Vol.66: e23220381, 2023
https://doi.org/10.1590/1678-4324-2023220381
ISSN 1678-4324 Online Edition

Article - Agriculture, Agribusiness and Biotechnology

Designing a P48-40 Chimeric Protein of *Mycoplasma agalactiae* and Highly Expression in *E. coli*, Applicable for Indirect ELISA

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Editor-in-Chief: Bill Jorge Costa
Associate Editor: Marcos Pileggi

Received: 21-May-2022; Accepted: 19-Jul-2022

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HIGHLIGHTS

- Chimeric protein P48-40 of *Mycoplasma agalactiae* was designed and expressed in E.coli BL21 (DE3).
- P48-40 protein was highly expressed in soluble form and purified with yield of 33mg/Lby using Nickel resin.
- Novel P48-40 protein showed high performance for detection specific antibodies with 100% sensitivity and specificity.

Abstract: *Mycoplasma agalactiae* is an economically important pathogen and the main etiological agent of contagious agalactiae (CA) in small ruminants with a relatively high prevalence in sheep and goat, mainly characterized by mastitis, conjunctivitis and arthritis as predominant symptoms of a localized infection. Two genes encoding p48 and p40 major surface lipoprotein are played a fundamental role in the pathogenesis of *Mycoplasma* agalactiae. In this study a chimeric protein P48-40 of *Mycoplasma agalactiae* was designed, expressed, purified and evaluated in indirect ELISA. Coding sequence of antigenic regions of two p48 and p40 proteins was cloned into the expression vector Pet32a+ then expressed successfully into the E. coli BL21 (DE3) at optimal temperature 22^{o}C after 4 hours with 0.1mM IPTG as an inducer. Affinity batch formation method was done for purification of recombinant P48-40 by using Nickel resin. The protein was highly expressed in soluble form and purified with yield of 33mg/L. Function of recombinant protein in indirect ELISA was evaluated and the results showed that 125ng of novel P48-40 protein could well detect specific

antibodies with 100% sensitivity and Specificity by indirect ELISA. We concluded that the recombinant p48-40 protein could be applied for serodiagnosis of Mycoplasma agalactiae or evaluation of antibody levels in vaccinated animal.

Keywords: *Mycoplasma agalactiae*; chimeric protein; P48; P40.

INTRODUCTION

Mycoplasma agalactiae is the main agent of Contagious Agalactiae (CA) in sheep and goats resulting in significant losses of milk and meat industry and classified by the World Organization for Animal Health (OIE) as a notifiable disease [1,2]. CA which has been known for a long time in Europe primarily occurs in Mediterranean countries and by the end of the 19th century the disease had become enzootic in many parts of the world such as, India, Australia, Turkey, Iran, Mongolia, Nigeria, Senegal, Iraq, and Spain [3-11].

Occasionally, the disease becomes chronic, which can escape from the host immune system due to altered surface lipoproteins. Another function of these surface lipoproteins is to attach the bacterium to the host cell and colonize. The role of these adhesions is to facilitate the transfer of bacterial metabolites such as super oxides and peroxides to the host cell, leading to tissue oxidative damage [12].

CA common symptoms are conjunctivitis, polyarthritis, mastitis and abortion [13].

Mycoplasmas totally lack the cell wall, so membrane lipoproteins are directly exposed to the environment, and the two main surface lipoproteins which are responsible for the disease are P48 (465aa) and P40 (359aa) [4,9].

The diagnosis is based on the isolation and identification of the infectious agent by common methods, such as cultivation, growth inhibition test, epi-immunofluorescence test, etc., as well as on recently developed molecular techniques such as polymerase chain reaction or even more specific and sensitive methods like protein extraction and purification. The routine serological method is the complement-fixation test and, recently, also immuno-enzymatic methods [2,3].

In this study the *p40* and *p48* genes of *Mycoplasma agalactiae* was chosen because according to the recent researches they are present in all *Mycoplasma agalactiae* strains, and their expression levels increases in inflammation and immune defense [14]. The purpose of this study was to design, expression and purification a chimeric protein applicable for a serological test such as rELISA.

MATERIAL AND METHODS

Bioinformatics studies

DATA collection and sequence analysis:

To design the DNA construct of a chimeric protein, all 50 protein and nucleotide sequences related to the two genes P48 (26 sequences) and P40 (24 sequences) since 07-11-2019 available in GenBank (ncbi.nlm.nih.gov/genbank/) were collected. Basic Local Alignment sequence tools (BLAST) of NCBI were used for comparing and identifying the percent of covering and identity among protein and nucleotide sequences. Multiple Alignments and phylogeny tree was designed by MegAlign 5.0 software, DNAstar Inc. package. Polymorphisms in nucleotide and protein levels include conserved and hyper variable regions, SNPs and percent of divergence and identity among 50 available sequences were analyzed.

