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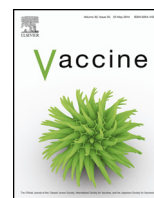
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Immunisation with bacterial expressed VP2 and VP5 of bluetongue virus (BTV) protect α/β interferon-receptor knock-out (IFNAR^{-/-}) mice from homologous lethal challenge

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ABSTRACT

BTV-4 structural proteins VP2 (as two domains: VP2D1 and VP2D2), VP5 (lacking the first 100 amino acids: VP5_{Δ1–100}) and full-length VP7, expressed in bacteria as soluble glutathione S-transferase (GST) fusion-proteins, were used to immunise Balb/c and α/β interferon receptor knock-out (IFNAR^{-/-}) mice. Neutralising antibody (NAb) titres (expressed as log₁₀ of the reciprocal of the last dilution of mouse serum which reduced plaque number by $\geq 50\%$) induced by the VP2 domains ranged from 1.806 to 2.408 in Balb/c and IFNAR^{-/-} mice.

The immunised IFNAR^{-/-} mice challenged with a homologous live BTV-4 survived and failed to develop signs of infection (ocular discharge and apathy). Although subsequent attempts to isolate virus were unsuccessful (possibly reflecting presence of neutralising antibodies), a transient/low level viraemia was detected by real time RT-PCR. In contrast, mice immunised with the two VP2 domains with or without VP5_{Δ1–100} and VP7, then challenged with the heterologous serotype, BTV-8, all died by day 7 post-infection.

We conclude that immunisation with bacterially-expressed VP2 domains can induce strong serotype-specific NAb responses. Bacterial expression could represent a cost effective and risk-free alternative to the use of live or inactivated vaccines, particularly if viruses prove to be difficult to propagate in cell culture (like BTV-25). A vaccine based on bacterially expressed VP2 and VP5 of BTV is also DIVA-compatible.

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1. Introduction

Bluetongue virus is the type species of genus *Orbivirus*, family *Reoviridae* [1,2]. Bluetongue viruses (BTV) are transmitted by adult *Culicoides* midges, causing 'bluetongue' (BT), a non-contagious but economically important disease of ruminants (sheep, cattle and some species of deer) [3,4]. Currently 26 BTV serotypes have been identified, 10 of which (BTV-1, 2, 4, 6, 8, 9, 11, 14, 16 and 25) have been detected in Europe since 1998 [5–7]. It is estimated that over one million sheep have died during repeated BT incursions into the Mediterranean basin between 1998 and 2005 [5]. An outbreak caused by BTV-8 that started in the Netherlands during 2006, subsequently spread across most of Europe, causing high levels of mortality in sheep (15–32%, reaching ~50% in some

areas), as well as significant clinical signs but low mortality (<1%) in cattle [8–13]. However, inactivated-virus vaccines were used successfully, leading to the rapid eradication of BTV-8 from the region. These inactivated vaccines, which were made available for serotypes 1, 2, 4 and 8 of BTV are thought to work primarily through generation of a protective serotype-specific neutralising-antibody response targeting the VP2 antigen [2,14–21].

The BTV particle is made of seven structural proteins (VP1–VP7) [2,22,23]. VP2 represents a primary target for neutralising antibodies [1,2,16,17] and determines virus serotype [24]. VP2 shows 22.4–73% aa sequence variation between BTV serotypes [24]. VP5 of BTV, the second most variable BTV protein (aa identity of 41–79% between BTV serotypes [25,26]) enhances neutralising antibody response to VP2 [1,2,14,27].

Selected structural-proteins of BTV-4, including two domains of VP2 (aa 63–471 and 555–956), VP5 (from which a coiled-coil sequence [amino acids 1–100] was deleted to improve solubility) and full-length VP7, were expressed in bacteria as soluble

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fusion-proteins with glutathione S-transferase (GST). We report the use of these proteins to immunise mice, generating neutralising-antibodies to the homologous BTV serotype.

2. Materials and methods

2.1. Cell lines, viruses and mice

KC cells (*Culicoides variipennis*) were grown at 28 °C in Schneider's Drosophila medium, supplemented with 10% foetal bovine serum (FBS). BHK-21 cells (European Collection of Animal cell Cultures: ECACC – 84100501), or BSR cells (a clone of BHK-21 a gift from Dr. Noel Tordo, Institut Pasteur) were grown at 37 °C in Glasgow's Minimum-Essential-Medium supplemented with 10% FBS.

BTV-4(SPA2003/01) was from blood of sheep showing severe clinical disease (Spain 2003). The virus was isolated in embryonated eggs then adapted to BHK-21 cells (E1/BHK4). BTV-4(SPA2003/01) was used for RNA extraction/cDNA synthesis for the purpose of generating protein expression constructs.

BTV-4-Italy03 and BTV-8-France-28 were isolated in embryonated eggs, from sheep-blood (Italy), or cow-blood (France), then adapted to BHK-21 cells (BTV-4-E1/BHK4 or BTV-8-E1/BHK2). These isolates were used for homologous and heterologous challenge of IFNAR^{-/-} mice.

Six weeks-old female Balb/cByJ mice were obtained from Charles River laboratories. Groups of six animals were immunised with proteins to assess NAb production.

Six weeks-old female IFNAR^{-/-} mice (genetic background: A129SvEvBrd) were obtained from B&K Universal Ltd. Groups of six animals were used for immunisation with soluble expressed-proteins followed by homologous or heterologous challenge with live BTV.

Immunisation protocols were approved by ethics committees at the Pirbright Institute (license number 70/6133) and ANSES (license number 12/04/11-5).

2.2. Construction of expression plasmids

Previous analysis has indicated that BTV-VP2 is potentially made of two related domains [18]. We used BTV-4(SPA2003/01) VP2 domains which encompassed amino acid sequences 63–471 (44.5 kDa) and 555–956 (46 kDa) (nucleotide positions: 187–1326 and 1663–2868, Genbank accession: KJ700442).

