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Optimizing and Expressing of Proteins Including PDHA, PDHB and PDHC of *Mycoplasma capricolum* sub sp. *capripneumoniae*

Ping Zhao, Ying He, Yuefeng Chu, Baoyu Li, Pengcheng Gao, Xuan Zhang, Haiyan Zhao, Youjun Shang and Zhongxin Lu

State Key Laboratory of Veterinary Etiological Biology, Key Laboratory of Epizootic Diseases of Grazing Animals of Ministry of Agriculture, Key Laboratory of Veterinary Public Health of Ministry of Agriculture, Engineering Research Center of Biological Detection of Gansu Province, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China

Corresponding Author: Zhongxin Lu, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730046, China

ABSTRACT

Mycoplasma capricolum sub sp. *capripneumoniae* (Mccp) is the causative agent of contagious caprileuropneumonia (CCPP) in goats. To develop more effective vaccines against CCPP, three protein sequences were optimized, in terms of codon usage bias, GC content, restriction enzymes, CIS-acting elements and repeat sequences, which are critical for efficient gene expression. These three proteins, respectively are the pyruvate dehydrogenase E1 component alpha subunit (PDHA), pyruvate dehydrogenase complex E1 component beta subunit (PDHB) and dihydrolipoylysine-residue acetyltransferase components of the pyruvate dehydrogenase complex (PDHC). The three optimized genes were expressed in *E. coli* and the relevant proteins were analyzed further by in vivo expression detection and antibody detection. The results showed the following: (1) A significantly higher level of serum antibodies against the three proteins were detected with convalescent sera and immunized sera, which demonstrated that the three proteins were expressed *in vivo*. (2) Goats antisera tests against the three proteins showed that they could all elicit humoral immunity with potential antigenicity and reactogenicity. (3) The results suggested that these three proteins (PDHA, PDHB and PDHC) have strong potential as vaccine candidates.

Key words: Antigenicity, optimization, PDHA, PDHB, PDHC

INTRODUCTION

Mycoplasma capricolum subsp. *capripneumoniae* (Mccp) is the causative agent of contagious caprileuropneumonia (MacOwan and Minette, 1976), a disease associated with cough, dyspnea, fever (40.5-41.5°C) (Thiaucourt *et al*., 1996). Attempts to control Mccp infection have been hindered by a lack of comprehensive knowledge regarding virulence factors and protective antigens. At present, the major vaccine against CCPP is inactivated vaccine of saponin adjuvant and the antigen using for diagnostic is the whole cell proteins and secretion polysaccharide. As is well-known, culture *in vitro* of *Mycoplasma* is very difficult and poor, so it is necessary to obtain effective recombinant protein. The membrane proteins of *Mycoplasma* have been considered as important targets for vaccine development and as diagnostic candidates (Boyce *et al*., 2006; Miniats *et al*., 1991; Nally *et al*., 2005). In the previous study, the authors identified nine novel antigenic OMPs in Mccp using an immunoproteomic approach (Zhao *et al*., 2012). In this study, the aim is to

optimize three of these OMPS, i.e., PDHA, PDHB and PDHC because of the high A+T content and the use of TGA as a tryptophan codon rather than as a stop codon in mycoplasmae and express them in *E. coli*. Further analyses detected a significantly high level of serum antibodies against the three proteins using convalescent sera and immunized sera. Tests with goats antisera against the three proteins showed that they all elicited humoral immunity, indicating their potential antigenicity and reactogenicity. These results should prove valuable for studies of pathogenicity and the development of vaccines in the future.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions: The Mccp strain was isolated from an infected goat in GanSu, China. The strain was cultivated at 37°C in KM_2 broth, containing 0.5% (w/v) Minimum Essential Medium, 0.5% (w/v) lactalbumin hydrolysate, 0.5% (w/v) yeast extract, 15% (v/v) horse serum, 0.08% (w/v) phenolsulfonphthalein, 0.01% (w/v) thallium acetate and 2000 IU L^{-1} penicillin. The pH was maintained at 7.4-7.6. Laboratory *E. coli* BL21 were used as nonadherent and noninvasive recipients of the recombinant gene-containing plasmid pET-32a (+).

