



Development of recombinant cells encoding surface proteins of *Corynebacterium pseudotuberculosis* against caseous lymphadenitis in goats

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Abstract

Caseous lymphadenitis is an infectious disease caused by an intracellular bacterium, *Corynebacterium pseudotuberculosis*. Control is via vaccination. This report describes construction of two recombinant cells; one that carried the putative surface-anchored protein, the SpaA (pET32/LIC-SP31) and the other the glyceraldehyde-3-phosphate dehydrogenase protein, the GAPDH (pET32/LIC-SP40). The recombinant cells were introduced into goats before antibody response by the goats and protective capacities of the recombinant cells were measured. Fifteen goats were divided into 3 groups. Group 1 was injected intramuscularly with PBS, Groups 2 and 3 were injected on days 0 and 14 with 10⁶ CFU/ml of recombinant pET32/LIC-SP31 and pET32/LIC-SP40 cells, respectively. Serum samples were collected weekly to determine the antibody levels using ELISA. Two weeks after the last vaccination, all goats were challenged subcutaneously with 10⁹ CFU/ml of live *C. pseudotuberculosis*. The results revealed that goats exposed to the recombinant cells showed significantly ($p < 0.05$) higher IgG level compared to the control that lasted for 11 weeks. Generally, the exposed groups showed similar antibody pattern although those exposed to pET32/LIC-SP40 showed insignificantly ($p > 0.05$) higher level in the first 7 weeks than the recombinant pET32/LIC-SP31. Following challenge at week 6, abscesses were observed in the lymph nodes of all groups while *C. pseudotuberculosis* was successfully isolated. The recombinant cells were able to induce humoral response but failed to protect the goats against challenge by live *C. pseudotuberculosis*.

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Introduction

Caseous lymphadenitis (CLA) is a chronic disease of goats and sheep that is caused by *Corynebacterium pseudotuberculosis* (Literak *et al.*, 1999). It is characterized by the formation of abscesses in the superficial and internal lymph nodes and occasionally in the internal organs (Centikaya *et al.*, 2002). The disease causes significant economic losses to goat and sheep industries due to decreased production and quality of milk and wool other than condemnation of carcass and skin in abattoirs (Simmons *et al.*, 1998; Hoelzle *et al.*, 2013). Vaccination has been used to reduce the spread and gradual decline the disease prevalence (Dorella *et al.*, 2006; Fontaine and Baird, 2008). There are several types of vaccine against CLA, which include inactivated whole cells, toxoid of *C. pseudotuberculosis*, live and DNA vaccines (Izgur *et al.*, 2010). However, the current commercially available vaccines are not effective in protecting against CLA (Guimarães *et al.*, 2011).

Gram-positive bacteria lack outer membrane proteins (Desvaux *et al.*, 2006) but possess surface proteins (Schneewind and Missiakas, 2014). Cell surface of Gram-positive bacteria displays proteins that are frequently considered as virulence factors that can potentially be used for vaccine development (Desvaux *et al.*, 2006). This paper describes the construction of recombinant cells carrying surface proteins of *C. pseudotuberculosis* and reports the antibody response and protective capacity provided by these inactivated recombinant cells against caseous lymphadenitis in goats.

Materials and methods

Bacterial strain, plasmid and culture condition

Two isolates of *C. pseudotuberculosis*, UPM J1 and UPM J2 were used in this study. They were obtained from local outbreaks of caseous lymphadenitis. The isolates were cultured on 5% blood agar for 48 h at 37°C before pure colonies of *C. pseudotuberculosis* were further subcultured into 15 mL of brain heart infusion broth (Oxoid, UK) and incubated for 48 h with gentle shaking at 37°C. A non-expression host,

the *E. coli* strains Nova-Blue Giga-Singles (Merck, Germany) and an expression host, the *E. coli* strain BL21 (DE3) (Merck, Germany) were used for cloning and expression. The expression vector, pET-32 Ek/LIC was obtained from Merck, Germany.

Preparation of the surface proteins of Corynebacterium pseudotuberculosis

Surface proteins of *C. pseudotuberculosis* were prepared according to Sabri *et al.* (2000). The bacterial isolate was grown in one litre of brain heart infusion broth (BHIB) in 250 rpm incubator shaker at 37 °C for 48 h. The bacteria cells were harvested by centrifugation at 5000 rpm for 20 min where the pellet was washed three times by centrifugation at 5000 rpm for 10 min. The pellet was resuspended in 20 ml of PBS (pH 7.2) and sonicated 3 times for 10 min on ice. The cell lysate was exposed to diethylether for 6 h before centrifuged at 6000 rpm for 30 min. The supernatant was further centrifuged at 28 000 rpm for 2 h at 4°C, the pellet was resuspended in 2 mL of 1% sodium lauryl sarcosinate (Sigma, UK) and incubated for 2 h. The suspension was centrifuged again at 28 000 rpm for 2 h at 4°C before the pellet was resuspended in 100 µl sterile PBS and stored at -20°C. The concentration of surface proteins was determined using the Qubit flourometer probes (Invitrogen, USA).