VP5 lacked aa 1–100 (used sequence encompassed nucleotide positions 289–1581, Genbank accession: AJ783908) while the full-length aa sequence of VP7 was used (nucleotide positions: 1–1050, Genbank accession: KJ700443). All cDNAs were cloned into pGEX-4T-2 (expressing GST). The resulting plasmids are pGEX-BTV4VP2D1, pGEX-BTV4VP2D2, pGEX-BTV4VP5 and pGEXBTV4VP7. Their sequences were confirmed by comparison to parental virus sequences. Theoretical sizes of the GST-fused proteins are 70.5 kDa (VP2 domain 1), 72 kDa (VP2 domain 2), 73 kDa (VP5 lacking aa 1–100) and 64.5 kDa for the VP7. The full-length ORFs of VP2, VP5 and VP7 were also cloned in the mammalian-expression plasmid pCIneo (pCIneo-BTV-4VP2, pCIneo-BTV-4VP5, or pCIneo-BTV-4VP7).

2.3. Protein expression and purification

pGEX-BTV4VP2D1, pGEX-BTV4VP2D2, pGEX-BTV4VP5 and pGEXBTV4VP7 were used to transform C41 bacteria, known to improve solubility of expressed proteins [28]. Overnight bacterial cultures were grown in 2XYT medium at 37 °C. On the day of expression bacterial cultures were grown until OD₆₀₀ reached 0.6, then fusion-protein expression was induced by addition of 0.5 mM IPTG and incubation of the cultures at 28 °C for 4 h with

shaking at 200 rpm. Bacteria were pelleted (3250 g) and lysed by sonication. Soluble proteins were purified from bacterial lysates by glutathione-affinity chromatography as previously described [29], then analysed by sodium-dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). GST-fused proteins from inclusion bodies (insoluble fraction) were dissolved in a CAPS buffer (CAPS 50 mM, DTT 1 mM and Sarkosyl 0.3%), hence denaturing the proteins [30]. The dissolved and denatured protein was dialyzed overnight against 20 mM Tris-HCl pH 8.5. Insoluble proteins dissolved in CAPS buffer/dialysed are referred to as 'CAPS-denatured proteins' throughout the text. Purified proteins were quantified by two different methods: (i) a Bradford assay at 595 nm and (ii) UV spectrophotometry at 280 nm (extinction coefficient determined from aa sequences of each fusion protein). Concentration measurements were consistent using both methods. Relative amounts of proteins to be injected were based on copy number considerations in a BTV particle, as determined by X-ray crystallography (780 copies for VP7, 360 copies for VP5 and 180 copies for VP2 [1]).

2.4. Immunisation of mice with soluble bacterial expressed proteins

2.4.1. Immunisation of Balb/c

Seven groups of six Balb/c mice were injected subcutaneously at days 0, 14 and 28 with 100 µl of soluble protein/Montanide ISA 50V emulsion (Table 1).

Three groups of six Balb/c mice were injected subcutaneously at days 0, 14 and 28 with 100 µl of CAPS-denatured protein/Montanide ISA 50V emulsion (Table 1).

A group of six Balb/c mice were injected subcutaneously at days 0, 14 and 28 each with 100 µl of Zulvac-4[®] Bovis. Sera were used for normalisation of ELISA results.

A group of six control Balb/c mice which were not immunised with any of the antigens was also included.

2.4.2. Immunisation and challenge of IFNAR^{-/-} mice

Six groups of six IFNAR^{-/-} mice were injected subcutaneously at days 0, 14 and 28 with: a mixture of VP2 domain 1 (VP2D1) and VP2 domain 2 (VP2D2) in Montanide, then challenged with (i) BTV-4 or (ii) BTV-8; or a mixture of VP2D1 + VP2D2 + VP5_{Δ1–100}/Montanide, then challenged with (iii) BTV-4 or (iv) BTV-8; or a mixture of VP2D1 + VP2D2 + VP5_{Δ1–100} + VP7/Montanide, then challenged with (v) BTV-4 or (vi) BTV-8 (Table 1). Blood samples were collected at day 0 and day 28.

The mice received an intravenous lethal [31] challenge on day 40, with 10³ pfu of BTV-4-italy03 (homologous-challenge), or 10 pfu of BTV-8-28 (heterologous-challenge). Blood was collected on the day of challenge (day 40), then at days 2, 3, 4, 5, 7, 10 and 12 p.i. Sera were tested for anti-VP2, anti-VP5 and anti-VP7 antibodies by ELISA and immunofluorescence and for NABs by PRNT.

Two groups of six IFNAR^{-/-} mice were injected subcutaneously with VP5_{Δ1–100} on days 0, 14 and 28. These groups were not challenged with BTV-4 or BTV-8. Two additional groups of six IFNAR^{-/-} mice were immunised with VP7 on days 0, 14 and 28, then challenged at day 40 with either BTV-4 or BTV-8.

Two groups of non-immunised mice were used as positive controls, to confirm lethality of BTV-4 or BTV-8 challenge-strains. Blood from these two groups was taken at days 0 and 40 (just before challenge) in order to assess reactivity of sera with BTV-4 ELISA and for PRNT.

2.5. Detection of BTV-specific antibodies

2.5.1. Western blot

Sera were analysed by western blotting using BTV-infected cell-lysate antigens, as previously described [29,30,32]. Anti-VP2,

Table 1
combinations of antigens used for immunisation (Balb/c or IFNAR^{-/-} mice) and levels of antibodies in mouse sera against BTV-4 (by PRNT and ELISA).