B-cell epitope prediction and DNA construction

Linear B cell epitopes in two major proteins P48 and P40 of *Mycoplasma agalactiae* were predicted by Bepipred Linear Epitope Prediction 2.0 and Kolaskar & Tongaonkar Antigenicity methods of Immune Epitope Database and Analysis Resource (http://www.iedb.org). From P48 protein with 465amino acids length, two antigenic regions were chosen, the first part between amino acid number 63 to 219 and the second from residues 265 to 335. In other hand, one antigenic region from amino acid25 to 215 has been chosen from P40 protein with 359 amino acids. Five amino acids EAAAK were consider between selected antigenic regions as a linker (Figure 1). The sequence is based on a protein which is reverse translated to a DNA sequence and then codon optimization was done according to the E. coli codon usage table (https://www.idtdna.com/CodonOpt). The nucleotide coding sequences was synthesized and cloned into the pET32a+expression vector (Figure 1).

Expression of P48-40 chimeric protein

The recombinant vector pET32-P48-40 was transformed into the competent cell *E. coli* BL21 (DE3) by Heat Shock method, and cultured on LB/Agar containing 50µg/mL ampicillin. Suspension was made by using a colony of bacterium from culture in 5mL2YT media with ampicillin and incubated at 37°Cwith shaking incubator for 16 hours. Induction was performed in two different temperatures (37°C and 22°C) by adding the entire suspension into a flask with 50 mL 2YT broth with ampicillin and incubation in 37°Cand 22°Cfor 2-4 hours until optical density reached 0.6 to 0.8. Recombinant protein was induced by adding 0.1mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) to the flask and samples were taken once before induction (T_0 or non-induced control) and every hour after it (T_1 to T_4) and the last sample after 16 hours (T_{16}). The induced cells were picked by centrifuge at 5000 g and 4°C, PBS (1X) was added on the remaining sample to suspend the pellets and sonication was done on ice 5 times for 1 minute with 1-minute intervals after all Phenyl Methyl Sulfonyl Fluoride (PMSF) was added to inhibit protease activity. The cell lysate was centrifuged at 15,000 RPM at 4°C for 15 minutes. Electrophoresis of 10% SDS-PAGE was performed on all samples before and after sonication to confirm the protein expression and accordingly temperature and IPTG amount. The gel was stained for 1 hour by standard Coomassie Brilliant Blue method.

Affinity purification of recombinant protein

Purification was done on post sonicated supernatant by affinity batch formation method with 100µL resin and three buffers with adjusted pH according to the protein PI (pH=8):200µLBindingbuffer containing 5mM Imidazole; 400µL wash buffer having 10mM Imidazole for three times and 15 minutes incubation to remove unwanted molecules. 100µLElution buffer with 300mM Imidazole and 50mM NaCl for 15 minutes incubation were used for elution of recombinant p48-40 protein.

Immunoblotting

Cell lysate after induction along with the prestainedprotein marker were electrophoresed into the 10%SDS-PAGE for 16 hours. In next step protein bands were transferred into the nitrocellulose membrane in transfer buffer for 2 hours with100 volt. The blocking step was performed by using 1% BSA in PBST (PBS containing 0.1% Tween20). After two washes with 1% PBST, the membrane was hybridized with a HRP conjugated antiHis-Tag antibodies (Abcam, USA) (diluted1:5,000).4-choloro-1-naphthol was used for developing the protein band.

Protein assay

Quantification of recombinant protein was performed by Bradford protein assay. To achieve a standard curve, various concentrations in range of 0 to 10 µg/µL protein from 0.25 mg/mL BSA (Bovine Serum Albumin) were utilized. 200µL Bradford reagent was added to all dilutions containing BSA and recombinant protein up to 1 mL final volume. Using the Microsoft office Excel, the formula (Y=29.035X-0.390and R2=0.9434) was obtained for calculating the amount of purified protein.

Enzyme-Linked Immunosorbent Assay (ELISA):

The optimal serum dilutions and recombinant P48-40 protein concentrations were established by a checkerboard titration. The micro plate (Nunc) was coated in different concentration from 500ng to 7.8 ng in 100µL carbonate-bicarbonate buffer (pH 9.6) per well followed incubation in 8°C for 16 hours. Unoccupied protein binding sites were blocked by adding 300 µL of blocking buffer (1% BSA to PBS) to each well and incubating the plate for 1 hour at 37°C. After incubation, the wells were washed three times with PBS-T (PBS plus 0.05 % Tween20). ELISA microplate was incubated 1 hour at 37°C with 100 µL of Different dilutions 1/10 to 1/1280 sera from immunized goat with Mycoplasma agalactiae vaccine (Razi Vaccine and Serum Research Institute). The plate was washed thrice with PBST. Anti-goat IgG 1/10000 dilution was added to each wells and incubated for 1 hour at 37°C. The plate was washed thrice with PBST. TMB substrate solution was added 100 µL per well and incubated for 10 min at room temperature in darkness. In final step, the reaction was stopped with H2SO4 (1M), and the absorbance was read at wave length A450 nm.