Convalescent sera and hyperimmune sera: Five Mccp-free goats were inoculated tracheally with Mccp at a dose of 2.5×10^{10} CCU $goat^{-1}$. Blood was collected seven weeks after the first injection and serum samples were evaluated by enzyme-linked immunosorbent assay ELISA (Zhou *et al*., 2009). Specific titers were obtained from 1:5,000 to 1:10,000. Pre-infection sera were collected for use as negative controls. Three goats were immunized by hypodermic injections of an emulsion containing equal parts of Mccp total protein extract (1 mg) and complete Freund's adjuvant (Sigma, USA), followed by boosters after 2 weeks using the same antigen formulation and incomplete Freund's adjuvant (Sigma, USA). Blood was collected on the 14th day after the booster immunization and serum samples were evaluated by ELISA (Zhou *et al*., 2009). Specific titers were obtained from 1:7,000 to 1:15,000. Pre-immune goat serum samples were collected for use as negative controls. The Institutional Animal Care and Use Committee of Lanzhou Veterinary Research Institute approved the study.

Gene synthesis and optimization: In mycoplasmae, there is the high A+T content and the use of TGA as a tryptophan codon rather than as a stop codon. In this study three genes were synthesized to optimize their codon usage bias, GC content, restriction enzymes, CIS-acting elements and repeat sequences which are critical for efficient gene expression. The above were performed by software GenScript OptimumGene™ Codon Optimization 10 (GenScript Nanjing China Co., Ltd).

Expression and purification of the three proteins: To express the proteins in *E. coli*, the three genes of interest were cloned into the pET-32a(+) expression vector and transformed into laboratory *E. coli* strain BL21. Positive clones were screened using LB-Amp medium (100 μg mL^{-1}). The integrity of cloned sequences was confirmed by DNA sequence analysis. Transformed *E. coli* containing the selected constructs were incubated at 37°C until the Optical Density (OD) reached 0.6 at 600 nm. Recombinant protein expression was induced by adding 0.25 mM IPTG and culturing for 6 h at 37°C. Cells were harvested by centrifugation for 10 min at 10,000×g at 4°C. The recombinant protein was purified from the supernatant using a Ni-NTA agarose (Qiagen GmbH, Germany) column with an AKATA FPLC (GE Healthcare, USA), according to the

manufacturer's instructions. The inclusion body pellets were suspended in 20 mL binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 6 M urea. After incubation on ice for 1 h to dissolve the protein completely, the suspension was centrifuged at 12,000×g for 30 min and dialyzed against 10 mM Tris-HCl (pH 7.9), 100 mM NaCl and 5 mM $MgCl_2$ to promote refolding.

***In-vivo* detection of the three proteins:** An indirect Enzyme-linked Immunosorbent Assay (ELISA) was used to measure the *in-vivo* expression of the three proteins, according to the method of Zhang *et al.* (2008). Sera collected from pre-infected or pre-immune goats were used as controls. In brief, microtiter plates (Costar, USA) were coated with 200 ng/100 μL of purified recombinant protein diluted in 50 mM sodium carbonate buffer (pH 9.6) overnight at 4°C, before washing three times with PBS containing 0.05% Tween-20 (PBS-T). The antigen-coated plates were blocked with 1% BSA in PBS-T buffer for 1 h at 37°C, followed by the addition of 50 μL goat sera (convalescent sera and immunized sera) diluted in 1:40 and incubation for 1 h at 37°C. Plates were washed four times, followed by the addition of 50 μL of rabbit anti-goat IgG (H+L)-HRP (1:5000) (Sigma, USA) and incubation for 1 h. Next, 50 μL of the activated substrate solution was added (sodium citrate buffer containing 0.4 mg mL^{-1} o-phenylenediamine and 0.15% H_2O_2). The reaction was stopped after 10 min by adding 50 μL of 2 M sulfuric acid to each well. The plates were read at 490 nm using an ELISA reader. This assay was repeated in duplicate on different occasions.