Antigen form	Antigen used in 1st, 2nd and 3rd injections (amount of antigen/animal)	Mouse strain (number of animals)	NA at day 40 (A)	Mean antibody titre $\pm$ SD (B)	Statistical groups (C)	ELISA mean NOD $\pm$ SD (D)
Soluble proteins	VP2D1 (15 μ g)	Balb/c (6)	1/32–1/40	1.569 $\pm$ 0.06	C	0.68 $\pm$ 0.04
Soluble proteins	VP2D2 (15 μ g)	Balb/c (6)	1/2–1/3	0.498 $\pm$ 0.05	D	0.59 $\pm$ 0.05
Soluble proteins	VP2D1 (15 μ g) + VP2D2 (15 μ g)	Balb/c (6)	1/64–1/128	2.007 $\pm$ 0.15	B	0.75 $\pm$ 0.05
Soluble proteins	VP2D1 (15 μ g) + VPD2 (15 μ g) + VP5 _{Δ1–100} (25 μ g)	Balb/c (6)	1/128–1/256	2.358 $\pm$ 0.12	A	0.78 $\pm$ 0.04
Soluble proteins	VP2D1 (15 μ g) + VPD2 (15 μ g) + VP5 _{Δ1–100} (25 μ g) + VP7 (40 μ g)	Balb/c (6)	1/128–1/256	2.258 $\pm$ 0.16	A	0.82 $\pm$ 0.06
Soluble proteins	VP5 _{Δ1–100} (25 μ g)	Balb/c (6)	NNT	N/A	N/A	0.63 $\pm$ 0.04
Soluble proteins	VP7 (40 μ g)	Balb/c (6)	NNT	N/A	N/A	0.67 $\pm$ 0.04
Insoluble proteins (dissolved in CAPS buffer)	VP2D1 (15 μ g)	Balb/c (6)	NNT	N/A	N/A	0.63 $\pm$ 0.05
Insoluble proteins (dissolved in CAPS buffer)	VP2D2 (15 μ g)	Balb/c (6)	NNT	N/A	N/A	0.59 $\pm$ 0.04
Insoluble proteins (dissolved in CAPS buffer)	VP2D1 (15 μ g) + VP2D2 (15 μ g)	Balb/c (6)	NNT	N/A	N/A	0.73 $\pm$ 0.04
Zulvac [®] -4	Zulvac [®] -4 (100 μ l)	Balb/c (6)	1/256–1/512	2.508 $\pm$ 0.15		
None	None	Balb/c (6)	NNT	N/A	N/A	0.05 $\pm$ 0.02
Soluble proteins	VP2D1 (15 μ g) + VP2D2 (15 μ g)	IFNAR ^{-/-} (12)	1–64–1/128	2.007 $\pm$ 0.15	B	0.73 $\pm$ 0.0
Soluble proteins	VP2D1 (15 μ g) + VPD2 (15 μ g) + VP5 _{Δ1–100} (25 μ g)	IFNAR ^{-/-} (12)	1/128–1/256	2.333 $\pm$ 0.14	A	0.78 $\pm$ 0.05
Soluble proteins	VP2D1 (15 μ g) + VPD2 (15 μ g) + VP5 _{Δ1–100} (25 μ g) + VP7 (40 μ g)	IFNAR ^{-/-} (12)	1/128–1/256	2.257 $\pm$ 0.16	A	0.82 $\pm$ 0.05
Soluble proteins	VP5 _{Δ1–100} (25 μ g)	IFNAR ^{-/-} (12)	NNT	N/A	N/A	0.58 $\pm$ 0.04
Soluble proteins	VP7 (40 μ g)	IFNAR ^{-/-} (12)	NNT	N/A	N/A	0.61 $\pm$ 0.06
None	None	IFNAR ^{-/-} (12)	NNT	N/A	N/A	0.04 $\pm$ 0.02

A: minimum and maximum dilutions of mice sera (per group of mice) collected at day 40 (12 days after receiving the third dose of antigen) which reduced the BTV-4 plaque number by $\geq$ 50%.

B: mean value of the log₁₀ ($\pm$ standard deviation: SD) of the reciprocal of the last dilution of mouse serum which reduced BTV-4 plaque numbers by $\geq$ 50%.

C: statistical information for groups of mice obtained using Tukey's method, 95.0% confidence. Mean antibody titres (shown in B) which do not share a letter are significantly different.

D: ELISA was conducted using heat inactivated BTV-4 only (not BTV-8).

N/A: not applicable; NNT: no neutralisation.

anti-VP5 or anti-VP7 antibodies were diluted at 1/50, while anti-mouse peroxidase-conjugated antibody was diluted at 1/750.

2.5.2. ELISA

Supernatant of BTV-4-infected BHK-21 cells was clarified by centrifugation at 3000 $\times$ g, then inactivated at 56 °C for 1 h. The inactivated BTV-4 virus suspension was mixed volume to volume with 100 mM sodium carbonate buffer pH 9.6 and 100 μ l was used to coat 96 well plates (4 °C for 16 h). Sera were diluted 1/100 in 5% skim-milk and ELISA were conducted as previously described [29,30,32]. A serum sample from Balb/c mice immunised with Zulvac-4[®]-Bovis (inactivated BTV-4, Zoetis) was identified as the 'standard' against which all OD readings were subsequently normalised. Normalised optical density (NOD) was calculated as NOD = [OD (sample) – OD (Blank reaction)]/[OD (standard) – OD (Blank reaction)]. An ELISA based on clarified supernatants from non-infected cells was also used.

2.5.3. Immunofluorescence

BSR cells were grown on coverslips in 24-well plates, transfected with pCIneo-BTV-4VP2, pCIneo-BTV-4VP5, or pCIneo-BTV-4VP7 and processed for immunofluorescence as previously described [22]. Cells were probed with anti-VP2, anti-VP5 or anti-VP7 antibodies diluted 1/500 in phosphate-buffered saline containing 0.5% bovine serum albumin.

2.5.4. Plaque-reduction neutralisation-tests (PRNT) and plaque assay

BSR cells were plated (1 $\times$ 10⁵ cells/well) in 48 well plates a day before PRNT initiated [33]. 50 pfu of BTV-4 or BTV-8, in 125 μ l of Eagle's minimum essential medium (EMEM), were incubated with 125 μ l of two-fold serial dilutions of mouse sera in EMEM, incubated at 37 °C for 2 h, then added to confluent BSR cell-monolayers. The supernatant was discarded and replaced with molten 1% low melting point agarose (Sigma) in EMEM. Plates were subsequently incubated at 37 °C for 5 days, fixed by addition of 2 ml of 10% formaldehyde in phosphate-buffered saline per well. After removal of agarose plugs, monolayers were stained with 0.1% naphthalene-black solution, then washed with deionised water and plaques counted.