Statistical evaluation

The results of the immune reactivity assay were analyzed and compared between groups using one way analysis of variance (ANOVA) and p-values <0.05 were considered statistically significant.

To establish a vaccinated and unvaccinated cut-off value for this test, 40 positive and negative serum samples were used. The optimal cut-off values for the ELISA with 95% confidence intervals (CIs) were established by receiver operating characteristics (ROC) curves with the software GraphPad Prism version 9.2.0.

RESULTS

Protein Sequences analysis observed more than 47% in P40 and 18% in P48 proteins divergence between M. agalactiae and M. bovis. 15 linear B-cell epitopes in P48 protein and 12 epitopes in P40 protein were identified (Table1). Eight of 15 predicted linear B-cell epitopesinP48 protein in two antigenic regions and nine of 12 predicted epitopes from P40 protein were considered for designing DNA construct (Figure 1). One specific antigenic region 25-215 in P40 and two polymorphic antigenic regions 63-219 and 265-335in P48 proteins were selected (Figure 1). According to the results from site http://www.iedb.org most antigenic regions with the highest score and coverage of epitopes were selected for each protein (Figure1, 2). Analysis of P48 antigen showed the first 21 amino acids are signal peptide, and the most antigenic regions are between amino acids 63 to 219 and also another site between amino acids 265 to 335. For P40 one antigenic area with highest score is between amino acids 25 to 215 (Table1, Figures 1, 2).

Table 1.A: Linear B-cell epitopes of p48 protein. B: Linear B-cell epitopes of P40protein which were predicted with IEDBserver.

A: predicted peptides in p48

NO.	START	END	Peptide	Length
1	6	28	FYLFLGAAPVLSVPLVAASCGDK	23
2	40	47	TISTLAHI	8
3	89	97	VHKVSYELG	9
4	*134*	142	RYIVLCGFT	9
5	144	151	QASLVGLD	8
6	165	171	FITVDFN	7
7	190	198	GHLVPVIFD	9
8	202	208	AAYIAGR	7
9	210	220	LADYFSQVYKD	11
10	235	241	WPAVSDF	7
11	285	300	INSWKATASYPVAGS	16
12	336	342	FTSVMKL	7
13	344	355	GQAVYNILADLY	12
14	390	397	YVGVATSG	8
15	414	422	ATAYYVQKK	9

B: predicted peptides in p40

NO.	START	END	Peptide	Length
1	8	27	LFGLLTASSFTAVPLLAAKC	20
2	60	66	GEIVLPK	7
3	73	84	ASSILESLVKTN	12
4	89	97	TSELEVSNI	9
5	116	122	SINVTFT	7
6	126	133	SDDWAKK	8
7	146	163	LTNFVFGSDLLEALKTDL	18
8	171	180	DDFQFTVDKL	10
9	187	193	GKLVIEA	7
10	201	215	TGTVILDIPRLWKP	15
11	274	280	SSQVKAK	7
12	311	321	NDFIAYYFTEV	11