Immunization and antibody detection: Fourty, 2-to 3-month-old goat were randomly assigned to five groups. Groups 1-3 are, respectively ten goats and other groups are respectively five goats. The 500 μg of each recombinant protein was mixed with the same volume of complete Freund's adjuvant (Sigma, USA), which was then used to subcutaneously immunize the goat in groups 1-3. Subsequent booster injections were applied on the 14th day using the same proteins emulsified in incomplete Freund's adjuvant (Sigma, USA). In group 4, goat inoculated with PBS emulsified in the same adjuvant were used as negative controls while group 5 goat were inoculated with PBS only as blank controls. All pre-immune goat sera were collected for use as controls. Sera were obtained from each group on the 14th day after the booster immunization. An indirect enzyme-linked immunosorbent assay (ELISA) was used for antibody detection. Microtiter plates (Costar, USA) were coated with 200 ng/100 μL of each purified recombinant protein. ELISAs were performed as described above. This assay was repeated in duplicate on different occasions.

Statistic analysis: Statistic analysis was performed on number and variety of samples. Effects of various factors parameters measured were analysed using SPSS (Statistic Packages for social sciences) and significant differences between samples were tested with Duncan's multiple range test.

RESULTS

Gene synthesis and optimization: The optimization results for codon usage bias and GC content are shown in Fig. 1-3. In terms of the codon adaptation index, PDHA was improved from 0.68 to 0.88, PDHB from 0.66 to 0.88 and PDHC from 0.65 to 0.87. In terms of the frequency of optimal codons (>90%), PDHA was improved from 49 to 70%, PDHB from 45 to 69% and PDHC from 41 to 66%. In terms of GC content adjustment, PDHA was improved from 30.91 to 47.64%, PDHB from 33.69 to 51.89% and PDHC from 32.69 to 50.58%. The results for restriction enzymes, CIS-acting