Preparation of rabbit hyper-immune serum against whole cell C. pseudotuberculosis

Hyperimmune serum against *C. pseudotuberculosis* was prepared according to Cameron and Maria (1971). *Corynebacterium pseudotuberculosis* was grown in 50 ml of brain heart infusion (BHI) broth and the concentration was determined as 10⁶ cfu/ml before resuspended in 50 ml of PBS (pH 7.2) with 0.5 % formalin and incubated overnight at 4 °C. The bacterial cells were then washed again and were mixed well with 50 ml PBS. Approximately 1 ml of the bacterial cells were mixed with 1 ml of complete Freund's oil adjuvant (Sigma, UK) and injected into rabbit subcutaneously. The injections were given on days 1, 7 and 14. The blood was collected on day 28 post-injection and the hyper-immune serum was stored in -20 °C until used.

Sodium deodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of the surface proteins

The surface proteins of *C. pseudotuberculosis* were subjected to the SDS-PAGE using the Mini-Protein® II Electrophoresis Cell (BIO-RAD, USA) (Paule *et al.*, 2004). A 12% (w/v) resolving gel solution was prepared before 4% (w/v) stacking gel solution was dispensed onto the top of the resolving gel and plastic comb was carefully inserted. Dispensing samples were prepared by mixing one part of the protein sample with one part of the SDS loading buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS containing 5% (v/v) 2-mercaptoethanol). Twelve µl of the sample and 5 µl of protein marker were loaded into the wells filled with 1x Tris-glycine running buffer (25 mM Tris; 250 mM glycine; 0.5% (w/v) SDS, pH 8.3). Voltage of 95V was applied and the gel was left running for 2 h. The gel was then stained with Coomassie blue solution (0.25% (w/v) Coomassie brilliant blue G (Sigma, UK); 45% (v/v) ethanol; 10% (v/v) acetic acid) overnight followed with staining with de-staining solution (45% (v/v) ethanol; 10% (v/v) acetic acid) for 30 min with gentle shaking. The protein bands were compared against standard of known molecular weight of protein marker.

For immunoblotting, the bands were transferred to the PVDF membrane in cold transfer buffer at 100 V, 300 mA for 2 h. Then the PVDF membrane was stained with Ponceau S for 15 min. The membrane with the transferred proteins was washed with PBST three times for 10 min each before incubated with blocking buffer for 1 h at 37°C. The membrane was washed for 10 min three times and incubated with hyperimmune serum against *C. pseudotuberculosis* (1:100) for 2 h at 37°C. The membrane was rinsed again with PBST three times for 10 min and goat anti-rabbit IgG (1:1000) was added, incubated for 2 h at 37°C. After incubation, the membrane was washed with PBST five times for 10 min and washed one time with TBS before being exposed with the TMB substrate solution (Promega, USA) for 2-3 min. Lastly, the membrane was washed with distilled water and dried before being used for N-terminal amino acid sequencing.

Amplification of the surface protein genes

Two set of primers were designed based on the published sequences of *C. pseudotuberculosis* FRC41 (accession number CP002097.1) that were specific for the 31 kDa and 40 kDa genes of interest. They were LIC-SP31F (5' GAC GAC GAC AAG ATG AAC AGG TTC TCT 3') and LIC-SP31R (5' GAG GAG AAG CCC GGT CTA GTT TTT AGC 3') for SP31, and LIC-SP40F (5' GAC GAC GAC AAG ATG ACG ATT CGC GTA 3') and LIC-SP40R (5' GAG GAG AAG CCC GGT TTA AAG GCG CTC 3') for SP40.

Polymerase chain reaction was carried out in 50 µl volume containing 10 µl of 5x PCR BIO reaction buffer (15mM MgCl and 5mM dNTPs), 2 µl forward and reverse primers respectively (10 µM), 2 µl DNA template (8ng/µl), 1U PCR BIO HiFi Polymerase (2U/µl) and 33.5 µl of sterile distilled water. The amplification of the DNA was performed using the Thermocycler (Appendorf, Germany) where the condition was set with initial denaturation step at 95°C for 1 min. The next 30 cycles were performed with 15 sec denaturation at 95°C, annealing at 66.5°C and 67°C for SP31 and SP40, respectively for 15 sec and extension at 72°C for 1 min, following final extension at 72°C for 10 min and hold at 4°C. After amplification, 5 µl sample mixed with 1 µl of loading dye (Fermentas, Lithuania) was subjected to electrophoresis in a 1% agarose gel in TBE at 80 V (Bio-Rad, Germany) for 1 h and stained with GelRed nucleic acid gel stain (Biotium, USA) to detect the presence of the amplified products. Gel was visualized under an ultraviolet light transilluminator (Bio-Rad, Germany) and photograph by photography system, KODAK.