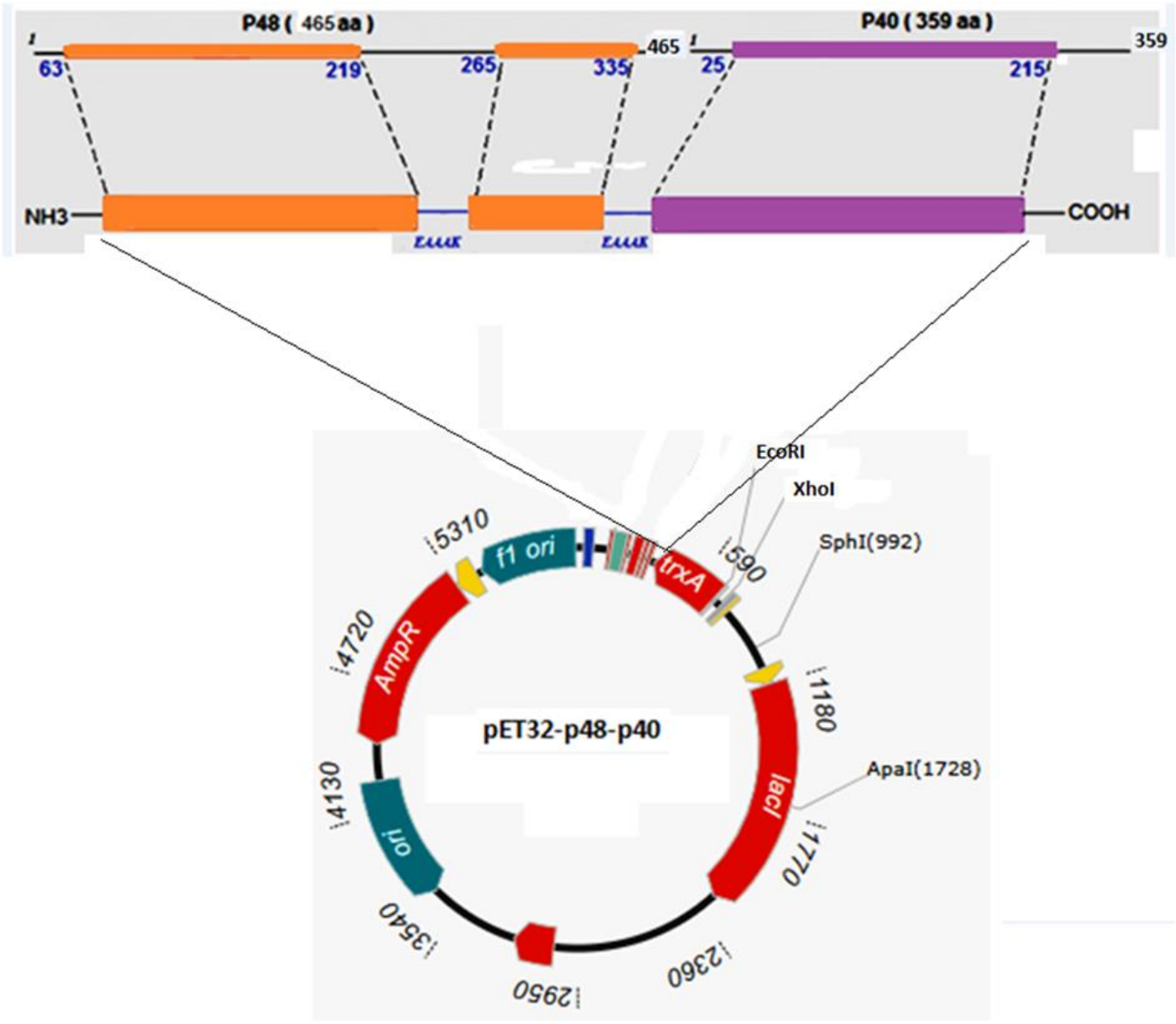

Figure 1. Schematic picture of the designed DNA construct to producing chimeric P48-40 protein, two antigenic regions of p48 (from amino acids # 63 to 219 and 265 to 335) and one part from p40 (from amino acids # 25 to 215) were fused together with linker EAAAK, inserted into the pET-32a+P48-40 Vector between *EcoR*I and XhoI recognition.