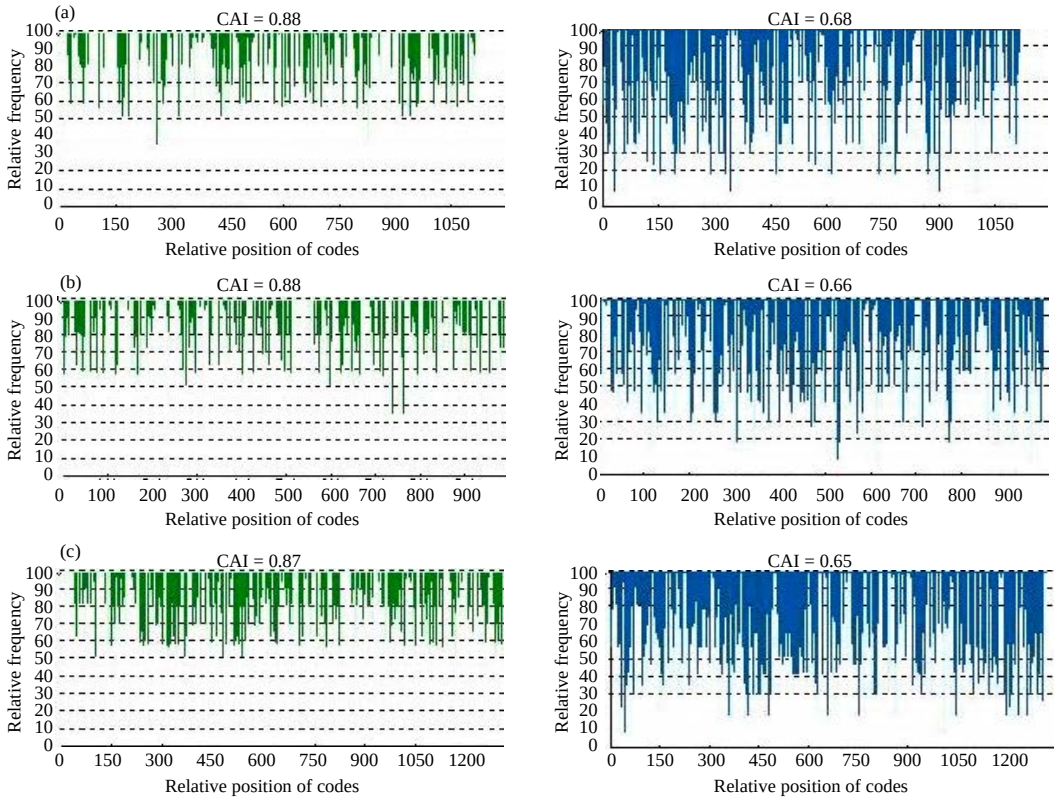

Fig. 1(a-c): Distribution of codon usage frequency along the length of the gene sequence; (a) PDHA, (b) PDHB and (c) PDHC after (left) and before (right) optimization, CAI = 1.0 was considered ideal for the desired expression by an organism, while CAI>0.9 was regarded as very good in terms of high gene expression

elements and removed repeat sequences are shown in Table 1. The optimized content included *E. coli* RBS, PolyAs, Max Direct Repeats, Max Inverted Repeats and Max Dyad Repeats. All of these elements are critical for efficient gene expression.

***In vivo* detection of the three proteins:** To analyze the *in vivo* expression of the three proteins and provide more information relevant to the development of efficient vaccines against Mccp, the specific antibody responses to the three recombinant proteins were assessed using sera collected from infected goats and immunized goats (The result of protein purification are shown in Fig. 4). All samples produced higher OD values than negative sera ($p<0.01$) (Fig. 5), which demonstrated that the three antigens were reactive with five convalescent sera. The presence of antibodies against these three proteins in five animals indicated that they were expressed *in vivo* and demonstrated that most individuals had a humoral immune response against these proteins. Both conditions are requirements for successful vaccine candidates. Compared with sera before immunization, specific antibodies against the proteins PDHA, PDHB and PDHC were significantly increased in the immunized goats ($p<0.01$) (Fig. 6). These results agreed with those obtained using convalescent sera.

Table 1: Results for restriction enzymes and CIS-acting elements and removed repeat sequences

Optimization content	Gene	Before optimization	After optimization
E.coli RBS(AGGAGG)	PDHA	2	0
	PDHB	0	0
	PDHC	0	0
PolyA (AAAAAAA)	PDHA	1	0
	PDHB	2	0
	PDHC	0	0
Max direct repeat	PDHA	Size:11, Distance:834,Frequency:2	Size:10, Distance:111, Frequency:2
	PDHB	Size:11, Distance:336, Frequency:2	Size:13, Distance:336, Frequency:2
	PDHC	Size:11, Distance:399,Frequency:2	Size:10, Distance:765,Frequency:2
Max inverted repeat	PDHA	Size: 10, Tm: 11.5, Start Positions: 458, 163	Size:11, Tm:38.4, Start Positions: 639, 70
	PDHB	None	None
	PDHC	Size: 11, Tm: 17.7, Start Positions: 910, 858	None
Max dyad repeat	PDHA	Size: 10, Tm: 19.3, Start Positions: 722, 844	None
	PDHB	None	None
	PDHC	Size: 10, Tm: 15.6, Start positions: 599,1041	None

