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RESEARCH ARTICLE

Production, Purification and Characterization of the Recombinant *Brucella abortus* rP17 Protein [1] [2]

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Summary

Immunoreactive cytosolic P17 protein of *Brucella abortus* was produced in *Escherichia coli* as 6xHistidine tagged recombinant protein (rP17) by cloning the *p17* gene into pColdI cold-shock expression vector. DNA sequence analysis of the cloned *p17* gene showed that the recombinant rP17 protein contains a total of 181 amino acids constituted of 83 hydrophobic, 42 hydrophilic, 35 basic and 21 acidic residues. Its theoretical isoelectric point was calculated as 6.42 and GRAVY index of -0.097 indicates its solubility. The instability index classifies the rP17 as a stable protein expressed in the transformed *E. coli* cells by inducing with IPTG. Lysate of the induced and non-induced bacteria was analyzed by SDS-PAGE showing expression of the rP17 with a relative molecular weight of 24 kDa. After two-step purification procedure, Ni-NTA affinity chromatography and elution from polyacrylamide gels following SDS-PAGE, the rP17 was highly purified and analyzed by Western blot. Preliminary results showed that the recombinant rP17 protein still preserves its immunoreactivity. In present, large scale production of the rP17 is carried out for evaluation of its diagnostic performance with a large panel of well-defined sera.

Keywords: *Brucella abortus, Bacterial expression, Purification, Recombinant P17 protein*

Rekombinant Brucella abortus rP17 Proteininin Üretilmesi, Saflaştırılması ve Karakterizasyonu

Özet

Sitozolik immunoreaktif *B. abortus* P17 proteini, pCold I soğuk-şok ekspresyon vektörüne *p17* geni klonlanarak *Escherichia coli*'de 6xHistidin kuyruklu rekombinant protein (rP17) şeklinde üretildi. Klonlanan *p17* geninin DNA sekans analizi, rekombinant rP17 proteininin 83 hidrofobik, 42 hidrofilik, 35 bazik ve 21 asidik olmak üzere 181 amino asitten oluştuğunu gösterdi. Teorik olarak izoelektrik noktası 6.42 olarak belirlendi. -0.097 olan GRAVY indeksi proteinin eriyebilir olduğuna işaret etmektedir. İnstabilite indeksi, IPTG ile indüklenerek transforme *E. coli* hücrelerinde eksprese edilen rP17 proteininin stabil olduğunu göstermektedir. İndüklenmiş ve indüklenmemiş bakteri lizatlarının SDS-PAGE analizleri göreceli moleküler ağırlığı 24 kDa olan rP17 proteininin ekspresyonunu göstermektedir. rP17 proteini, Ni-NTA affinite kromatografisi ve SDS-PAGE sonrası poliakrilamid jelden elusyonu içeren iki aşamada saflaştırıldı ve Western blot yöntemi ile incelendi. Ön sonuçlar rekombinant rP17 proteininin immün reaktifliğini koruduğunu göstermiştir. Bu aşamada, tanımlanmış serumlardan oluşan kapsamlı bir koleksiyon ile diyagnostik performansının değerlendirilmesine yönelik olarak rP17 proteininin büyük ölçek üretimi gerçekleştirilmektedir.

Anahtar sözcükler: *Brucella abortus, Bakteriyel ekspresyon, Saflaştırma, Rekombinant P17 proteini*

INTRODUCTION

Brucellosis is an economically important zoonotic infectious disease of livestock with worldwide distribution [1]. Surveillance, control and eradication of the disease are very important not only for public and animal health purpose but also for its economic consequences. Therefore, several serological tests are widely used for

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brucellosis surveillance by detecting the presence of anti-*Brucella* antibody in cattle, sheep and goat [2]. Serological tests used in the diagnosis of animal brucellosis mostly aim to detect antibodies directed to lipopolysaccharide (LPS), the most immunodominant antigen of *Brucella* spp. However, cross-reactions due to some Gram negative bacteria sharing similar antigenic determinants can be observed in LPS-based serological tests, especially when the prevalence of the disease is low [3,4].