Construction and transformation of recombinant plasmid

To construct the recombinant plasmid, the gene of the surface protein was treated with T4 DNA polymerase to ensure ligation between the insert and the vector pET32 Ek/LIC. After ligation, the recombinant plasmids were transformed into cloning host, NovaBlue GigaSingle competent *E. coli* cells (Merck, Germany).

Positive clones were screened by PCR using vector specific primers, forward primer s-tagF (5'- ATG GAT AGC CCG GAT CTG GGT ACC-3') and reverse primer T7terR (5'- TTA GTG GCC CCA AGG GGT-3') (Nur-Nazifah, Sabri, & Siti-Zahrah, 2014).

Positive clones of the two recombinant plasmids were sent for DNA sequencing (1st Base, Malaysia) to confirm the presence of the cloned fragments before being used for transformation into *E. coli* BL21 (DE3) strain for expression study. The clones were screened using vector specific primers and subcultured onto Luria Bertani (LB) medium containing 50 mg/ml ampicillin overnight at 37°C with gentle shaking. The bacterial culture was mixed with 80% glycerol and stored at -80 °C until further used.

Isopropyl-beta-D-thiogalactopyranoside (IPTG) induction

The induction was performed according to the manufacturer's protocol (Novagen, USA). A positive recombinant cell colony was picked and inoculated into 3 ml of LB broth containing 50 mg/ml ampicillin overnight at 37°C. The culture was then added into 100 ml LB broth containing 50 mg/ml ampicillin. Before induction, the culture was split into 2x 50 ml culture where one mM IPTG was added into one of the 50 ml culture and the other culture was used as un-induced control. The cultures were incubated at 37 °C for 16h with shaking at 250 rpm. The expressed broths were centrifuged at 10 000 xg at 4 °C for 10 min to harvest the cells. The harvested pellets were re-suspended in 5 ml/g of Bugbuster Protein Extraction (Merck, Germany).

The supernatants that contained the soluble proteins were used for analysis and detection of the expressed protein using SDS-PAGE (Bio-Rad, USA) and Western immunoblot (Bio-Rad, USA).

Preparation of inactivated recombinant cells

Following induction, cultures of recombinant *E. coli* BL21 (DE3) expressing the surface protein genes were harvested and killed in 0.5% formalin (PBS, pH 7.4; Sigma, USA) overnight at 4 °C.

This was followed by washing three times in sterile PBS by centrifugation at 5 000 xg for 10 min at 4°C to ensure that formalin was completely removed. The inactivated recombinant cells were re-suspended in sterile PBS and the inoculums were prepared to the final concentration of 10⁶ cfu/ml using McFarland method.

Experimental design

Fifteen goats were divided into three groups. Group 1 was the unvaccinated control injected intramuscularly with PBS while Groups 2 and 3 were exposed intramuscular with 1 ml of inactivated recombinant cells prepared earlier (SP31 and SP40, respectively).

Respective booster dose was given two weeks after the first exposure. Serum sample were collected from all goats prior to and at weekly intervals post-vaccination until week 12 and subjected to ELISA to determine the antibody levels. Two weeks after booster dose, all goats were challenged with live virulent *C. pseudotuberculosis* and all surviving goats were killed at week 12. The Institutional Animal Care and Use Committee, Universiti Putra Malaysia approved the experiment (Approval No. R077/2014).

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were subjected to direct enzyme-linked immunosorbent assay (Paule *et al.*, 2003). The microtitre plates were coated with 50 µl of suspension containing 10⁶ cfu/ml of antigen diluted in citrate coating buffer and incubated overnight at 4°C. The plates were then added with 200 µl of blocking buffer and incubated at 37 °C for 1 h. After washing, 100 µl goat serum (1:500) was added and incubated at 37°C for 1 h. The plates were washed again before 100 µl rabbit anti-goat IgG-horse radish peroxidase (Nordic-MUBio, Netherland) diluted 1:8000 was added into each well and incubated for 1 h at 37°C. The plates were washed three times and 100 µl of TMB one solution substrate (Promega, USA) was added and incubated for 30 min at 37°C. Lastly, 50 µl of stopping buffer solution (2.5 M sulphuric acid) was added and the optical density was measured at 450 nm wavelengths.

