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Secretory expression of bovine herpesvirus type 1/5 glycoprotein E in *Pichia pastoris* for the differential diagnosis of vaccinated or infected cattle

Bianca S. Siedler [a, *], Bárbara C. Roloff [a], Gizele L. de Sá [a], Alessandra Neis [a], Fabrício R. Conceição [a], Daiane D. Hartwig [b], Sibele Borsuk [a], Odir A. Dellagostin [a], Fabrício S. Campos [c], Paulo M. Roehe [c], Claudia P. Hartleben [a], Alan J.A. McBride [a, d]

[a] Núcleo de Biotecnologia - CDTec, Universidade Federal de Pelotas, Campus Universitário, Caixa Postal 354, 96010-900, Pelotas, RS, Brazil
[b] Instituto de Biologia, Departamento de Microbiologia e Parasitologia, Universidade Federal de Pelotas, Campus Universitário, Caixa Postal 354, 96010-900, Pelotas, RS, Brazil
[c] Instituto de Ciências Básicas e da Saúde, Universidade Federal do Rio Grande do Sul, Av. Sarmento Leite 500, 90050-170, Porto Alegre, RS, Brazil
[d] Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, BA, Brazil

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ABSTRACT

Bovine herpesvirus (BoHV) glycoprotein E (gE) is a non-essential envelope glycoprotein and the deletion of gE has been used to develop BoHV-1 and BoHV-5 differential vaccine strains. The DIVA (Differentiation of Infected from Vaccinated Animals) strategy, using marker vaccines based on gE-negative BoHV strains, allows the identification of vaccinated or infected animals in immunoassays designed to detect *anti*-gE antibodies. In this study a codon optimized synthetic sequence of *gE* containing highly conserved regions from BoHV-1 and BoHV-5 was expressed in *Pichia pastoris*. Following expression, the recombinant gE (rgE) was secreted and purified from the culture medium. The rgE was identified by Western blotting (WB) using sera from cattle naturally infected with BoHV-1 and/or BoHV-5, or sera from bovines experimentally infected with wild-type BoHV-5. Sera collected from cattle vaccinated with a BoHV-5 gI/gE/US9 marker vaccine failed to recognise rgE. Expression of rgE, based on a sequence containing highly conserved regions from BoHV-1 and BoHV-5, in *P. pastoris* enabled the production of large quantities of rgE suitable for use in immunoassays for the differentiation vaccinated or infected cattle.

1. Introduction

Bovine herpesviruses types 1 (BoHV-1) and 5 (BoHV-5) are important pathogens associated with respiratory, reproductive and neurological disorders in cattle [1], that cause significant economic losses in the beef and dairy industry. Both viruses belong to the *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* [2]. Following a typical herpesvirus pattern, they can establish long-term latent infections in their hosts, with sporadic episodes of viral shedding [3]. BoHV-1 is distributed worldwide [4] while BoHV-5 is restricted to a number of countries in South America including Brazil [5], Argentina [6], Colombia [7] and Uruguay [8]. In the northern hemisphere, the occurrence of BoHV-5 infections is rare [9,10]. Inactivated and modified live vaccines have been used worldwide in an attempt to control herpesvirus infections in cattle [11], and most of these are based on BoHV-1 strains [12,13]. Cross-protection against BoHV-1 and BoHV-5 may occur as the proteomes are highly conserved, average 82.0% [14,15]; however, this is not always the case [16]. In addition, animals may be infected with both virus types [17].

Glycoprotein E (gE) is a non-essential glycoprotein which has been used as an antigenic marker for BoHV-1 and BoHV-5 vaccines [18–23]. The DIVA (Differentiation of Infected from Vaccinated Animals) strategy is based on using marker vaccines, where the *gE* gene has been deleted from the vaccine strain. This allows protection against the disease while enabling identification of vaccinated or infected animals in immunoassays based on the detection of *anti*-gE antibodies [20,24]. The ability to differentiate between vaccinated and infected animals is critical for trading restrictions and surveillance in countries that are running control programs for

* Corresponding author.
E-mail address: bssiedler@gmail.com (B.S. Siedler).