To overcome cross-reactions, several studies have been conducted to analyze the antibody response against *Brucella* and to identify the immunoreactive molecules as candidate specific diagnostic antigens. Several cytoplasmic (P15, P17, P18 and P39), periplasmic (BP26) and outer membrane proteins (OMPs) have been identified as immunodominant molecules [5-14]. Immunoreactive P15, P17, BP26 and P39 proteins were produced as recombinant antigens in bacterial expression systems and used as diagnostic antigens for brucellosis diagnosis in indirect enzyme-linked immunosorbent assay (ELISA) [9,10,12,14].

Indirect and competitive ELISA as screening methods and complement fixation test (CFT) as confirmatory test for brucellosis diagnosis are very sensitive and specific but require adequate infrastructure and professional expertise. However, the development of sensitive and specific diagnostic tools applicable in the field and limited laboratory conditions is still needed. Recently, we have developed qualitative and quantitative ELISA models [15] as well as field diagnostic tools based on the enzymatic and non-enzymatic rapid immunofiltration assays [16] and evaluated for brucellosis diagnosis. To improve the specificity and sensitivity of these tools, purified immunodominant *Brucella* specific antigens are required. For this purpose, production of the P17 protein as recombinant protein (rP17) was aimed in the first step because it was found to be one of the most promising diagnostic antigens [7,10]. This study also aimed to purify and characterize the rP17 protein expressed in *E.coli* bacterial system.

MATERIAL and METHODS

Bacterial Strain, Expression Vector and Monoclonal An.body

Brucella abortus S19 vaccine strain (Biovar 1) obtained from Pendik Veterinary Control and Research Institute (Istanbul/Turkey) was used for genomic DNA extraction. *E. coli* strain DH5α (Takara, Paris, France) grown at 37°C in LB (Luria-Bertani) medium with 50 µg ampicillin/ml was used for transformation and expression procedures. For production of recombinant proteins, the *p17* gene encoding P17 protein was cloned into multiple cloning site of pCold I bacterial expression vector (Takara). Monoclonal mouse anti-HisTag antibody (His-probe (H-3): sc-8036, IgG_1 Santa-Cruz Biotechnology Inc.) was used to confirm expression of the recombinant protein.

Genomic DNA Extrac.on

Genomic DNA from the inactivated (0.5% formaldehyde) *Brucella abortus* S19 strain grown in BHI agar was purified using "Genomic DNA Purification Kit" (Qiagen GmbH, Hilden, Germany) as recommended by the manufacturer and analysed by separation in agarose gel electrophoresis (1%). The concentration and purity of DNA were measured by absorbance at 260 and 280 nm wavelengths and stored at -80°C until use.

PCR Amplifca.on and Cloning of Brucella abortus p17 Gene

B. abortus p17 gene encoding the P17 protein was amplified by PCR using genomic DNA as template. The cloning primers were designed in accordance with the *B. abortus p17* gene sequence deposited in GenBank. For *p17* gene amplification, forward and reverse primers 5' CGTGAGGAATTCATGAACCAAAGCTGTCC 3' and 5' CGGCTGCGGTCGACCACGATCTGCAAG 3' contained *EcoRI* and *SalI* restriction enzyme sites, respectively. Each 50 µl of PCR mixture contained 200 ng of genomic DNA, 1x PCR buffer, 1.5 mM of $MgCl_2$, 1 mM of each dNTP, 30 pmol of each primer and 2 U/ml of Taq DNA polymerase (Fermentas UAB). The amplification reaction was carried out in a DNA thermal cycler (Biometra/TPersonal). The initial denaturation at 94°C for 5 min followed by 35 cycles of 30 s of denaturation at 94°C, primer annealing for 30 s at 66°C, 1 min 40 s of extension at 72°C, with a final 10 min extension step at 72°C. The amplicon was purified from agarose gel (1.5% w/v) using QIAquick Gel Extraction Kit (Qiagen GmbH). Wide range DNA Ladder (Fermentas UAB) was used as a DNA size marker. The purified *p17* gene fragment was digested by *EcoRI-SalI* restriction enzymes and cloned into the multiple cloning site of pColdI expression vector linearized with *EcoRI-SalI* enzymes as previously described [17]. Cohesive-end ligation of the restricted amplicon was performed with the aid of T4 DNA ligase (Fermentas UAB) and the resultant vector was termed as pCold-p17.