Bacterial isolation and identification

Samples of lymph nodes (submandibular, preescapular, preferences, supramammary and mesenteric) and organs (lung, liver kidney and spleen) were collected and subcultured onto blood agar before incubated at 37°C for 48 h. Suspected colonies of *C. pseudotuberculosis* were confirmed by PCR according to Centikaya *et al.* (2002) using 16S rRNA primers.

Statistical analysis

Statistical analysis of the data was carried out using univariate analysis of variance (ANOVA) and Post Hoc (Turkey test) with SPSS 16.0 software (IBM, USA). The graph shows the standard error mean (SEM) for each group. The significant difference were determine when the P values <0.05.

Results

SDS-PAGE and Immunoblotting of the Surface Proteins of *C. pseudotuberculosis*

SDS-PAGE of the surface proteins of both strains revealed 11 bands of 140, 78, 75, 68, 64, 60, 50, 40, 36, 31 and 25 kDa. The major proteins were 31 and 40 kDa (Fig. 1). Immunoblot revealed positive reaction by protein bands 75, 40, 31 and 25 kDa (Fig. 2) with 40 and 31 kDa bands showed intense reactions. Further analyses revealed that the 31 kDa band showed 83% homology with the putative surface-anchored protein (PSA) while the 40 kDa band showed 100% homology with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *C. pseudotuberculosis* FRC41 (accession number CP002097.1).

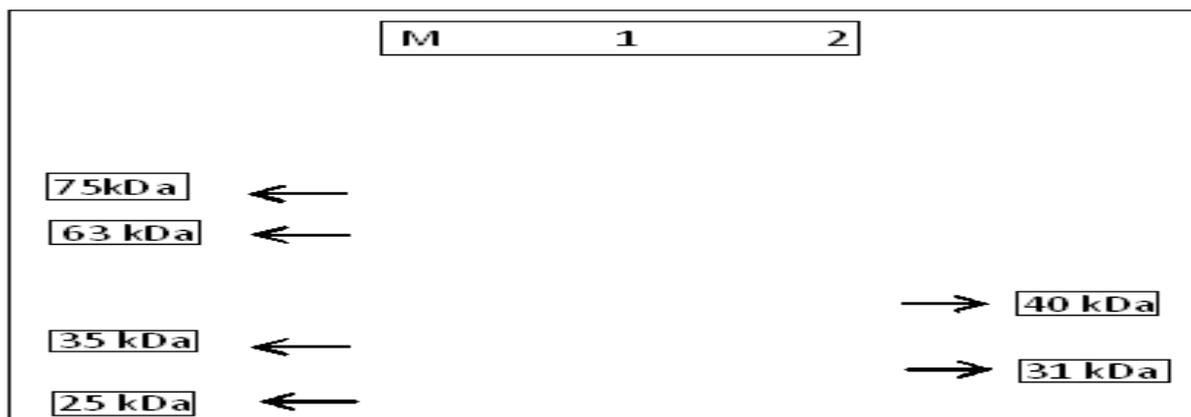


Fig. 1. Distribution of surface proteins of *C. pseudotuberculosis* showing 11 bands with 31 and 40 kDa being the major bands. Lane 1 is UPM J1 and lane 2 is UPM J2 strains of *C. pseudotuberculosis*. Lane M is the protein marker (M).