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Abbreviations used

BoHV	bovine herpesvirus
gE	glycoprotein E
DIVA	differentiation of infected from vaccinated animals
rgE	recombinant gE
WB	western blotting
LB	Luria-Bertani
YPD	yeast extract peptone dextrose
BMGY	buffered glycerol complex medium
BMMY	buffered methanol complex medium
TCA	trichloroacetic acid
TE	Tris-EDTA buffer
EDTA	ethylenediaminetetraacetic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
BSA	bovine serum albumin
PBST	phosphate-buffered saline with 0.05% Tween 20
HRP	horseradish peroxidase
mAb	monoclonal antibody
LipL32	leptospiral major outer membrane protein.

the eradication of BoHV-1 [25]. Furthermore, it is possible to monitor wild-type virus transmission in animal populations and to determine the true prevalence of infection in herds, regions, and countries [20], since gE seroconversion means infection post-vaccination.

In Brazil, BoHV-1 and BoHV-5 *gE*-deleted marker vaccines have been developed and tested but are not yet commercially available [22,23,26–31]. Marker vaccines (live or inactivated) represent a good choice for disease control as they protect cattle clinically in case of infection and markedly reduce the subsequent shedding of wild-type virus [22,23,32]. A recombinant BoHV-5 lacking thymidine kinase and *gE* genes was evaluated as a live experimental vaccine and conferred protection upon homologous and heterologous (BoHV-1) challenge [22]. Campos and co-workers showed that a BoHV-5 gI/gE/US9^{-} inactivated marker vaccine was capable of conferring protection against encephalitis when vaccinated cattle were challenged with a large infectious dose of the parental wild-type BoHV-5 [23]. Furthermore, Romera and colleagues developed and evaluated a gE-deleted BoHV-1 marker vaccine strain as an inactivated and a live attenuated vaccine preparation. Both formulations elicited an efficient and protective immune response against challenge with virulent wild-type BoHV-1 [32]. Importantly, once the marker vaccine was administered there was no evidence of horizontal or vertical spread. Importantly, marker and conventional BoHV vaccines can prevent disease but not latent infection. Accordingly, an interesting safety feature that comes from using live attenuated marker vaccines based on gE-deleted viruses is that the virus cannot spread in an anterograde direction from the sensory neurons in the trigeminal ganglia to the respiratory mucosa [32,33]. The implementation of a DIVA strategy requires diagnostic assays for the detection of *anti*-gE antibodies. However, commercial gE antibody detection assays are rarely used in Brazil due to the high costs associated with importation, justifying the development of new diagnostic tools.

Truncated forms of gE from BoHV-1 have been expressed successfully using Baculovirus-insect systems [34–36], mammalian cells [37] and prokaryotic expression methods [35,38]. However, as gE contains several antigenic domains, detection of *anti*-gE antibodies is likely to be enhanced using the whole protein [39,40]. In addition, the yeast expression system offers several advantages relative to other mammalian expression systems including: high growth rates of *P. pastoris* that are associated with improved protein yield; reduced costs, the growth medium components (glycerol and methanol, biotin, salts, trace elements) are simple and inexpensive; and as *P. pastoris* is cultured in media with a relatively low pH and methanol it is less likely to become contaminated [41]. Furthermore, *P. pastoris* represents an excellent alternative to *E. coli*, since it possesses the eukaryotic machinery needed for posttranslational modifications for processing signal peptide sequences (both pre and prepro types), folding, disulphide bond formation and O- and *N*-linked glycosylation. Consequently, many proteins sequestered in inclusion bodies in prokaryote expression systems can be produced as biologically active molecules in *P. pastoris* [42]. In addition, the expression vector pPICZαB uses the secretion signal sequence from *Saccharomyces cerevisiae* α-factor prepro-peptide (leader) and consequently, heterologous proteins are secreted into the medium, which serves as a substantial first step purification, since *P. pastoris* secretes only low levels of endogenous proteins [43].