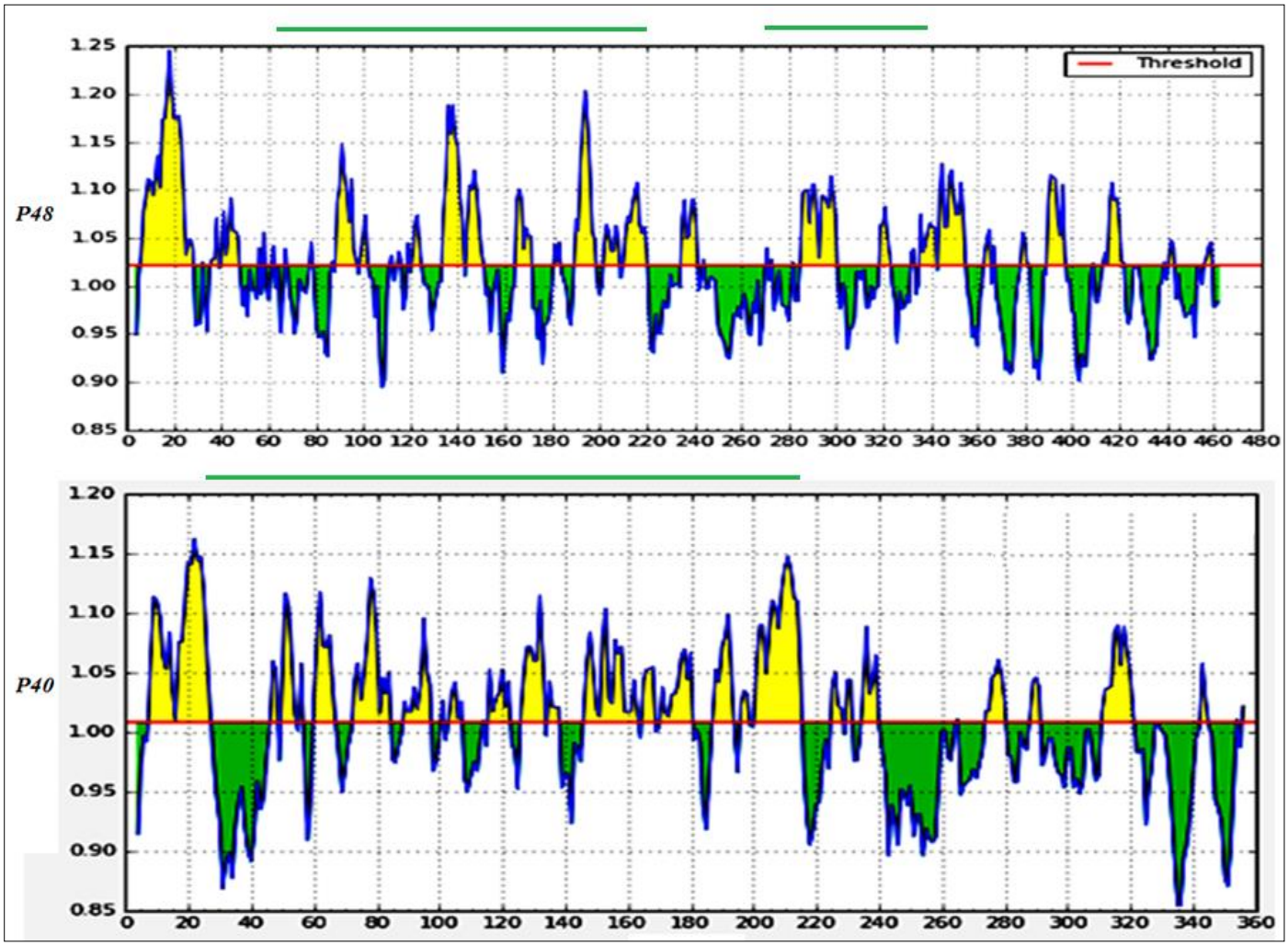

Figure 2. Antigenic regions of P48 and P40 proteins predicted with Kolaskar & Tongaonkar antigenicity method of IEDB Analysis Resource.

The SDS-PAGE electrophoresis of cell lysated protein was demonstrated a band of recombinant protein that corresponded to successful induction of 65kDa P48-40 chimeric protein. However, maximum expression of the P48-40 chimeric protein was observed after 4hours post induction at 22 °C in the presence of 0.1 mMIPTG (Figure 3). Analysis of the post-sonication cell lysate showed that the recombinant protein was mostly found in the soluble phase.

The protein was highly purified in soluble form using Affinity Batch Formation method with yield of 33 mg/L (Figure 3). Western blot analysis confirmed the presence of 65 k Dachimeric protein (Figure3).

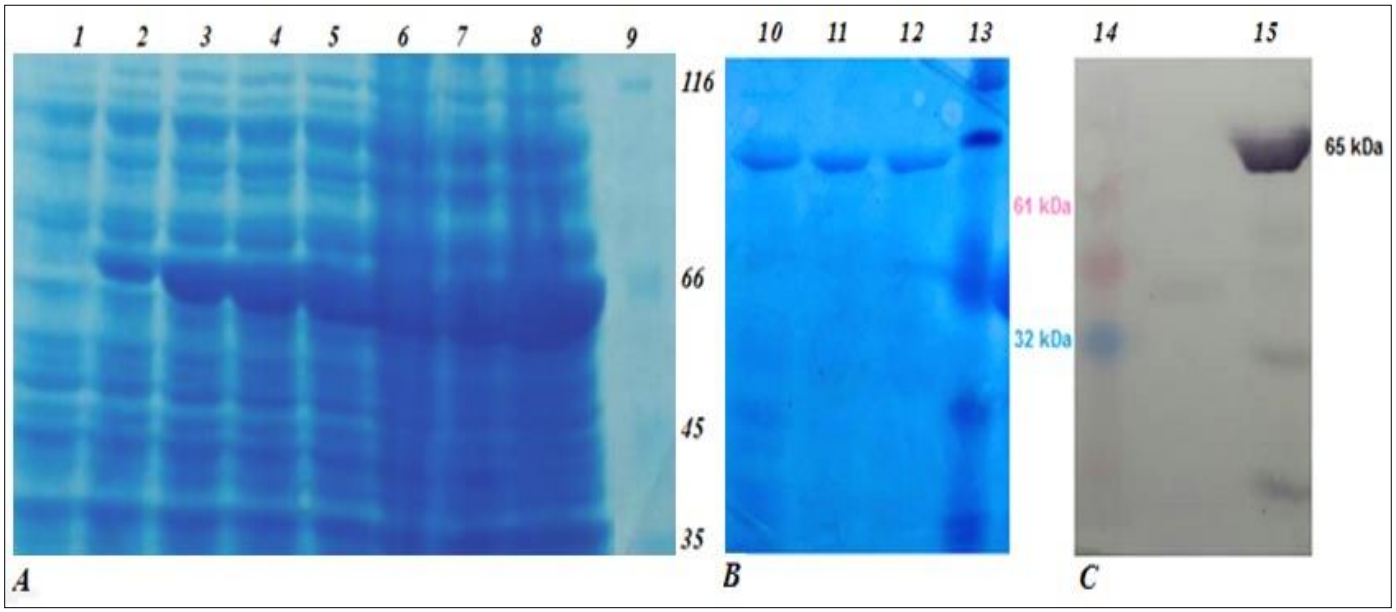

Figure 3.A: 10% SDS-PAGE analysis of expressed chimeric protein, 1; Uninduced cell, 2-5; induced cells after 2, 4, 5 and 16 hours, 6; post sonication, 7; post sonication cell lysate, 8; post sonication supernatant and 9 is protein marker. **B:** Purified P48-P40 protein with Affinity Batch Formation Method (10 to 12), in 10% SDS-PAGE, 5: Unstained protein molecular weight marker. **C:** Western blot analysis 14; Prestained protein molecular weight marker, 15; Recombinant P48-40 protein.