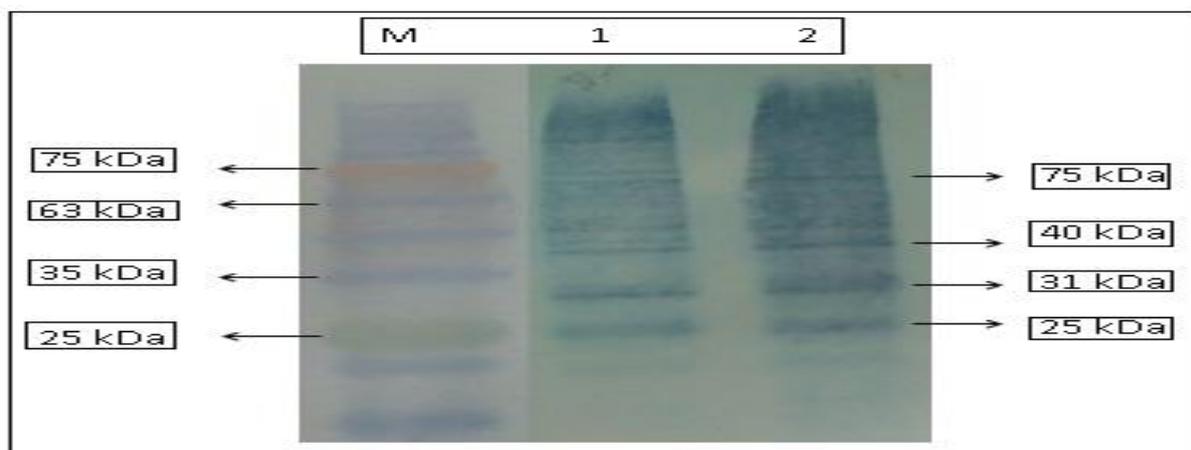


Fig. 2. Immuno-detection of the antigenic surface proteins of *C. pseudotuberculosis* showing positive bands. Lane 1 is the UPM J1 and lane 2 is the UPM J2 strains. Lane M is the protein marker.

Development of the recombinant plasmid and expression

Amplification and DNA sequencing of the SP31 gene of *C. pseudotuberculosis* resulted in fragment size of

approximately 1443 bp encoding 480 amino acids (Fig. 3) whereas SP40 revealed fragment size of approximately 996 bp encoding 331 amino acids (Fig. 4).

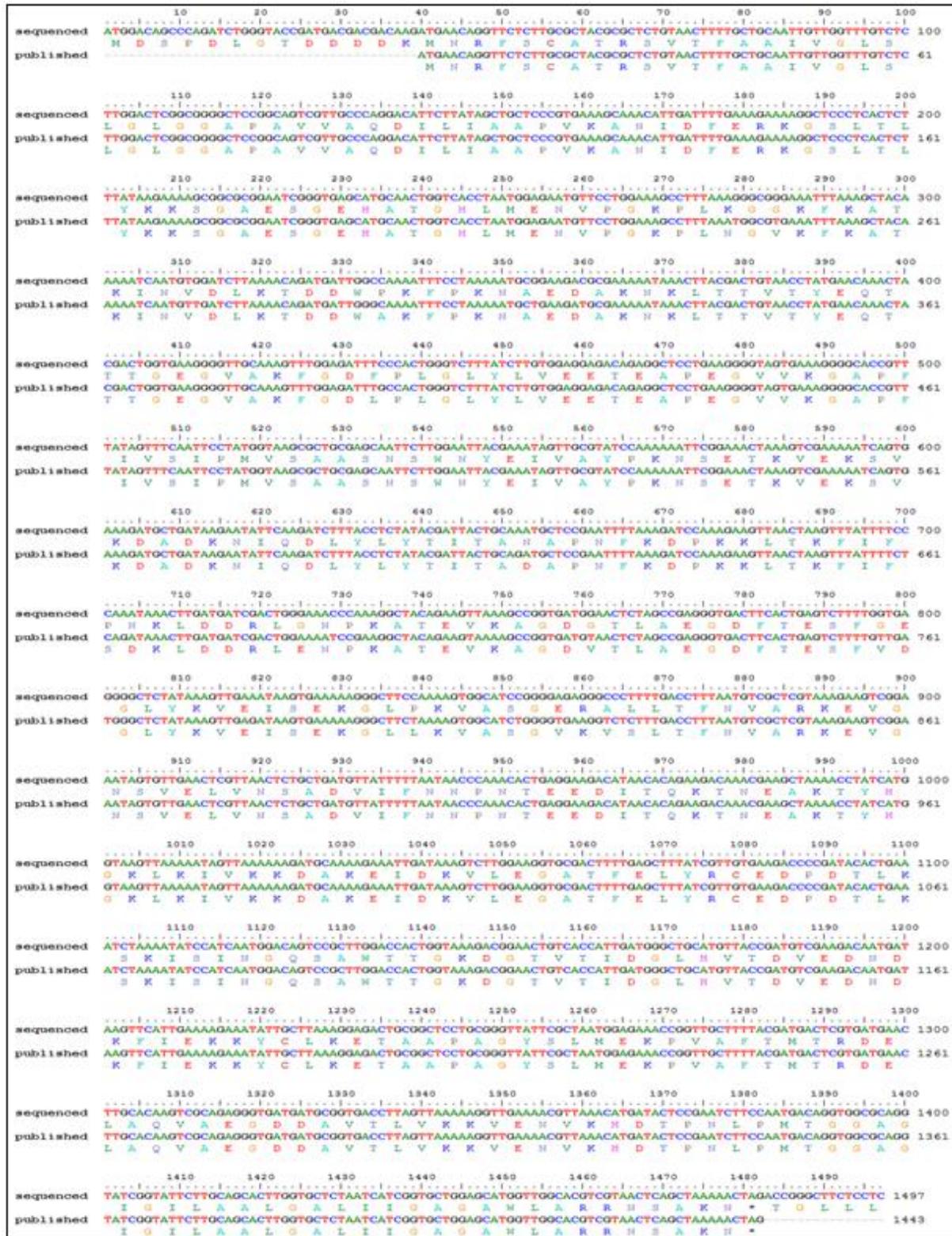


Fig. 3. Alignment of the pET32/LIC-SP31 sequence and published nucleic acid of putative surface-anchored protein (SpaA) of *Corynebacterium pseudotuberculosis*.