In the present study, a synthetic *gE* gene, containing highly conserved regions from BoHV-1 and BoHV-5 and optimized for yeast codon usage, was designed, cloned and expressed in the methylotrophic yeast *P. pastoris*. Recombinant gE (rgE) was evaluated by Western blotting (WB) and could differentiate between sera collected from vaccinated and BoHV infected cattle.

2. Materials and methods

2.1. Strains and growth conditions

Escherichia coli DH5α was grown in Luria-Bertani (LB) low-salt medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl - pH 7.5) at 37 °C with the addition of zeocin (25 μg/ml). *Pichia pastoris* strain KM71H (MutS, Invitrogen) was grown in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% D-glucose) supplemented with 100 μg/ml of zeocin at 28 °C.

2.2. Cloning of the gE gene

Following the alignment of six gE proteins (GenBank accession numbers: NP_045372.1, AAA67214.1, ADE08265.1, AAF71143.1, YP_003662535.1, AAF34744.1) we identified a polypeptide that included highly conserved regions in both BoHV-1 and BoHV-5. This was encoded by a 1682 bp sequence from BoHV-1 and was named BoHV-1/5 gE. The codon usage of the *gE* sequence was optimized for *P. pastoris* (based on the codon usage database at http://www.kazusa.or.jp/codon). Restriction enzyme sites for *Eco*RI and *Kpn*I, at the 5'- and 3'-termini, respectively, were included in the *gE* sequence for subsequent cloning into the pPICZαB expression vector (Invitrogen, Brazil). The presence of the insert was determined by restriction enzyme digestion with *Eco*RI and *Kpn*I. The pPICZαB/BoHV-1/5 gE plasmid was propagated in *E. coli* DH5α, purified (Purelink Quick Mini 50 Kit, Invitrogen, Brazil) and linearized with *Sac*I restriction enzyme (Invitrogen, Brazil). Linear plasmid DNA was purified by phenol-chloroform extraction and DNA precipitation as previously described [44]. *P. pastoris* competent cells were transformed with 10 μg of linear plasmid DNA by electroporation (25 μF, 200 Ω, 2 kV).

2.3. Animal sera

Sera samples were collected from cattle: naturally infected with BoHV-1 and/or BoHV-5 (n = 10, named S1-S10); experimentally infected with a wild-type BoHV-5 strain (n = 10, named S11-S20)

[23]; vaccinated with a BoHV-5 gI/gE/US9 vaccine strain (n = 10, named S21-S30) [28] and a negative control serum sample was collected from a healthy calf (ELISA negative for BoHV antibodies). The sera samples (S1-S10 and the negative control sample) were provided by the Virology Laboratories, Federal University of Pelotas and the Federal University of Rio Grande do Sul (S11-S30), RS, Brazil.

2.4. Screening for P. pastoris clones expressing rgE

Recombinant *P. pastoris* clones (Rcs) were screened for the presence of rgE by colony blotting (150 colonies were tested) and dot blotting (59 recombinant clones were screened by spotting 5 μl of each supernatant sample onto a nitrocellulose membrane), as described previously [44]. The presence of rgE was detected using anti-Histidine mAb (cat. No. H1029, Sigma-Aldrich, Brazil) at a dilution of 1:6.000 in PBST, and pooled serum samples from BoHV infected cattle (1:10.000 in PBST). The anti-Histidine mAb and the cattle sera were pooled prior to incubation with the blots. Using the 10 highest expressing clones, a dot-blot time-course study (24, 72 and 144 h) was performed to determine optimal protein secretion. The relative integrated density of each dot was measured using ImageJ software (version 1.50i, National Institutes of Health, USA).