Checkerboard titration

Checkerboard titration was carried out for determining the optimal antigen concentration and serum dilution. The threshold value was estimated to be at approximately 125 ng concentration of the antigen and 1:40 dilution of test sera (Figure 4).

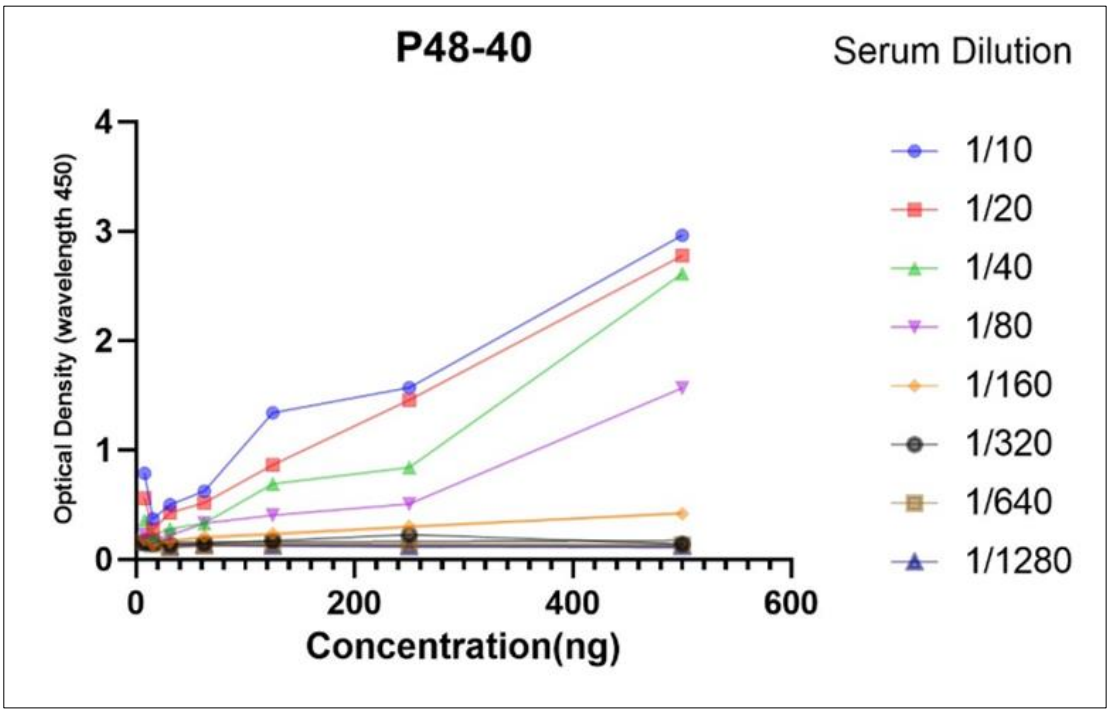

Figure 4. Graph showing the optical density for various serum and antigen dilutions. The threshold value was estimated to be at approximately 125 ng concentration of the antigen and 1:40 dilution of test sera.

Indirect ELISA results with 125ng the coated recombinant p48-40 protein showed that this protein could well detect antibodies produced in the serum of immunized host with agalactiae vaccine (Figure 5). The

results of the immune reactivity assay were analyzed and compared between groups using one way analysis of variance (ANOVA) and results were considered statistically significant (Table 2).

Cut-off values, sensitivity and specificity were calculated based on two groups of immunized and non-immunized with agalactiae vaccine serum samples using GraphPad Prism version 9.2.0 software by drawing a ROC curve. The amount of >0.950 for cut-off and 100% sensitivity and specificity were observed (Figure 6).