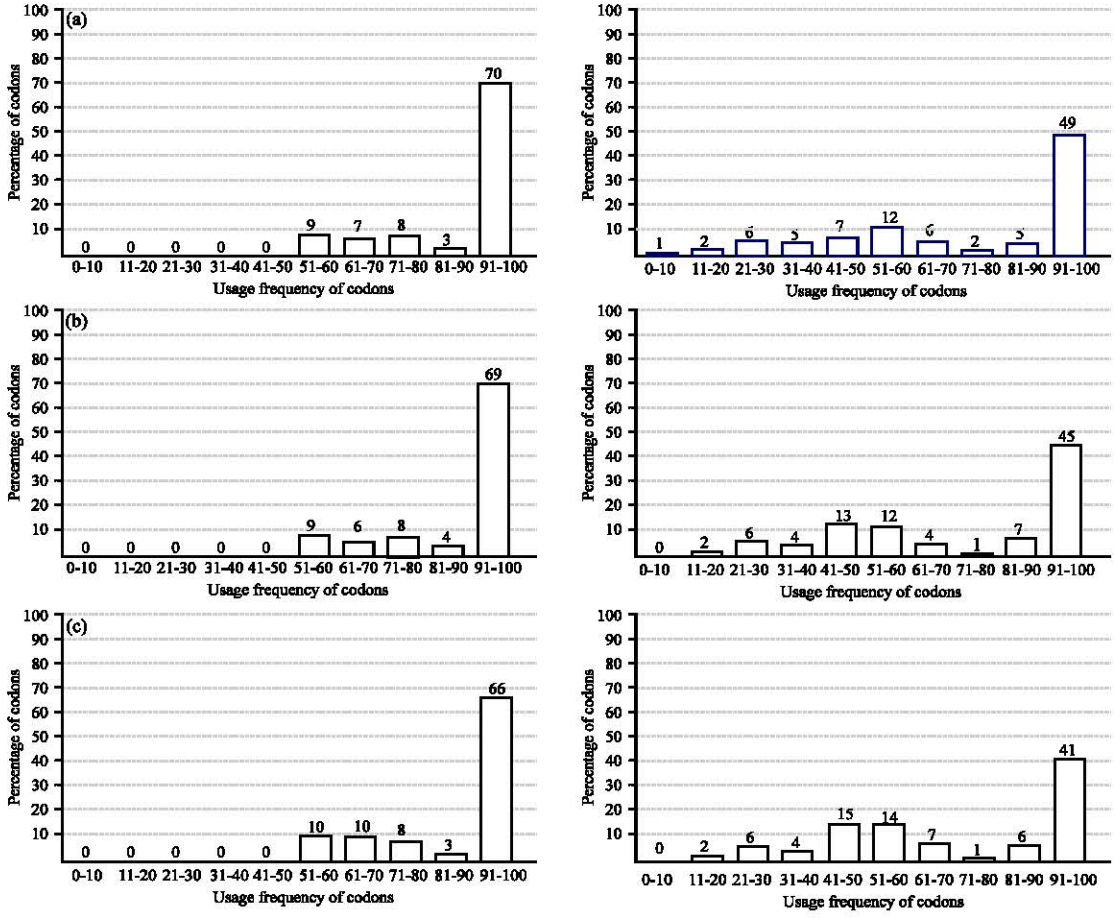

Fig. 2(a-c): Percentage distribution of codons in computed codon quality groups, (a) PDHA, (b) PDHB and (c) PDHC after (left) and before (right) optimization, A value of 100 was set for the codon with the highest usage frequency for a given amino acid in the desired expression organism