2.5. Expression and purification of rgE in P. pastoris KM71H

The recombinant clone with the highest expression level of rgE was used to inoculate 25 ml of BMGY (2% glycerol) in a baffled flask and was incubated in an orbital shaker at 28 °C, 250 rpm, to an OD600 of 2–6. The cells were harvested by centrifugation and resuspended in 100 ml of BMMY to a final OD_{600} of 0.5. Expression was induced for 96 h by adding methanol to a final concentration of 0.5% every 24 h, until the culture reached an OD600 of 5, whereupon methanol (1% final concentration) was added every 12 h to maintain expression of rgE, regardless of evaporation and consumption levels. The supernatant containing the secreted rgE was collected by centrifugation and stored at −80 °C.

The rgE in the supernatant (100 ml) was purified and concentrated by precipitation with 20% trichloroacetic acid (TCA) as described previously [45,46]. The protein pellet was resuspended in 1 × TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA, pH 8.0). Samples were suspended in loading buffer (1% SDS, 250 mM Tris pH 6.8, 1% bromophenol blue, 50% glycerol and 1% 2-mercaptoethanol) and boiled for 10 min prior to analysis by 10% SDS-PAGE. The supernatant of untransformed *P. pastoris* KM71H (20 ml) was precipitated with 20% TCA and used as a negative control. The gel was stained with Coomassie Brilliant Blue R250. The concentration of the purified rgE was determined by Bradford assay (Bio-Rad, USA) using BSA in a standard curve.

2.6. Western blotting (WB) to detect gE antibodies

The ability of rgE to be recognized by sera collected from cattle naturally infected, or experimentally infected with BoHV-1 and/or BoHV-5, was evaluated by WB. The rgE was separated by 10% SDS-PAGE (~2 μg/lane) and transferred to a nitrocellulose membrane as described previously [44]. Briefly, 31 membrane strips containing rgE were blocked (PBST, 1% foetal bovine serum), each strip was individually incubated for 1 h at room temperature with the following sera: one of 10 samples (S1-S10) from naturally infected cattle; one of 10 samples (S11-S20) from experimentally infected cattle; one of 10 samples (S21-S30) from cattle vaccinated with a BoHV-5 gI/gE/US9 marker vaccine; and a negative serum sample. Each serum sample was diluted 1:150 in PBST, 1 ml/strip, in a Western blot tray. A wild-type BoHV-5 strain was included as a positive control antigen (10 μl of whole viral extract – concentrated and semi-purified BoHV-5 isolate containing 10^7 $TCID_{50}$/ml–

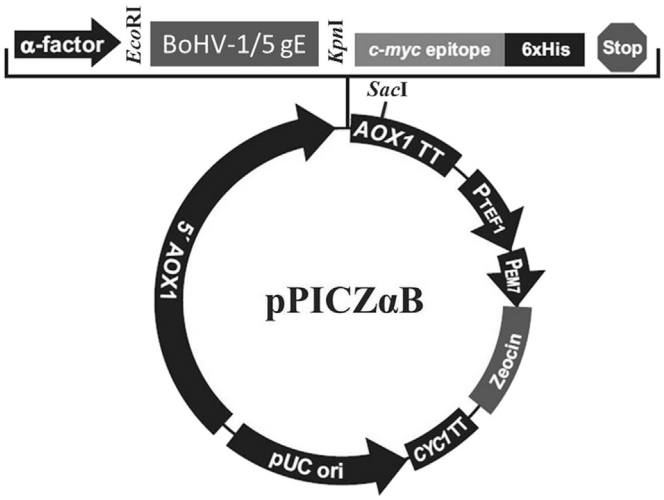

Fig. 1. Construction of the pPICZαB/BoHV-1/5 gE plasmid. The BoHV-1/5 *gE* gene was cloned into the *Eco*RI and *Kpn*I restriction enzyme sites located in the multiple cloning region of the pPICZαB vector, in frame and downstream of the α-factor signal peptide sequence. The gE amino acid sequence contained a 6 × His tag followed by a stop codon at the carboxy-terminus (see Supplementary data Fig. 3); the *c-myc* epitope and 6 × His tag from pPICZαB were not expressed in the rgE.