DNA Sequencing

The *p17* gene fragment was amplified by PCR using the pCold-p17 vector as template for DNA sequencing with primers as previously described [17]. DNA sequencing was performed with ''ABI 310 Capillary DNA Sequencer'' (Global Medical Instrumentation Inc., Minnesota, USA)

and ''ABI PRISM BigDye Terminator Cycle Sequencing Kit'' (Applied Biosystems Inc, California, USA). The homology search of the nucleotide and deduced amino acid sequences of the integrated *p17* gene was performed by the BLAST program (NCBI).

Transforma.on of Competent E. coli DH5α Cells

To prepare competent *E. coli* cells, bacteria were treated with 50 mM $CaCl_2$ in the ice bath for 30 min and centrifuged (4000 rpm, 10 min). The bacterial pellet was resuspended in 50 mM $CaCl_2$ and stored at 4°C overnight. Then, 10 ng of the pCold-p17 circular vector was added in 100 µl of the competent *E coli* suspension. The mixture was kept in the ice bath for 30 min and incubated for 45 sec at 42°C. Thereafter, the bacteria were again transferred in the ice bath for 2 min. One ml of SOC medium pre-incubated at 37°C was added and incubated by shaking for 1 hour at 37°C. Transformed bacteria were plated onto LB agar containing 50 µg/ml of ampicillin. The recombinant pCold-p17 vectors were purified from the resistant colonies. Then, the bacterial clones containing *p17* gene were determined by *EcoRI-SacI* restriction.

Expression of Recombinant rP17 Protein in E. coli DH5α

The transformant bacteria were cultured until they reached 0.2-0.3 absorbance at 600 nm. Expression was performed by induction with 0.5 mM isopropyl-β-D thiogalactopyranoside (IPTG) (Fermentas UAB) at 15°C for 24 h with shaking (200 rpm). Non-induced *E. coli* DH5α cells were also incubated at the same conditions. Bacteria were harvested by centrifugation at 4000 x g for 20 min and washed in 0.15 M NaCl solution. Bacteria suspended in lysis buffer (125 mM Tris-HCl pH 6.8, 2% SDS) were boiled for 15 min and centrifuged at 20 000 x g for 20 min. The supernatants were analysed for the presence of the recombinant proteins by SDS-PAGE and Western blot.

Purification of the Recombinant rP17 Protein

The solubilised recombinant rP17 protein was purified by Ni-NTA (Ni-nitrilotriacetic acid) affinity chromatography as recommended by the manufacturer (Qiagen GmbH). The supernatant from extract of the transformant bacteria dialyzed against PTU buffer (0.1 M NaH_2PO_4, 10 mM Tris, 8 M urea pH 8.0) was incubated with 1 ml of Ni-NTA agarose beads at room temperature for 1 h and loaded into a column. After washing with PTU buffer at pH 6.3 and 5.9, the rP17 was eluted by the same buffer at pH 4.5. The rP17 protein presence was determined by Western blot using monoclonal anti-His-Tag antibody. The recombinant proteins were further purified by elution from the polyacrylamide gel in elution buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA pH 7.5) with shaking.

