goats, and cattle) meaning, in effect, that three separate tests have been produced and evaluated. Reliable methods for large scale production and purification of the recombinant antigen have been established, and stocks of purified protein have been prepared and stored for future use.

As outlined in the previous sections, the project objectives are therefore considered to have been achieved successfully. An ELISA, based on a viral recombinant integral membrane protein, with no requirement for infectious reagents, has been developed and undergone validation to Stage 2 of the OIE Assay Validation Pathway (Jacobson and Wright, 2014). However, importantly, this testing was done using different samples that were available at each collaborating laboratory, by the same operator. The next step (Stage 3) in the validation pathway requires the reproducibility of the assay to be determined, which is the ability of the ELISA to provide consistent results when applied to aliquots of the same samples tested in different laboratories (at least three, preferably in different countries), by different operators, using the identical protocol, reagents and controls (a proposal to extend the project for this purpose has been submitted for consideration by the MLA Donor Company). Subject to satisfactory performance, this is a critical component of the assay validation pathway that needs to be undertaken for the test to be considered as "validated for the original intended purpose".

Determination of assay reproducibility would fulfil a key condition in the diagnostic test validation pathway and would be required to obtain international acceptance and recognition of the test, which would be vital for re-establishing disease free status and expediting the resumption of trade in livestock and livestock products in the event of an outbreak. Subject to determining the reproducibility of the assay in multiple laboratories, the ELISA offers the prospect of a reliable and standardised high-throughput antibody detection capability, without the requirement for infectious reagents, to support post-outbreak surveillance and proof of freedom testing in sheep, goats, and cattle. This would address a major deficiency in AAHL's capability and preparedness for undertaking effective post-outbreak sero-surveillance for sheeppox, goatpox, and lumpy skin disease, which would be critical to minimising the impact to the livestock industries in the event of a capripoxvirus outbreak.

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CSIRO Australian Animal Health Laboratory: Dr Axel Colling and Mr Ross Lunt.

National Centre for Foreign Animal Disease (Canada): Drs Shawn Babiuk, Hani Boshra and Tangh Nuen.

The Pirbright Institute (UK): Dr Eeva Tuppurainen and Ms Caroline Chambers

7 Appendices

iELISA Method for Detection of Antibodies to Capripoxviruses

Equipment

Nunc F MaxiSorp 96-well ELISA immuno plates
Nunc F 96-well cell culture plates (for serum dilutions)
Plate shaker
ELISA plate reader (Multiskan EX) with 450 nm filter
Plate washer
Incubator (37 °C)
Fridge (4 °C) / freezer (-20 °C) / freezer (-80 °C)
Balance
pH meter
Glassware: cylinders / bottles
Pipettes and tips

Reagents

Carbonate-Bicarbonate Buffer Capsules (Sigma, C3041-50CAP)
For use in coating plates: dissolve one capsule in 100 ml deionized water to yield a 0.05 M buffer solution.

Dulbecco's Phosphate-Buffered Saline (DPBS) (137.9 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl and 1.5 mM KH_2PO_4) (Life Technologies, 21600-044, powder for 50 L), 0.05% (v/v) Tween 20 (Merck, 8221840500, 500 ml) (PBST)

To make a 10X stock solution of PBST: add powdered medium to 4.5 L of deionised water. Stir gently until dissolved and adjust pH to 7.4. Add 25 ml Tween 20 and adjust final volume to 5 L with deionised water.

To make 1X working solution of PBST: dilute 1000 ml of 10X stock solution to 10 L with deionised water.

Blocking / Dilution Buffer (Stock: 10X Blocking buffer (Sigma, B6429-500ML))
Dilute 100 ml of 10X Blocking buffer to 1000 ml with deionised water.

Conjugate: Recombinant Protein G-HRP (Life Technologies, 10-1223)
Dilute 1:128 000 (v/v) in 1X blocking buffer.

Substrate: 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma, T0440-1L)
Equilibrate required volume to room temperature before use.

1 M H_2SO_4 (Sigma, 339741-100ML)

SLOWLY add 55.6 ml concentrated H_2SO_4 to ~900 ml deionised water (adjust final volume to 1 L with deionised water).

ELISA Procedure

1. Coat a NUNC MaxiSorp ELISA plate with recombinant protein Cp 25 (final concentration 0.6 ng/µl) diluted in 0.05 M carbonate-bicarbonate ELISA coating buffer (100 µl or 60 ng per well).
2. Incubate plate overnight at 4 °C; cover with plate sealer.
3. Wash plate 4x with PBST using plate washer/robotic platform.
4. Block plate by adding 200 µl/well of blocking buffer (Sigma blocker).
5. Incubate for 1 h at 37 °C on a plate shaker.
6. Wash plate 4x with PBST using plate washer/robotic platform.
7. Dilute test sera and controls in blocking buffer (Sigma blocker) and add 100 µl/well.
8. Incubate for 1 h at 37 °C on a plate shaker, cover with plate sealer.
9. Wash plate 4x with PBST using plate washer/robotic platform.
10. Dilute Protein G-HRP conjugate 1:128 000 in blocking buffer and add 100 µl/well.
11. Incubate for 1 h at 37 °C on a plate shaker, cover with plate sealer.
12. Wash plate 4x with PBST using plate washer/robotic platform.
13. Add 100 µl/well TMB substrate and incubate at room temperature for 10 min.
14. Stop reaction with 100 µl/well 1 M H_2SO_4.
15. Read Optical Density at 450 nm on Plate Reader (Multiskan EX).