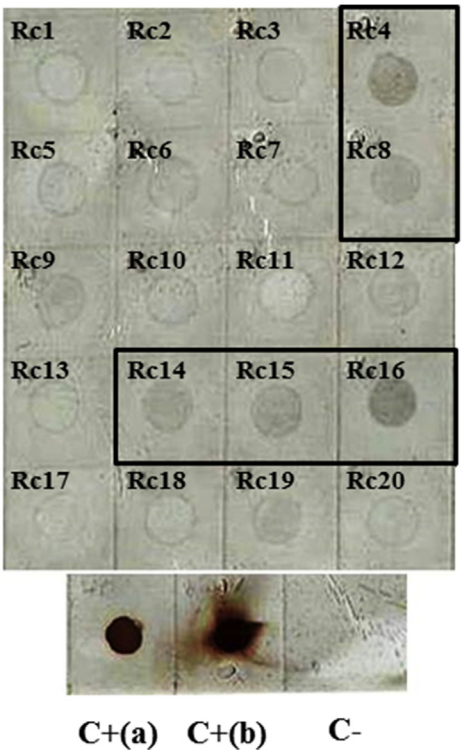

Fig. 2. Screening for *P. pastoris* recombinant clones expressing rgE. Expression was evaluated by dot blotting using anti-Histidine mAb and pooled sera from cattle naturally infected with BoHV-1 and/or BoHV-5. A representative number (20/59) of clones (Rc1-20) are shown and the five clones shown, as indicated by the black boxes (Rc4, Rc8, Rc14-16), demonstrated the highest levels of rgE expression. The following controls were included: (C-) Untransformed *P. pastoris* KM71H was used as a negative control; C+(a) Recombinant LipL32 [51] was included as a positive control for the anti-Histidine mAb (2.5 μl containing ~0.7 μg/dot); C+(b) BoHV-5 (2.5 μl of whole viral extract) was used as the positive control for the sera collected from BoHV-1 and/or BoHV-5 infected cattle.

diluted 1:5). The strips were incubated with anti-bovine IgG horseradish peroxidase (HRP) conjugate (1:10.000).

3. Results and discussion

3.1. Cloning and expression of rgE in P. pastoris

In this study, a *gE* sequence containing highly conserved regions from BoHV-1 and BoHV-5 was codon optimized and artificially synthesized (see Supplementary data). There are several advantages to using a synthetic codon-optimized *gE* gene. There is no need to culture the virus, extract DNA, amplify the DNA target by PCR and its subsequent cloning. In addition, the synthetic production of the gene encoding gE allowed the codon-usage to be optimized for *P. pastoris*. Several authors have explored gene optimization in order to improve protein expression in *P. pastoris* [47,48]. Chang et al. [49] obtained a protein expression level 4.6-fold higher using a codon-optimized gene construct when compared to the non-codon optimized production level. Codon optimization has also been shown to reduce protein production time [48,49].

After cloning, the pPICZαB/BoHV-1/5 gE plasmid (Fig. 1) was linearized and used to transform *P. pastoris* KM71H, the resultant clones were screened by colony and dot blotting for the expression of rgE. Of these clones, 59/150 (39.3%) colonies expressed rgE, and of these 59 colonies, 13 (22.0%) recombinant clones were identified as positive by dot blotting, see Fig. 2. Expression levels of rgE among the recombinant clones varied, even though the colonies were approximately the same size. This was probably due to differences in copy number of the integrated vector, since high copy number integration has been shown to be important for high expression [50]. Of note, the same gE coding sequence expressed and purified from *E. coli* was not recognized by sera from any of the infected animals (data not shown). Suggesting that post-translational modification of rgE is necessary for recognition by native gE antibodies. A time-course evaluation (at time-points 24, 72 and 144 h) revealed detectable amounts of the recombinant protein after 24 h induction, maximum expression was obtained after 72 h (Rc4, Rc8 and Rc15) and 144 h of induction (Rc14 and Rc16), see Fig. 3.