The nucleotide sequence of SP31 gene showed 98% homology with putative surface-anchored protein (fimbrial subunit), SpaA gene, meanwhile SP40 show 99% homology with

glyceraldehyde-3-phosphate dehydrogenase gene. The purified PCR products were successfully cloned into pET-32 Ek/LIC prokaryotic expression vector.

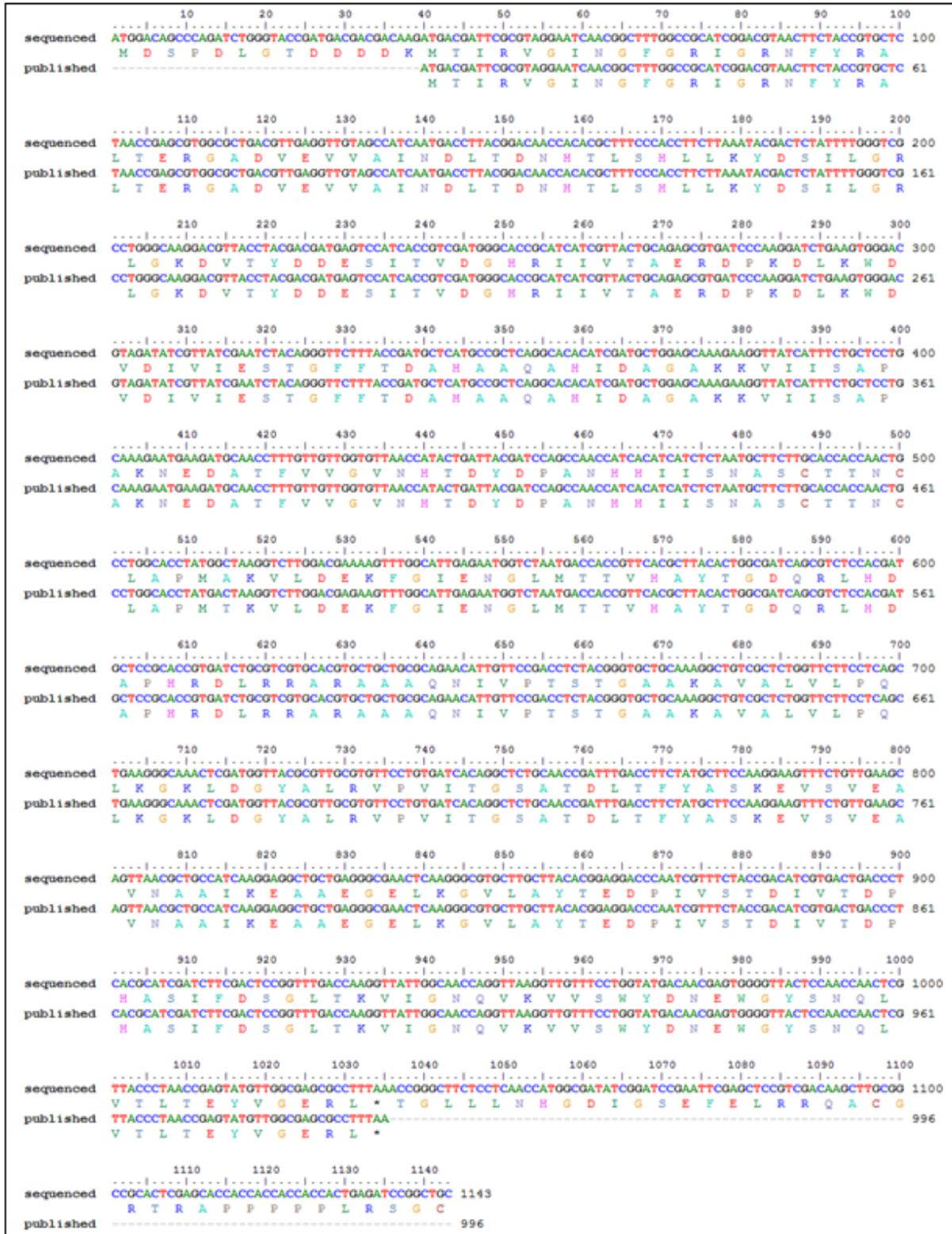


Fig. 4. Alignment of the pET32/LIC-SP40 sequence and published nucleic acid of glyceraldehyde-3-phosphate dehydrogenase of *Corynebacterium pseudotuberculosis*.