For plaque assay, the number of plaque-forming units (PFU) was determined using the same approach, while omitting the use of mouse serum.

2.6. RNA extraction and real-time PCR

BTV-4(SPA2003/01) infected BHK-21 cells were harvested at day 4 post-infection. Cells were centrifuged at 2000 $\times$ g and pellets were extracted with 'RNA Now' (Biogentex) [34].

Blood from challenged IFNAR^{-/-} mice was extracted using 'RNA Now' as previously described [35,36]. This extraction method results in high sensitivity for viral RNA detection in mouse blood [36].

Supernatants from BTV-4 or BTV-8 infected cell-cultures were clarified at $2000 \times g$, concentrated 10-fold using Vivaspin® concentrators (MWCO 100K) then treated with RNase-A and benzonase to remove non-encapsidated nucleic acids. Ten-fold serial dilutions of the concentrated virus were prepared in serum-free GMEM, for titration in triplicate by PRNT (as described above).

A dilution series of concentrated supernatant was also prepared in GMEM and added to non-infected mouse blood, then extracted with 'RNA Now', to determine the correlation between PFU and real-time RT-PCR 'cycle threshold' (Ct) values (to allow estimates of PFU-equivalents, only when BTV RNA was detected by RT-PCR but no virus could be isolated from blood samples).

The presence of viraemia was 'assessed' by BTV serogroup-specific real-time RT-PCR targeting Seg-1 [37] and virus isolation on BSR and KC cells.

2.7. Statistical analyses

Analysis of variance (ANOVA) between groups of mice, was carried out using Minitab-16 software (Minitab Inc., UK), or the Systat-5.03 program (Systat Inc., Evanston, IL). Statistical significance between groups was assessed by a general linear model using Tukey's test (differences are considered as statistically significant when $P < 0.05$).

3. Results

3.1. Expression VP2 domains, VP5 and VP7

Expression of GST-fused domains VP2D1 (aa 63–471) and VP2D2 (aa 555–955) in C41 bacteria at 28°C enhanced their solubility ($\sim 30\%$ soluble proteins) (Fig. 1A). The yields of soluble GST-fused VP2 domains were similar batch to batch at ~ 0.5 mg/ml (1 ml of protein from 100 ml of bacterial culture).

Deletion of aa 1–100, which forms part of the coiled-coils NH2-terminal structure (VP5 $_{\Delta 1-100}$) dramatically increased solubility (Fig. 1B) ($\sim 60\%$ soluble protein), yielding 1.5 mg/ml of protein (1 ml of protein from 100 ml of bacterial culture). Deletion of residues beyond aa 100 caused no further improvement in solubility.

The expressed BTV-4-VP7(T13)/GST-fusion protein was soluble (Fig. 1C) at a concentration of ~ 1 mg/ml (1 ml of protein from 100 ml of bacterial culture).

3.2. Relating cycle-threshold (Ct) values to PFU-equivalents of BTV-4 or BTV-8

Standard curves were generated to compare Ct values from real-time RT-PCR assays, with virus titres (PFU/ml) for BTV-4 and BTV-8 preparations. Both curves show a high correlation (R^2 values of 0.988 and 0.997 respectively). The number of PFU-equivalents for BTV-4 or BTV-8 in mouse blood can be calculated from the formulas $y = -1.667\ln(x) + 37.874$ (BTV-4) or $y = -1.772\ln(x) + 38.082$ (BTV-8), where y is the Ct value determined by real time PCR assay and x is the number of PFU-equivalents/ml. The value of x will be $x = e^{(y-37.874)/(-1.667)}$ for BTV-4, or $x = e^{(y-38.082)/(-1.772)}$ for BTV-8, where $e = 2.71828$ is the base of natural logarithm. Results were consistent when BTV-4 or BTV-8 were grown in different batches of BSR cells. Otherwise, number of PFU was determined by virus isolation on BSR cells.

3.3. Immunisation of mice, PRNT and ELISA

3.3.1. Immunisation of Balb/c

CAPS-denatured BTV-4 VP2 domain 1 and 2/GST-fusion proteins raised antibodies which detected a ~ 110 kDa protein (corresponding to VP2) in a BTV-4(SPA2003/01) infected-cell lysate, by

Western-blotting (Fig. 1d). They also detected inactivated BTV antigen in ELISA (Table 1), but failed to neutralise BTV-4(SPA2003/01).

Soluble BTV-4 VP2 domain 1 and 2/GST-fusion proteins, VP5 $_{\Delta 1-100}$ and VP7, all raised antibodies that gave positive results using inactivated BTV-4 ELISA (Table 1). These antibodies also detected bands of the predicted size for VP2 (~ 110 kDa), VP5 (~ 60 kDa) and VP7 (~ 38 kDa) in BTV-4(SPA2003/01) infected cell lysates by western-blotting (Fig. 1e, f, g). In contrast to expressed proteins that had been 'CAPS-denatured', antisera against the soluble amino terminal domain of VP2 contained NABs with titres of 1.505–1.602 (Table 1), giving $\geq 50\%$ plaque reduction. Lower titres of neutralising antibodies (0.301–0.477, $P < 0.05$) were found in antisera against the carboxy-terminal domain. Sera from mice immunised with: VP2D1 + VP2D2; VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$; or VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ + VP7, all neutralised the homologous BTV-4(SPA2003/01) at higher titres (1.806–2.408) but (as expected) failed to neutralise BTV-8 (Table 1).

Neutralising antibody titres generated by Balb/c mice immunised with VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ or VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ + VP7 were not significantly different, but were significantly higher ($P < 0.05$) than those immunised with VP2D1 + VP2D2 (Table 1).

3.3.2. Immunisation of IFNAR $^{-/-}$

Neutralising antibody (NAb) titres of 1.806–2.017 were detected in mice immunised with VP2D1 + VP2D2; with 2.017–2.408 in those immunised with VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ or VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ + VP7 (Table 1), supporting previous studies indicating that VP5 may play a significant role in generation of NABs [38–40]. There was no statistical difference between immunisation with VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$, or VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ + VP7, but a significant difference compared to immunisation with VP2D1 + VP2D2 only ($P < 0.05$) (Table 1).