The clone with the highest expression levels of rgE (Rc15) was cultured in a glycerol-based medium to generate biomass, followed by cultivation in a medium containing methanol to induce expression of rgE. As the rgE was expressed as a secreted protein there was no need for complex chromatographic purification procedures, rather the rgE was recovered by precipitation using 20% TCA. The total protein yield in the precipitated supernatant of transformed *P. pastoris* was 140 mg/L.

Following purification and concentration of the supernatant, several (5) secreted proteins were visualized by SDS-PAGE, including a major band (~80 kDa) corresponding to rgE and several unknown proteins (Fig. 4). The predicted molecular mass (Mm) of rgE was 59.2 kDa while the apparent Mm was ~80 kDa. This was not unexpected as previous studies reported gE with an apparent Mm of ~94 kDa [52]. This difference was attributed to posttranslational modifications, principally *N*-glycosylation, the rgE amino acid sequence contained several potential glycosylation sites and disulphide bonds (data not shown). Furthermore, there are reports of rgE with an apparent Mm of ~80 kDa when expressed in a Baculovirus-insect system [34,36].

3.2. Differential diagnosis of infected and vaccinated cattle

All of the sera (10/10) from cattle naturally infected with BoHV-1 and/or BoHV-5 (S1-S10) and from bovines experimentally infected with a BoHV-5 strain (10/10, S11-S20) recognized rgE by WB, see Fig. 5A and B. Furthermore, rgE was not detected by sera (0/10)

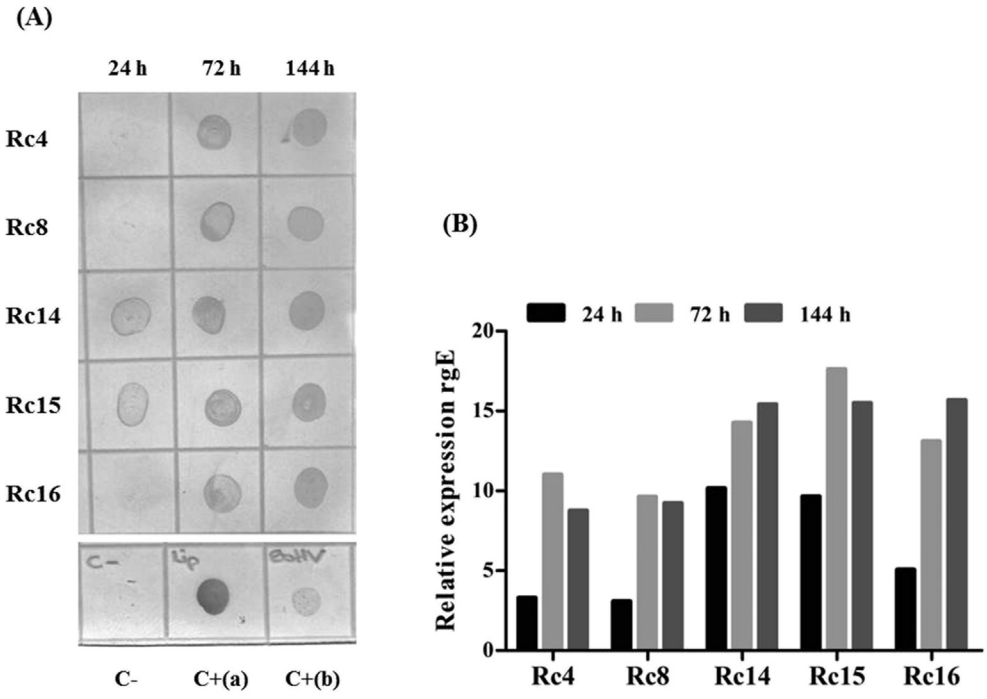

Fig. 3. Time-course study (24, 72 and 144 h post-induction) of rgE expression and secretion based on five of the highest expressing clones. rgE expression was induced by the addition of methanol (0.5% final concentration), at time points 24, 48, 72, 96 and 120 h. (A) Dot blotting was performed using anti-Histidine mAb and pooled sera from cattle naturally infected with BoHV-1 and/or BoHV-5. (C-) Untransformed *P. pastoris* KM71H was used as a negative control; C+(a) Recombinant LipL32 (2.5 μl containing ~0.7 μg/dot) was included as a positive control for the anti-Histidine mAb; C+(b) BoHV-5 (2.5 μl whole viral extract diluted 1:5) was the positive control for the sera collected from BoHV-1 and/or BoHV-5 infected cattle. (B) Histogram showing relative expression levels of rgE by clones Rc4, Rc8, Rc14-16.