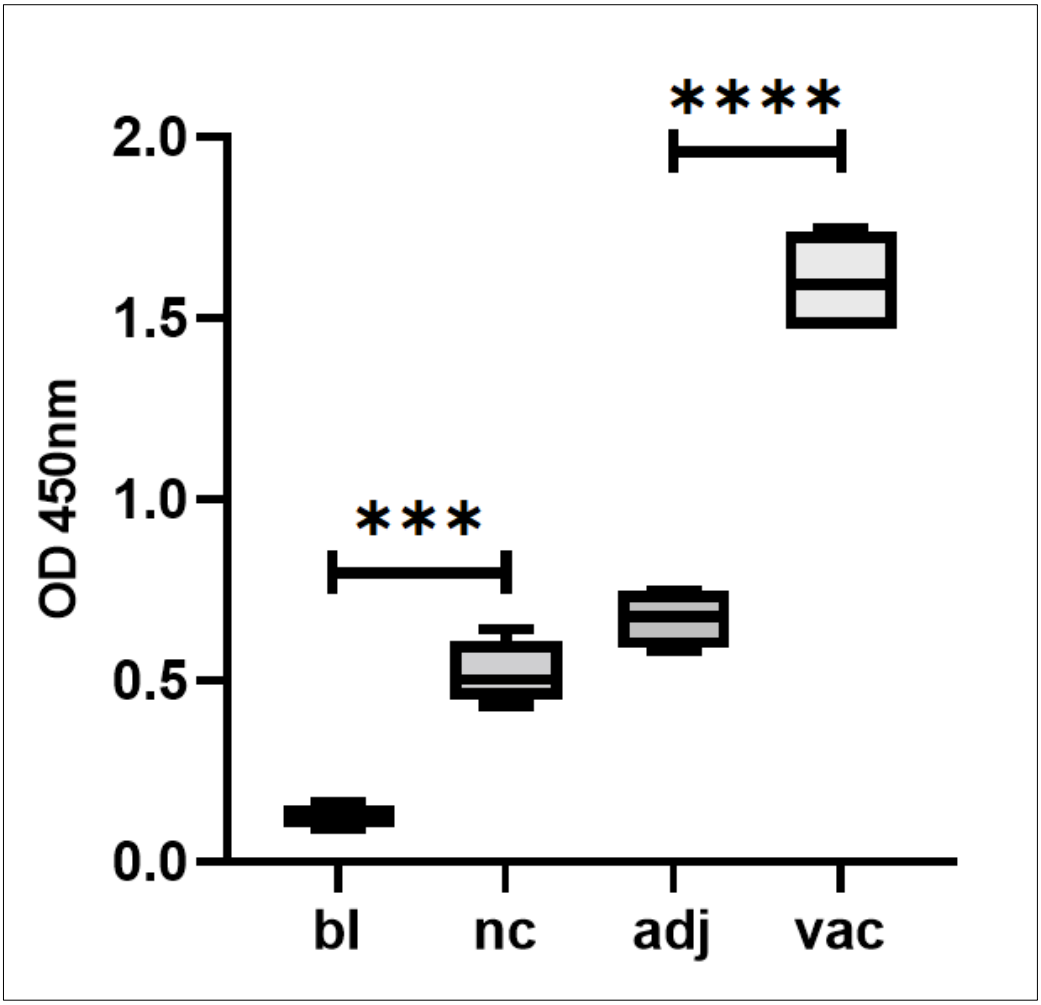

Figure 5. Effective of rP48-40 protein (125ng) coated on ELISA Micro plate for detection of specific antibody in vaccinated goat by indirect ELISA (wave length 450nM). Vac: Antibody level in immunized goat with vaccine, Nc: non-immunized sera (negative control). bl: Blank, adj: Optical density in immunized animal with adjuvant. Significant differences are indicated by *.

Table2. Statistical analysis of groups by GraphPad Prism software version 9.2.0, Multiple comparison One way ANOVA test method.

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of Diff.	Below threshold?	Summary	Adjusted P Value	
Blank vs. control	-0.3988	-0.6041 to -0.1934	Yes	***	0.0004	A-B
Blank vs. adjuvant	-0.5505	-0.7558 to -0.3452	Yes	****	<0.0001	A-C
Blank vs. vaccine	-1.481	-1.686 to -1.275	Yes	****	<0.0001	A-D
Control vs. adjuvant	-0.1518	-0.357 to 0.05356	NO	ns	0.1800	B-C
Control vs. vaccine	-1.082	-1.287 to -0.8767	Yes	****	<0.0001	B-D
Adjuvant vs. vaccine	-0.9303	-1.136 to -0.7249	Yes	****	<0.0001	C-D