Sera from IFNAR $^{-/-}$ mice immunised with recombinant VP2D1 + VP2D2, VP5 $_{\Delta 1-100}$ and VP7, either singly or in different combinations, all reacted with BTV-4 by ELISA (Table 1). The specificity of the antibodies was also confirmed by immunofluorescence (supplementary figure).

Sera from non-immunised mice did not neutralise BTV-4 nor show cross reactivity with BTV-4 ELISA.

3.4. Challenge of IFNAR $^{-/-}$ mice and viraemia

Mouse survival times p.i. provide a relative measure of protection afforded by vaccination.

Blood samples collected on days 2, 3, 4, 5, 7, 10 and 12 p.i., and tested.

3.4.1. Immunised IFNAR $^{-/-}$ challenged with BTV-4

Mice immunised with VP2D1 + VP2D2, VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ or VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ + VP7, then challenged with BTV-4, all survived until the end of the experiment on day 52 (12 days p.i.) (Fig. 2A). Two mice immunised with VP2D1 + VP2D2 were positive (Ct value of 34) on day 4 p.i. with BTV-4. Because no virus could be isolated from blood on KC cells or by plaque assay using BSR cells (possibly reflecting the presence of neutralising antibodies), we calculated PFU-equivalents using the formula linking Ct values to PFU numbers. A low PFU-equivalents/ml was calculated (~ 0.3 –9). Two mice in each group immunised with VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$, or VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ + VP7, were also potentially viraemic on day 5 p.i. (Ct values ~ 39), although no virus could be isolated on KC cells or by plaque assay on BSR cells (Fig. 2B). None of the animals in groups immunised with VP2D1 + VP2D2 showed any signs of infection (ocular discharge and apathy [31]) post-challenge with BTV-4 and no