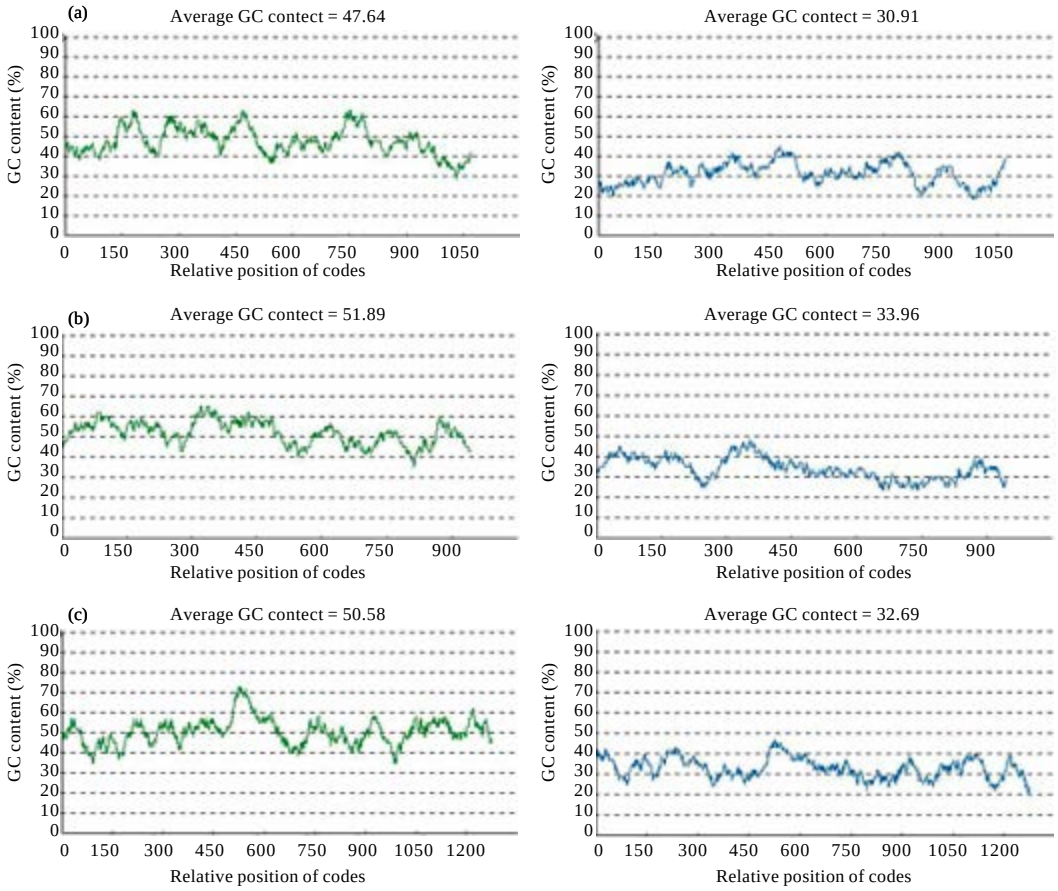

Fig. 3(a-c): The ideal percentage range for the GC content was 30-70%, %GC content peaks have been removed in a 60 bp window, (a) PDHA, (b) PDHB and (c) PDHC after (left) and before (right) optimization

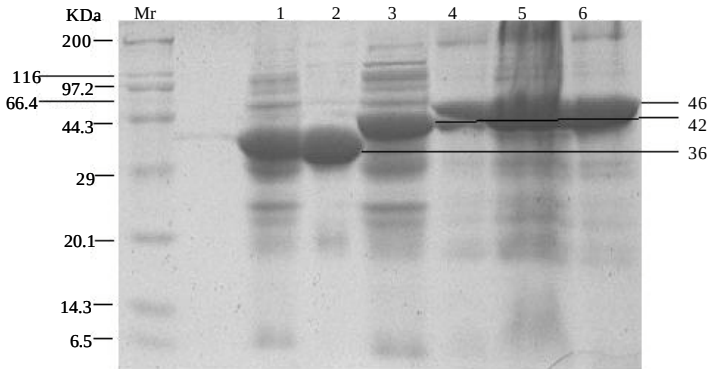

Fig. 4: SDS-PAGE analysis of purified recombinant expression protein, Mr: Protein molecular weight marker, 1: PDHB unpurified recombinant protein, 2: PDHB purified recombinant protein, 3: PDHA unpurified recombinant protein, 4: PDHA purified recombinant protein, 5: PDHC unpurified recombinant protein and 6: PDHC purified recombinant protein

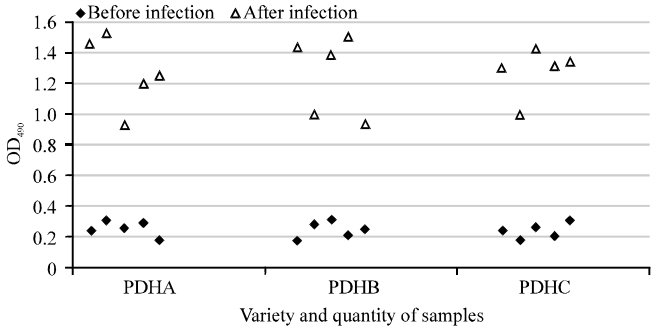

Fig. 5: *In-vivo* detection of novel proteins, The antibody responses against the proteins PDHA, PDHB and PDHC had higher ELISA OD values than negative sera from infected goats, which indicated that the three proteins were expressed *in-vivo*