The expression of the SP31 and SP40 in the expression vector *E. coli* BL21 (DE3) showed the presence of single band of 67 kDa and 54 kDa, respectively (Figs. 5 and 6).

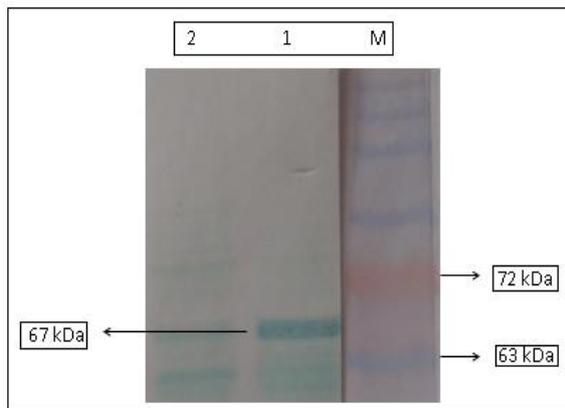


Fig. 5. Western immunoblotting of soluble fusion protein of recombinant SP31 after expression in *E. coli* BL21(DE3) using His.Tag Mab as primary antibody. Lane M: protein marker, lane 1: soluble cell protein of *E. coli* BL21 (DE3) expressing pET32/LIC-SP31 and lane 2: soluble cell protein of *E. coli* BL21 (DE3) expressing pET32/LIC without insert.

Immune response

At the start of the experiment, all groups showed low level of antibody against *C. pseudotuberculosis*. Following exposure, Groups 2 and 3 showed significantly ($p < 0.05$) high antibody level as early as week 1 (Fig. 7).

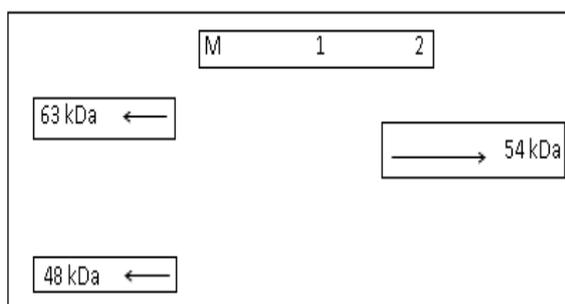


Fig. 6. Western immunoblotting of soluble fusion protein of the recombinant SP40 after expression in *E. coli* BL21(DE3) using His.Tag Mab as primary antibody. Lane M: protein marker, lane 1: soluble cell protein of *E. coli* BL21 (DE3) expressing pET32/LIC-SP40 and lane 2: soluble cell protein of *E. coli* BL21 (DE3) expressing pET32/LIC without insert

Following booster, Group 3 remained insignificantly ($p > 0.05$) higher than Group 2 until week 7 but both exposed groups showed significantly ($p < 0.05$) higher antibody levels than the control Group 1 until week 12 (Fig. 7). At the time of challenge on week 4, both exposed groups showed significantly ($p < 0.05$) high antibody levels than the control.

Gross pathology and bacterial isolation

Majority (14/15) of the challenged goats either exposed or non-exposed showed lesions of abscessation in the lymph nodes, except one goat of Group 2. *Corynebacterium pseudotuberculosis* was successfully isolated from all exposed goats of Group 2 and unexposed goats of Group 1. Two of the three (67%) goats of Group 2 have abscess in the lymph nodes and were positive for *C. pseudotuberculosis*.

Discussion

Generally, the efficacy of the vaccines in sheep is different when used in goat since the side effects are more intense with formation of lesions at the injection site, fever, malaise and reduced in milk production (Ribeiro *et al.*, 2014). Nevertheless, surface proteins are believed to play important role in host-pathogen interaction and have shown to be highly immunogenic when used for vaccine preparation (Pacheco *et al.*, 2011). In this study, we showed that the 31 kDa and 40 kDa surface proteins were good antigens when they cross-reacted with the hyperimmune serum against whole cell *C. pseudotuberculosis*.