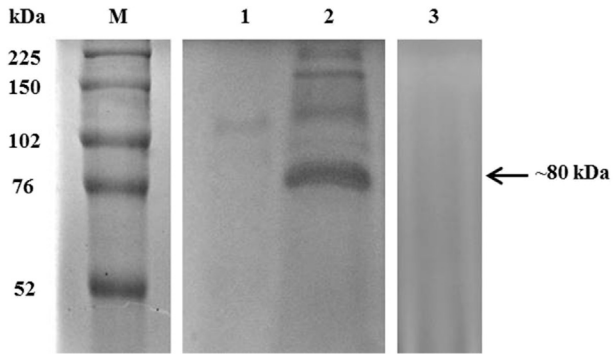

Fig. 4. SDS-PAGE analysis of rgE expression in *P. pastoris*. Lanes M: Full-Range Rainbow Molecular Weight Marker (GE Healthcare), the apparent Mm (kDa) of each band is indicated; 1: Negative control - supernatant of untransformed *P. pastoris* KM71H after methanol induction and precipitation with 20% TCA; 2: Purified rgE following precipitation with 20% TCA (16 μl, ~2 μg), the apparent mass of ~80 kDa is indicated (arrow); 3: Supernatant containing the secreted rgE prior to TCA precipitation.

collected from bovines vaccinated with the BoHV-5 gI/gE/US9 marker vaccine strain (S21-S30) (Fig. 5C). The presence of *anti*-BoHV antibodies in animals immunised with the marker vaccine was demonstrated using wild-type BoHV-5 virus as a positive control (C+), Fig. 5. The negative control serum (uninfected) did not recognise rgE, see Fig. 5. Of note, in the Western blots S1-20 (Fig. 5A and B), in addition to the rgE band, an additional band was revealed with an apparent Mm of 160 kDa. This band is possibly a dimer of rgE, since it was recognized by sera from cattle naturally infected with BoHV-1 and/or BoHV-5 and sera from bovines experimentally infected with wild-type BoHV-5.

4. Conclusion

In the present study, a recombinant *gE* gene, based on highly conserved regions from BoHV-1 and BoHV-5, was codon-optimized, synthesized and cloned into the *P. pastoris* expression vector pPICZαB. Following induction, rgE was expressed and