8 References

Babiuk, S., Bowden, T.R., Boyle, D.B., Wallace, D.B., Kitching, R.P., 2008a. Capripoxviruses: An emerging worldwide threat to sheep, goats and cattle. Transbound.Emerg.Dis. 55, 263-272.

Babiuk, S., Bowden, T.R., Parkyn, G., Dalman, B., Hoa, D.M., Long, N.T., Vu, P.P., Bieu, D.X., Copps, J., Boyle, D.B., 2009a. Yemen and Vietnam capripoxviruses demonstrate a distinct host preference for goats compared with sheep. J.Gen.Virol. 90, 105-114.

Babiuk, S., Bowden, T.R., Parkyn, G., Dalman, B., Manning, L., Neufeld, J., Embury-Hyatt, C., Copps, J., Boyle, D.B., 2008b. Quantification of lumpy skin disease virus following experimental infection in cattle. Transbound.Emerg.Dis. 55, 299-307.

Babiuk, S., Parkyn, G., Copps, J., Larence, J.E., Sabara, M.I., Bowden, T.R., Boyle, D.B., Kitching, R.P., 2007. Evaluation of an ovine testis cell line (OA3.Ts) for propagation of capripoxvirus isolates and development of an immunostaining technique for viral plaque visualization. J.Vet.Diagn.Invest. 19, 486-491.

Babiuk, S., Wallace, D.B., Smith, S.J., Bowden, T.R., Dalman, B., Parkyn, G., Copps, J., Boyle, D.B., 2009b. Detection of antibodies against capripoxviruses using an inactivated sheeppox virus ELISA. Transbound.Emerg.Dis. 56, 132-141.

Bowden, T.R., Babiuk, S.L., Parkyn, G.R., Copps, J.S., Boyle, D.B., 2008. Capripoxvirus tissue tropism and shedding: A quantitative study in experimentally infected sheep and goats. Virology 371, 380-393.

Bowden, T.R., Coupar, B.E., Babiuk, S.L., White, J.R., Boyd, V., Duch, C.J., Shiell, B.J., Ueda, N., Parkyn, G.R., Copps, J.S., Boyle, D.B., 2009. Detection of antibodies specific for sheeppox and goatpox viruses using recombinant capripoxvirus antigens in an indirect enzyme-linked immunosorbent assay. J.Virol.Methods 161, 19-29.

Davies, D.H., Liang, X., Hernandez, J.E., Randall, A., Hirst, S., Mu, Y., Romero, K.M., Nguyen, T.T., Kalantari-Dehaghi, M., Crotty, S., Baldi, P., Villarreal, L.P., Felgner, P.L., 2005. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. Proc.Natl.Acad.Sci.U.S.A. 102, 547-552.

Embury-Hyatt, C., Babiuk, S., Manning, L., Ganske, S., Bowden, T.R., Boyle, D.B., Copps, J., 2012. Pathology and viral antigen distribution following experimental infection of sheep and goats with capripoxvirus. J.Comp Pathol. 146, 106-115.

Garner, M.G., Lack, M.B., 1995. Modelling the potential impact of exotic diseases on regional Australia. Aust.Vet.J. 72, 81-87.

Ito, M., Murakami, T., Hayakawa, Y., Shintani, E., Inoshima, Y., 2011. Serological and epidemiological studies of parapoxvirus infection in cattle in Ishikawa Prefecture. Jpn.Agr.Res.Quart. 45, 123-127.

Jacobson, R.H., Wright, P., 2014. Chapter 1.1.5. Principles and methods of validation of diagnostic assays for infectious diseases, Office International des Epizooties, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris.

Kitching, R.P., Hammond, J.M., Black, D.N., 1986. Studies on the major common precipitating antigen of capripoxvirus. J.Gen.Virol. 67, 139-148.

Stubbs, S., Oura, C.A., Henstock, M., Bowden, T.R., King, D.P., Tuppurainen, E.S., 2012. Validation of a high-throughput real-time polymerase chain reaction assay for the detection of capripoxviral DNA. J.Virol.Methods 179, 419-422.

Tuppurainen, E., 2014a. Chapter 2.4.14. Lumpy skin disease, Office International des Epizooties Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris.

Tuppurainen, E., 2014b. Chapter 2.7.14. Sheep pox and goat pox, Office International des Epizooties, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris.

Wright, P.F., Nilsson, E., Van Rooij, E.M., Lelenta, M., Jeggo, M.H., 1993. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. Rev.Sci.Tech. 12, 435-450.