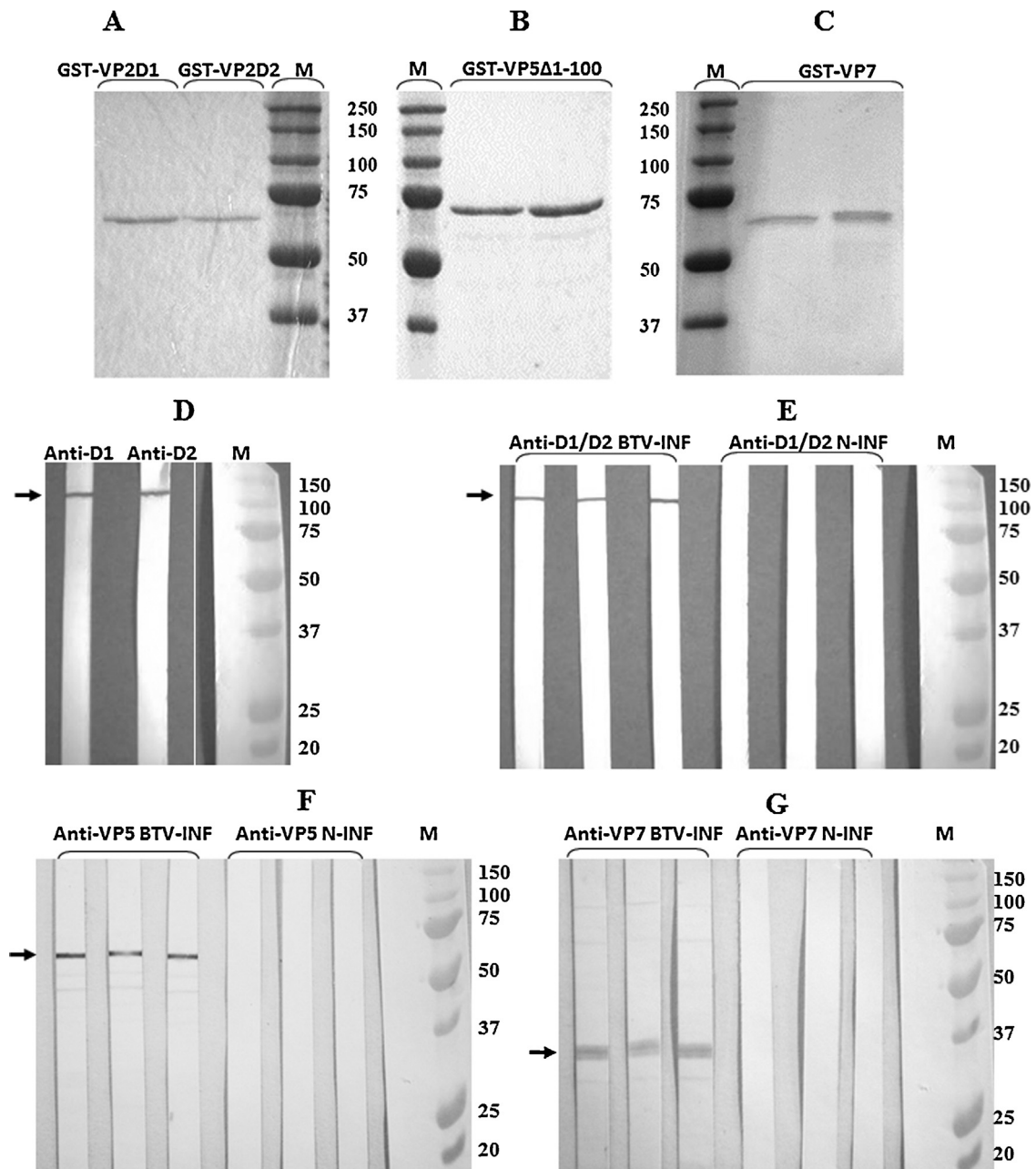


Fig. 1. VP2D1, VP2D2, VP5 $_{\Delta 1-100}$ and VP7 expression in bacteria and western blots using BTV-4 infected BSR cell lysates as antigens. (A–C) Bacterial expression of recombinant proteins. Gels were stained with Coomassie blue. Proteins were expressed in C41 bacteria, purified by glutathione-affinity chromatography and analysed by SDS-PAGE. (A) Soluble VP2 domains 1 (GST-VP2D1) and 2 (GST-VP2D2) fused to GST. (B) Soluble GST-VP5 $_{\Delta 1-100}$. (C) Soluble full-length GST-VP7. (D–G) Western blots using Balb/c mouse antibodies generated against the recombinant proteins VP2D1 + VP2D2, VP5 $_{\Delta 1-100}$ or VP7 tested with BTV-4-infected cell lysates. Lane M: size marker labelled in kDa. The position of the protein band revealed by western blot is indicated by an arrow. (D) Antibodies against VP2D1 and VP2D2 from inclusion bodies (insoluble proteins dissolved in CAPS buffer prior to immunisation). (E) Antibodies to mixtures of soluble VP2D1 + VP2D2 tested against lysates of BTV-4-infected (INF) or non-infected (N-INF) BSR cells. (F) Antibodies to VP5 $_{\Delta 1-100}$ tested against lysates of BTV-4-infected (INF) or non-infected (N-INF) BSR cells. (G) Antibodies to full-length VP7 tested against lysates of BTV-4-infected (INF) or non-infected (N-INF) BSR cells.

viraemia was detected by RT-PCR after day 7 p.i. All animals survived to the end of the experiment (Table 2, Fig. 2A).

3.4.2. Immunised IFNAR $^{-/-}$ challenged with BTV-8

Mice immunised with VP2D1 + VP2D2, or VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ of BTV-4, but challenged with BTV-8, showed signs of infection by day 3 p.i., and all had died by day 5 p.i. (Fig. 2C). Ct values of 20.7–22.4, and virus titres calculated by plaque assay were 7×10^3 – 2×10^4 pfu/ml on day 4 p.i. In contrast, time of death was delayed (day 5–7 p.i. [$P < 0.05$]) by addition of VP7 to this immunisation regime (BTV-4 VP2D1 + VP2D2 + VP5 $_{\Delta 1-100}$ + VP7) (Fig. 2C),

with Ct values on day 5 p.i. of 22.4–23.7 (virus titres calculated by plaque assay: 3×10^3 – 7×10^3 pfu/ml, Fig. 2D).

3.4.3. Controls

The two non-immunised control-groups, challenged with BTV-4(italy03), or BTV-8(BTV-8-28) were all positive by RT-PCR on day 3 p.i. and all died by day 5 p.i. (Fig. 2A and C) with Ct values 20.9–22.7. Virus was successfully isolated from these animals on both KC cells and BSR (BSR plaque assay titres: 5×10^3 – 3×10^4 pfu/ml (Fig. 2B and D)).