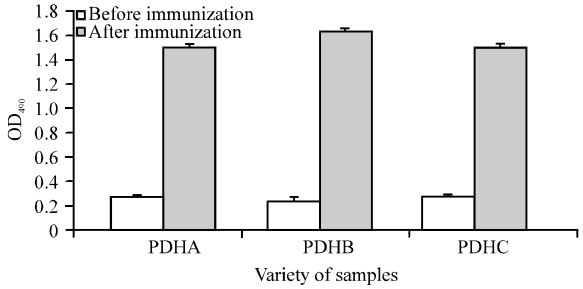

Fig. 6: Comparison of the specific antibody levels before and after immunization in goats, The antibody responses against the proteins PDHA, PDHB and PDHC had higher ELISA OD values than negative sera from the immunized goats

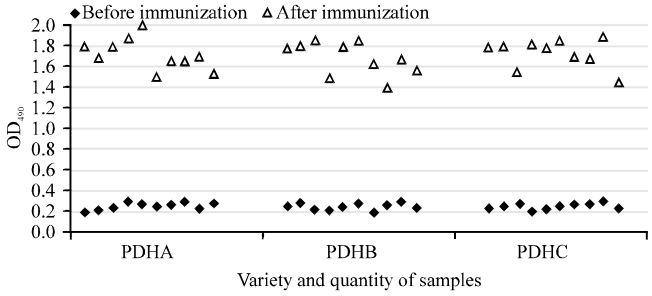

Fig. 7: Antibody detection of recombinant PDHA, PDHB and PDHC, The antibody responses against recombinant protein produced higher ELISA OD values than negative sera in immunized goats, This demonstrated that the three antigens could elicit a humoral immune response and their potential antigenicity and reactogenicity

Antibody detection: To assess the antigenic characteristics, the specific antibody responses to the three recombinant proteins were evaluated using polyclonal antisera against each recombinant protein. They all produced higher OD values than pre-immune sera ($p<0.01$) (Fig. 7), while no significant differences were found in the antibody levels against negative controls and blank controls (data not shown). This showed that the three antigens could elicit a humoral immune

response, thereby demonstrating their antigenicity and reactogenicity. This showed that the three proteins could potentially be used in the development of new vaccines.

DISCUSSION

In this study, the codon usage bias, GC content, restriction enzymes, CIS-acting elements and repeat sequences of three Mccp proteins (PDHA, PDHB and PDHC) were optimized, which are critical for efficient gene expression. This is important for producing sufficient antigen with a vaccine.

The authors assessed the effectiveness of PDHA, PDHB and PDHC as candidates for a vaccine and the specific antibody response against them was significantly increased in convalescent and immunized sera. The results indicate that these antigens are expressed *in vivo* and they can elicit an immune response, which are requirements for a potential vaccine candidate. The antigenicity of these proteins has been demonstrated in many bacteria (Lopez *et al*., 2005; Olson *et al*., 1991; Pinto *et al*., 2007; Regula *et al*., 2001; Wallbrandt *et al*., 1992).

In the present study, in vivo detection test analysed the immune response of goat inoculated with recombinant PDHA, PDHB and PDHC. The results showed that the three antigens could elicit a humoral immune response, thereby demonstrating their potential antigenicity and reactogenicity. OMPs could be highly advantageous for the eventual development of vaccines and diagnostic reagents (Meens *et al*., 2006). In the study, three OMPs, i.e., PDHA, PDHB and PDHC were expressed, which were demonstrated to be immunogenic antigens of Mccp for the first time. PDHA, PDHB, PDHC and dihydrolipoamide dehydrogenase (PDHD) are a series of genes, but PDHD is not expressed by *E. coli* under the same conditions as PDHA, PDHB and PDHC. It is possible that better conditions may be found for PDHD. Future experiments should focus on connecting the expression of PDHA, PDHB, PDHC and PDHD in an epitope vaccine to assess the dynamics of humoral immune responses during the course of disease, as well as the differences between infected animals that suffer acute disease and those that recover.

CONCLUSION

At present, the functions of only a few Mccp proteins have been reported. Thus, the OMPs described here will facilitate comparative studies to define their localization and their role in colonization during this important disease.

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