The recombinant cells that carry the 31 kDa surface protein were recognized as putative surface-anchored protein (fimbrial subunit) SpaA gene. This putative surface-anchored protein contains 1442 nucleotides that encodes 480 amino acids and could be detected in pathogenic *Corynebacterium*. The gene is present in the pili of pathogenic *Corynebacterium* sp. that assists in attachment of the bacterium to the host cell (Rogers *et al.*, 2011). Furthermore, it was reported that the adhesive pili of *C. pseudotuberculosis* FRC41 consisted of major pilin subunit SpaA, suggesting the SpaA as a potential virulence factor (Trost *et al.*, 2010).

Protein expression of the recombinant cells revealed the presence of single band of approximately 68 kDa corresponding to the recombinant fusion protein of 17 kDa tagged protein and 51 kDa recombinant SpaA.

Recombinant cells carrying the 40 kDa protein consisted of 996 bp nucleotides that encodes 332 amino acid. This recombinant protein encoded glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, a glycolytic enzyme that involves in glycolytic pathway (Oliveira *et al.*, 2012).

The GAPDH is one of the newly identified extracellular proteins that can be a target in prevention against CLA as it is found in the surface protein of various pathogens with an ability to modulate the host immune system during infection (Silva *et al.*, 2013). The protein expression revealed presence of single band of 53 kDa, which corresponded to 36 kDa of GAPDH and 17 kDa fusion proteins.

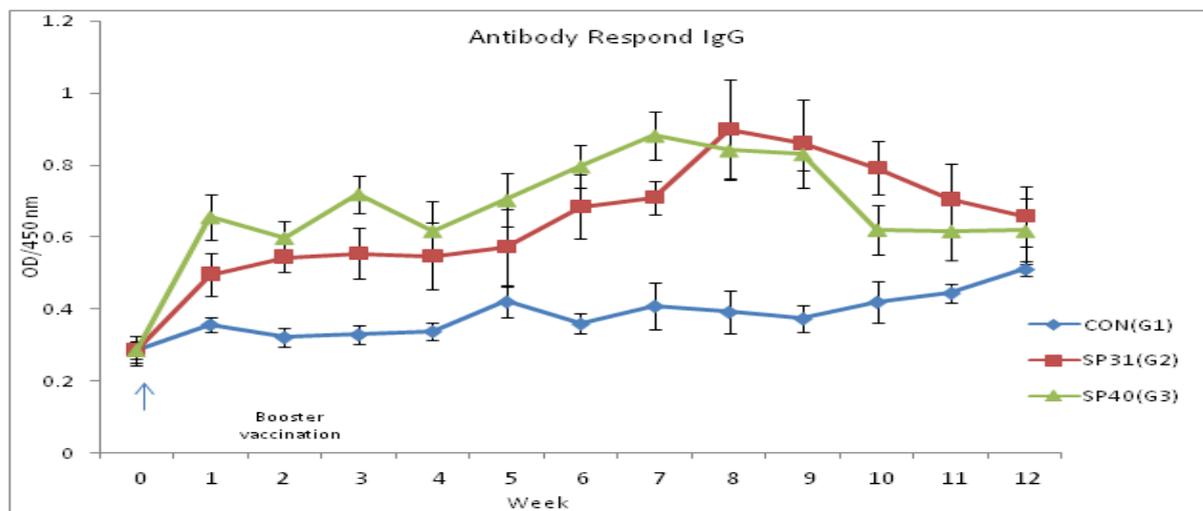


Fig. 7. Serum IgG levels of goats against *C. pseudotuberculosis* following exposures to inactivated recombinant cells. Generally, the exposed goats of Groups 2 and 3 showed significantly ($p < 0.05$) high antibody levels than the control Group 1.

Following development of recombinant cells, they were used to determine the ability to enhance immunity and prevent infection in goats.

Exposures to the recombinant cells increased the IgG levels as early as week 1 post-vaccination and remained significantly high following booster dose until the end of the 12-week study period. However, following challenge with live *C. pseudotuberculosis*, almost all exposed and unexposed goats developed abscessation in the lymph nodes while *C. pseudotuberculosis* was successfully isolated from all groups. These suggest that the recombinant cells carrying the surface protein are able to enhance humoral immune response but not protective against infection by live *C. pseudotuberculosis*. Similarly, vaccinating alpacas with cell wall components of *C. pseudotuberculosis* resulted in a robust humoral

reaction against *C. pseudotuberculosis* but gave no protection with the presence of abscess in internal and regional lymph nodes (Braga, 2007). It is suggested that effective vaccination against caseous lymphadenitis requires stimulation of the host reticuloendothelial system since *C. pseudotuberculosis* is an intracellular organism (Izgur *et al.*, 2010).

Furthermore, most vaccines that are based on recombinant protein present weak or poor immunogenicity when given alone thus require an adjuvant to elicit the protective and long lasting immune response (Nascimento and Leite, 2012).

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