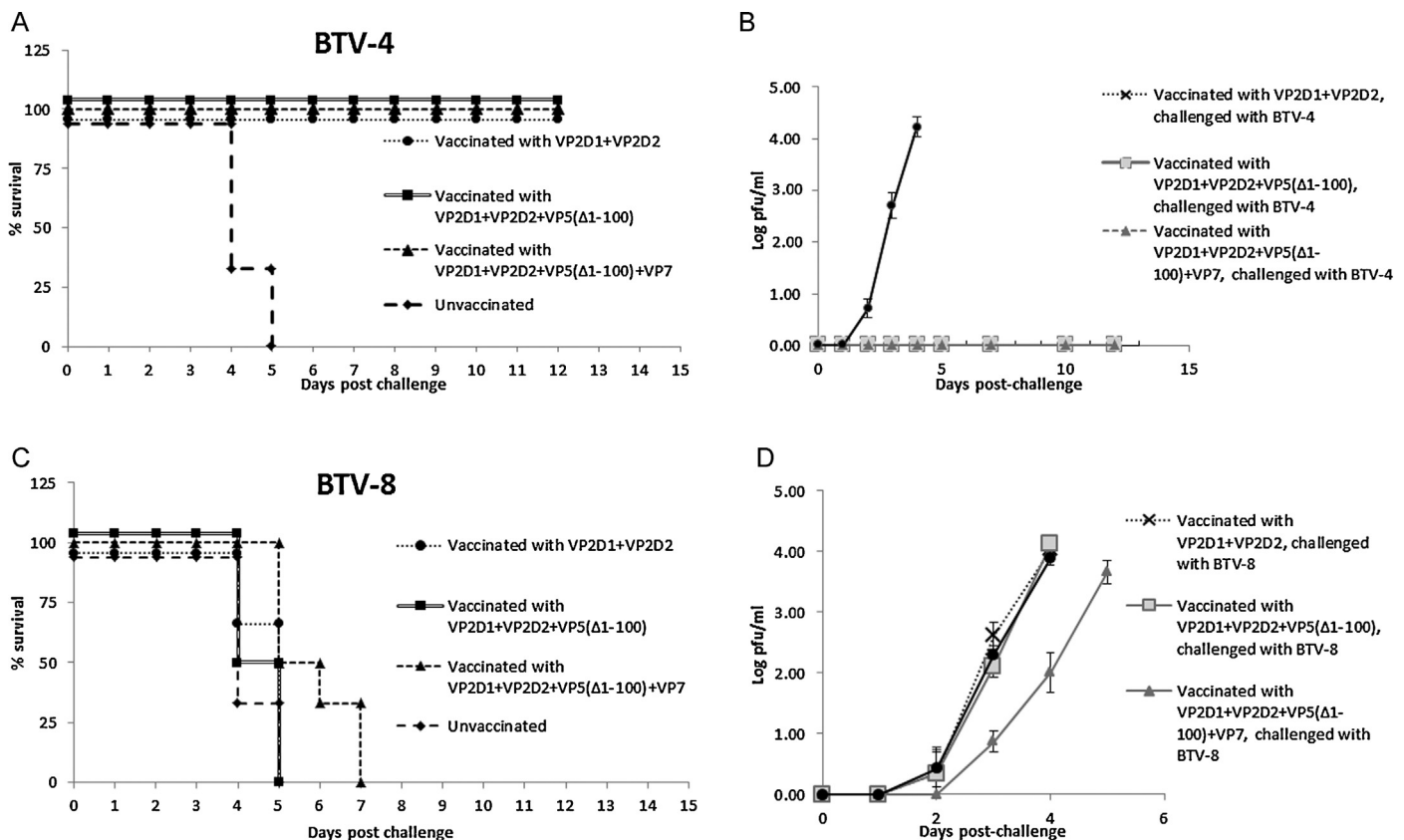


Fig. 2. Survival of IFNAR^{-/-} mice challenged with BTV-4 or BTV-8 and virus titres expressed as log₁₀ plaque forming units (PFUs were determined by plaque assay in BSR cells). Groups of IFNAR^{-/-} mice included non-immunised mice and mice immunised with VP2D1 + VP2D2, VP2D1 + VP2D2 + VP5_{Δ1-100}, or with VP2D1 + VP2D2 + VP5_{Δ1-100} + VP7. (A) Mice in the non-immunised group challenged with BTV-4 died by day 5 post-challenge while immunised animals were protected and all animals survived until the end of the experiment. (B) Virus titres (PFU as determined by plaque assay in BSR cells) in the groups of non-immunised and immunised groups challenged with BTV-4. Immunised animals survived until the end of the experiment and no virus was isolated by cell culture. (C) Groups of IFNAR^{-/-} challenged with BTV-8 all died by day 5–7 post challenge. (D) Virus titres (PFU as determined by plaque assay in BSR cells) in the groups of non-immunised and immunised groups challenged with BTV-8.

Animals in the group immunised with VP5_{Δ1-100} were not challenged because initial studies with Balb/c mice showed that sera of mice immunised with VP5_{Δ1-100} only, did not neutralise virus infectivity.

All animals in the groups immunised with VP7 only, died by day 5 p.i. with levels of BTV-specific RNA in blood similar to non-immunised mice (BSR plaque assay titres: 4×10^3 – 2.7×10^4 pfu/ml). This suggests that increased survival times of mice immunised with VP2D1 + VP2D2 + VP5_{Δ1-100} + VP7 is not due to VP7 alone, but may be an effect of combining these different proteins.

4. Discussion

Several inactivated mono- and multivalent vaccines for BTV serotypes 1, 2, 4 or 8, have been authorised via the European Medicines Agency for use in ruminants, particularly cattle and sheep [41,42]. These relatively un-purified vaccine antigens raise antibodies to all virus structural and non-structural proteins, making it impossible to distinguish infected from vaccinated animals (DIVA) by serological assays.

Previous studies exploring recombinant-expressed BTV structural proteins as subunit-vaccine candidates have evaluated crude lysates of recombinant-baculovirus-infected insect cells expressing BTV VP2 and VP5 [43–45]. Immunisation of sheep with these proteins, protected the animals and raised significant NAb titres (up to 2.408), with transient or undetectable viraemia after a subsequent homologous-BTV challenge [43]. Recently, it was shown that baculovirus-expressed and purified VP2 induced neutralising

antibodies [45] and is stable at +4 °C as well as –80 °C for almost 2 years [46]. Immunisation with virus-like particles (VLPs) containing capsid-proteins (VP3, VP7, VP2 and VP5) also protected sheep and raised NABs (titres of upto 2.107) generating sterile immunity post-homologous-challenge [44]. However, VLPs are thought to be relatively unstable and have a limited shelf life.

Other experimental subunit-vaccines for BTV include vectored-virus vaccines such as modified vaccinia Ankara (MVA), capripox virus, canarypox virus, bovine herpes virus, equine herpesvirus or myxomavirus [43,44,47–54]. However, simple bacterial expression systems have not been fully explored, due to difficulties generating larger BTV proteins (such as VP2 ~112 kDa) in a native and soluble form for use as subunit-vaccine antigens [55].

Previous findings suggested that VP2 of BTV (~110 kDa), evolved through duplication and may therefore exist as two related domains, VP2D1 and VP2D2 [18]. Sera from Balb/c mice immunised with the soluble recombinant VP2D1 of BTV-4, neutralised the homologous virus, while significantly lower NAB titres were observed with sera of mice immunised with soluble VP2D2. This suggests that the majority of the dominant neutralising epitopes are located in the amino terminal half of VP2. However, when both domains were mixed together on an equimolar basis, higher titres of neutralising antibodies were elicited. There is published evidence that neutralisation epitopes are located in the first ~350 amino acids (domain 1) of VP2 of BTV-10 [56]. IFNAR^{-/-} mice immunised with VP2D1 + VP2D2 and challenged with live BTV-4 survived until the end of the experiment with a transient viraemia (~0.3–9 pfu/ml detected by RT-PCR only) which was cleared subsequently. It was not possible to isolate virus in cell cultures from

Table 2Detection of BTV-4 or BTV-8 RNA in blood samples from IFNAR^{-/-} mice post-challenge.