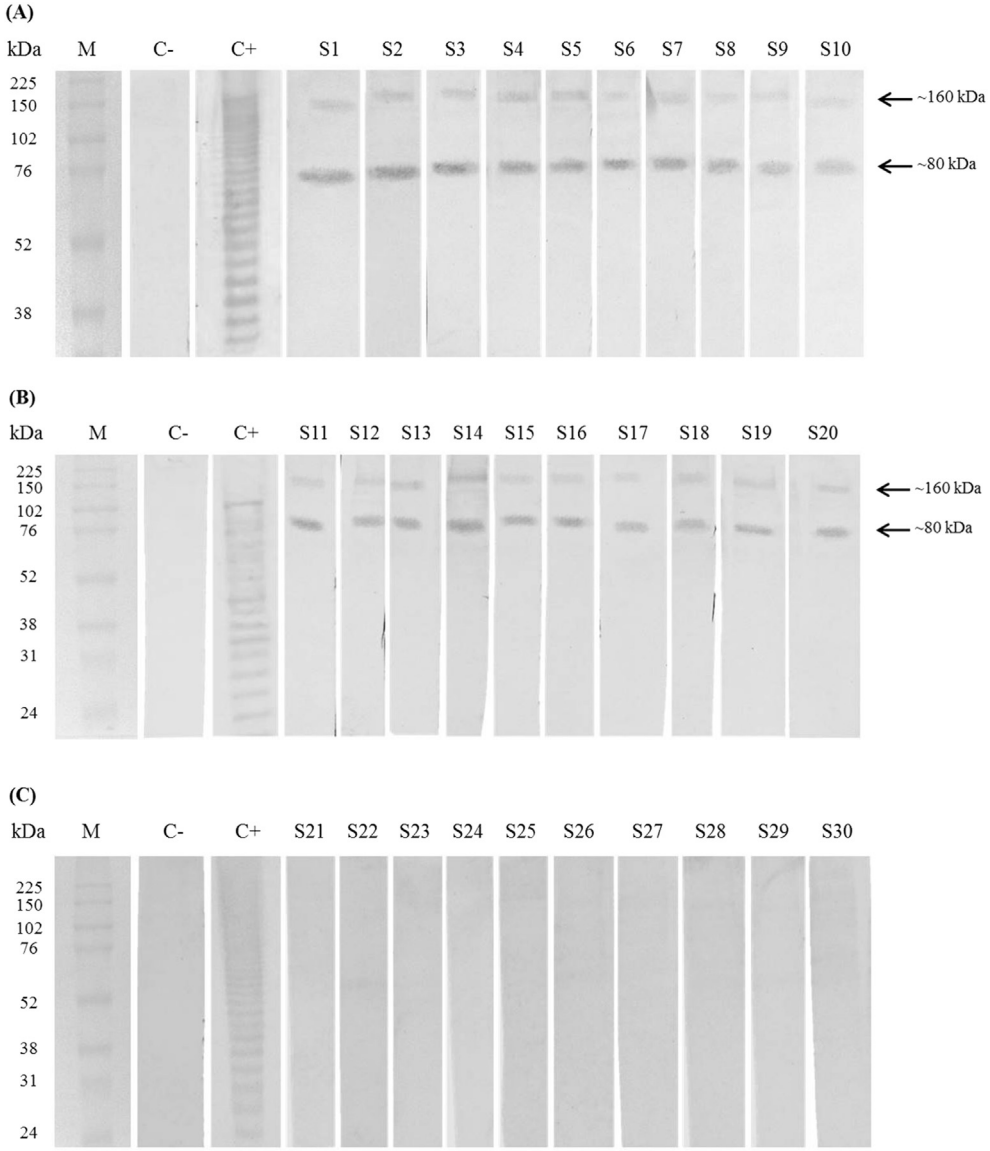

Fig. 5. WB analysis of rgE expressed in *P. pastoris*. (A) Lanes S1-S10, rgE (~2 μg/strip) and sera from BoHV-1 and/or BoHV-5 infected cattle; (B) Lanes S11-S20, rgE and sera from cattle experimentally infected with a wild-type BoHV-5 strain; (C) Lanes S21-S30, rgE and sera from cattle immunised with a BoHV-5 gI/gE/US9 marker vaccine; (M) Full-Range Rainbow Molecular Weight Marker (GE Healthcare), the Mm (kDa) of each band is indicated; (C-) rgE and negative control serum (ELISA negative for the presence of BoHV antibodies); (C+) Positive control, wild-type BoHV-5 antigen and sera from vaccinated animals. The apparent masses of ~80 kDa (rgE) and 160 kDa (possibly a dimer of rgE) are indicated by arrows.

secreted into the culture supernatant, which significantly improved protein recovery and purification, compared to gE expression in *E. coli*. Importantly, rgE retained the antigenic properties of the native protein in WBs, rgE was recognized by sera collected from naturally or experimentally BoHV infected cattle. Of note, rgE was not recognized by sera from cattle vaccinated with a marker vaccine. This suggested that rgE can be used as a diagnostic tool to differentiate between vaccinated and infected cattle. Further studies will focus on optimization of expression of this glycoprotein and its employment as immunodiagnostic reagent in different assay systems.

Conflict of interest statement

The authors herein declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2016.09.018.

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