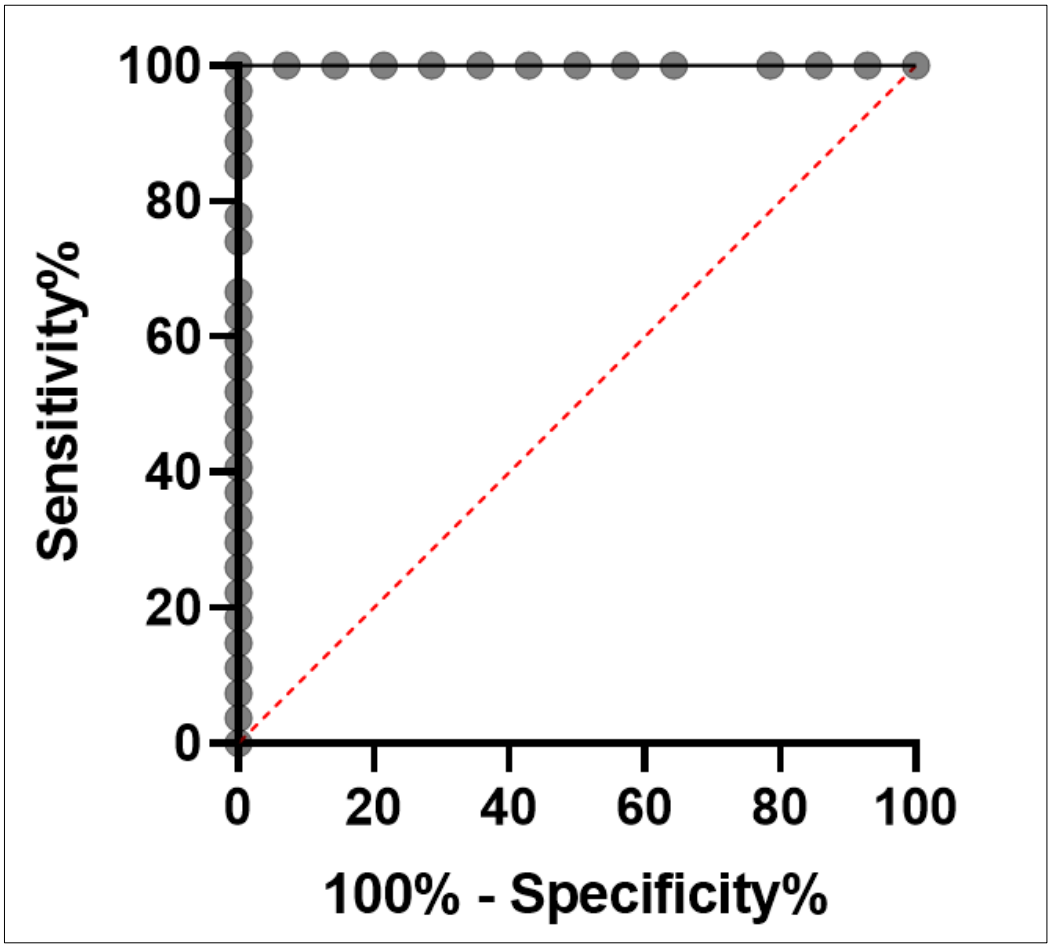

Figure 6. Diagram presenting the ROC analysis. The amount of >0.950 for cut-off was observed and sensitivity and specificity of ELISA were 100%. The value of the area under the curve (AUC) 1.0 indicated that the ELISA was also highly accurate.

DISCUSSION

Contagious agalactiae (CA), which is reported in many countries, is an economically important disease in small ruminants with a relatively high prevalence [15]. Antibiotic therapy can result in symptomatic improvement, but cured animals may remain carriers as a result of antibiotic resistance. To control CA the first step is to detect the infected animals by an effective, definite and accurate method. Vaccination is the only effective way to prevent and control the disease, especially in domestic areas.

P48 surface lipoprotein plays an important role in the immune response of infected animals and stimulates macrophages [16]. Also, P40 surface lipoprotein, which is involved in the binding of bacteria to the host cell membrane, is present in most M. agalactiae except for serotype C strains [17]. This protein is not expressed continuously; in fact, it is only expressed in the chronic stage of infection [12].

In this study, a novel chimeric protein P48-40 was designed by choosing the three immunogenic regions of protein p48 and p40 (Figure1 and 2). This peptide was designed based on conserved regions which were responsible for contagious agalactiae, with maximum population coverage.

rELISA is an accurate test in diagnosis and evaluation of vaccine as well as antibody levels of vaccinated animals and will say when a re-vaccination is needed. rELISA also can estimate how long vaccinated animal will stay immune, as well as evaluating the efficacy of existing vaccines.

The designed protein was highly purified which after further study and evaluation of its function it might be used to produce recombinant vaccine and also high sensitive diagnostic kit such as rELISA that will be used to prevent and detect the disease in a timely manner [18].

The existing diagnostic commercial kit is designed based on the manufacturer's country sequences, which are expensive and difficult to access and differ from the native Iranian sequences, so the need for a more cost-effective native kit is felt domestically.

To conclude, this study confirmed divergence between Iranian P48 and P40 protein sequences and foreign M. agalactiae (The basis of chimeric protein design). Designed construct was highly expressed in E.

coli BL21(DE3) and purified with good efficiency by Affinity Batch Formation method. The presence of purified protein was confirmed with SDS-PAGE analysis and western blotting. Chimeric p48-40 protein will be useful in detecting the presence of antibodies in sera of vaccinated small ruminant such as goat and sheep. When ROC analysis was performed, a cut-off of 0.950 gave the optimum sensitivity and specificity of 100%.

CONCLUSION

The rELISA developed here is a specific, sensitive, and reliable assay for the detection of antibodies in vaccinated animal against M. agalactiae. However, the serological diagnosis potential of this recombinant protein is recommended in main host by indirect ELISA method.

Acknowledgments: This study was supported by Grant No. 2-18-18-011-980243 from Razi Vaccine and Serum Research Institute.
Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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