Group number	Mouse	Ct Day 2	Ct Day 3	Ct Day 4	Ct Day 5	Ct Day 7	Ct Day 10	Ct Day 12
Group 1	1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Immunogen: VP2D1 + VP2D2	2	Neg	Neg	34.1	Neg	Neg	Neg	Neg
Challenge virus: BTV-4	3	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	4	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	5	Neg	Neg	34.5	Neg	Neg	Neg	Neg
	6	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Group 2	1	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Immunogen: VP2D1 + VP2D2 + VP5	2	Neg	Neg	Neg	39.7	42.3	Neg	Neg
Challenge virus: BTV-4	3	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	4	Neg	Neg	Neg	40.6	42.9	Neg	Neg
	5	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	6	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Group 3	1	Neg	Neg	Neg	38.7	Neg	Neg	Neg
Immunogen: VP2D1 + VP2D2 + VP5 + VP7	2	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Challenge virus: BTV-4	3	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	4	Neg	Neg	Neg	39.1	Neg	Neg	Neg
	5	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	6	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Group 4	1	39.1	28.8	20.8	D			
Immunogen: VP2D1 + VP2D2	2	36.5	27.1	22.4	D			
Challenge virus: BTV-8	3	35.8	27.6	D				
	4	37.4	28.2	21.5	D			
	5	39.9	26.5	21.8	D			
	6	34.1	26.3	D				
Group 5	1	34.9	29.5	20.7	D			
Immunogen: VP2D1 + VP2D2 + VP5	2	36.8	28.9	D				
Challenge virus: BTV-8	3	38.4	30.1	21.4	D			
	4	34.3	28.5	D				
	5	34.7	29.6	D				
	6	38.6	30.3	21.6	D			
Group 6	1	41.3	34.8	29.5	22.4	D(day6)		
Immunogen: VP2D1 + VP2D2 + VP5 + VP7	2	42.4	33.6	28.4	D			
Challenge virus: BTV-8	3	40.1	35.1	30.2	D			
	4	39.3	34.5	31.8	23.7	D		
	5	41.6	35.4	28.7	D			
	6	Neg	33.8	30.7	22.6	D		
Group 7 (BTV-4 Control)	1	36.3	26.7	21.4	D			
Immunogen: none	2	37.1	27.2	D				
Challenge virus: BTV-4	3	35.7	26.1	20.9	D			
	4	34.6	28.3	D				
	5	35.1	25.9	D				
	6	34.7	28.5	D				
Group 8 (BTV-8 Control)	1	37.6	29.1	D				
Immunogen: none	2	36.9	28.7	D				
Challenge virus: BTV-8	3	38.1	28.5	D				
	4	35.3	27.9	22.7	D			
	5	34.8	28.1	21.6	D			
	6	35.4	29.8	D				

RNA was extracted from mouse blood samples and tested using a generic BTV Seg-1 real-time PCR assay. D: animal died.

these blood samples, potentially reflecting presence of neutralising antibodies.

The CAPS-denatured (from insoluble fraction) VP2 domains did not raise any neutralising antibody response as compared to the soluble domains in bacteria. This strongly suggests that at least some neutralisation epitopes are conformational, which have been lost by dissolving the insoluble VP2 domains in a detergent such as CAPS. Several studies identified linear epitopes in VP2 which are serotype specific, some of which when used in the form of peptides prevented virus neutralisation [57–59].

Although BTV-VP2 is the primary determinant of serotype, the smaller outer capsid protein VP5, stimulates the neutralisation response, possibly through interactions with VP2 in the virus capsid [14,15]. Mice vaccinated with a combination of expressed VP5_{Δ1–100} and VP2 domains of BTV-4, generated higher neutralising antibody titres ($P < 0.05$) (against BTV-4, but not BTV-8)

and delayed the transient viraemia (detected by RT-PCR, while no virus could be isolated by KC or BSR cell cultures) observed in some animals after homologous challenge than mice vaccinated with VP2 domains alone. However, addition of VP5 did not have significant differences in terms of protection. The absence of neutralisation of BTV-8 is an indication that the neutralising antibodies elicited by BTV-4 VP2 domains are serotype-specific.

VP7(T13) is an immuno-dominant orbivirus-species/serogroup-specific antigen [51,60,61]. Antibodies to VP7 can neutralise the infectivity of BTV core-particles, but do not significantly neutralise intact virus particles [62]. The incorporation of baculovirus-expressed VP7 in previously reported vaccination studies using VP2 and VP5, also failed to enhance NAb responses in sheep [43]. However, vaccination with BTV-VP7 has been shown to induce a partially-protective cytotoxic T-cell response that may reduce

viraemia [63]. Capripoxvirus expressing VP7 was shown to confer cross-protection [51].

Although vaccination with baculovirus-expressed BTV core-like-particles (CLP – containing VP3 and VP7) did not prevent clinical signs of the disease, it did reduce their severity [44].

The addition of expressed VP7 to vaccination antigens (with VP5_{Δ1–100} and soluble domains of VP2) failed to increase neutralising antibody titres (against BTV-4) and failed to protect IFNAR^{−/−} mice from lethal challenge with BTV-8. Regardless of the antigen combination which we used, there was no protection from the heterologous BTV-8 lethal challenge. These results show that the response to immunisations is serotype-specific and that VP2 is the main protective component in the three combinations of antigens.

The results presented show that soluble BTV-VP2 domains and VP5 can be expressed in bacteria, suggesting that they adopt a native conformation/fold in this system.

The aim of this study was to assess bacterially-derived BTV structural-proteins as candidates for a DIVA-compatible subunit-vaccination-strategy, using Balb/c mice and the well-established BTV animal-model, IFNAR^{−/−} mice. DIVA-compatible BTV vaccines could be based on a subset of the viral proteins, with detection of antibodies to the remaining protein(s) as surveillance markers for previous infections. Our results demonstrate potential for a bacterial-expressed BTV-subunit DIVA vaccine, based principally on VP2 and VP5. The exclusion of VP7, which does not seem to influence protection, provides a mean for DIVA.

The two expressed VP2 domains, VP2D1 and VP2D2 combined on equimolar basis, generated high titres of neutralising antibodies with similar titres in both Balb/c and IFNAR^{−/−}. Although a transient viraemia was observed in mice immunised with VP2D1 + VP2D2, post-challenge with BTV-4, this was rapidly cleared and they survived without signs of infection throughout the experiment. This indicates that soluble bacterial-expressed antigens are protective and do not require more complex eukaryotic expression systems.

The use of bacterial-expressed protein antigens, could provide a safe and scalable alternative to live-attenuated BTV vaccines. Bacterial expression could represent an alternative to inactivated vaccines, particularly if viruses prove to be difficult to propagate in cell culture (like BTV-25 [7]). These antigens could be used in different combinations (from multiple-serotypes) or with 'virus-vectored' and/or DNA vaccines to create cross-serotype, or stronger protective responses.

The ability of bacterially-expressed BTV subunit-vaccines to induce NABs and protect sheep and cattle (natural hosts of BTV) will require further validation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.05.056>.

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