SDS-PAGE and Western Blo.ng

SDS-PAGE and Western blot were performed according to the method of Laemmli [18] and Towbin et al. [19], respectively. The transformant *E. coli* cell extracts and the recombinant proteins were separated on 12% SDS-PAGE and stained with Coomassie Blue or electro-transferred to polyvinyldifluoride membrane (PVDF, Immobilon P, Sigma-Aldrich) for 1 h at 0.8 mAmp/cm^2 using a semi-dry transblotter. The membrane strips blocked with 1% fish gelatin in PBST (PBST/FG, pH 7.4; 0.1% Tween 20) were incubated with monoclonal anti-His-Tag antibody at a dilution of 1:200 for 90 min at room temperature with gently shaking. The membranes were then incubated with AP conjugated goat anti-mouse γ chain specific antibody (Sigma-Aldrich) solution for 1 h at room temperature with shaking. Color reaction was developed with the addition of BCIP/NBT for membrane (5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium, Sigma-Aldrich) and stopped by washing with water.

RESULTS

PCR Amplifca.on and Cloning of Brucella abortus p17 Gene

The purified *p17* gene amplified by PCR was digested with restriction enzyme couple *EcoRI-SacI* and ligated into the linearized pColdI vector. The resultant pCold -p17 vector was transformed to competent *E.coli* DH5α cells. From selected transformant bacteria, sufficient quantity of the purified pCold-p17 vector was digested with *EcoRI-SacI* and the presence of *p17* insert was clearly detected by electrophoretic analysis *(Fig. 1)*.

Characteris.cs of the p17 Gene and Recombinant rP17 Protein

DNA sequencing showed that the *p17* gene was composed of 462 nucleotides encoding 154 amino acids *(Fig. 2)*. The amino acid sequence of the rP17 revealed high homology (>96%) with that of the wild-type p17 protein from *Brucella* strains. The first 27 amino acids at the N-terminal end correspond to 6xHisTagged fragment of pColdI expression vector. Thus, the rP17 protein contains a total of 181 amino acids corresponding to a theoretical molecular weight of 20.2 kDa with iso-electric point of 6.42. Characteristics of the rP17 have been evaluated by the bioinformatic tools (ExPASy Proteomics Server) showing that it is mainly composed of

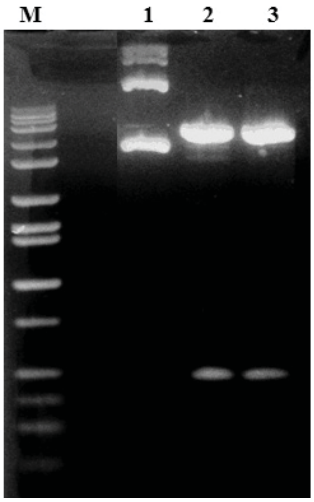

Fig 1. Confirmation of the cloning of *B. abortus p17* gene into pColdI expression vector

The undigested pColdI-p17 contains circular, coiled and supercoiled forms with different sizes (lane 1). Restriction of the pColdI-p17 vectors with *EcoRI-SacI* allows detecting the linearized pColdI and the *p17* gene (lanes 2, 3). M: DNA size marker (Fermentas UAB)

Şekil 1. *B. abortus p17* geninin pColdI ekspresyon vektörüne klonlandığının doğrulanması

Kesilmeyen pColdI-p17 vektörü farklı boyutlarda sirküler, sarmal ve super sarmal formlar içermektedir (hat 1). pColdI-p17 vektörlerinin *EcoRI-SacI* ile kesimi, lineer pColdI ve *p17* geninin tespitini sağlamaktadır (hatlar 2, 3)

alanine (11.6%), histidine (10.5%), valine (10.5%), glutamic acid (7.7%), glycine (6.6%), isoleucine (6.1%), phenylalanine (6.1%) and leucine (5.5%). The rP17 contains 83 hydrophobic (45.9%), 42 hydrophilic (23.2%), 35 basic (19.3%) and 21 acidic (11.6%) amino acids. The GRAVY index computed to be -0.097 indicates hydrophilic property of the rP17. Its high hydrophilic regions are mainly localized at the amino acids positions of 1-16, 53-58, 110-115 and 144-154 potentially accessible to the host immune response. Its aliphatic index (87.29) signifies increased thermostability of the rP17 containing 61 residues with aliphatic side chains.

Expression and Purifca.on of the Recombinant rP17 Protein

Whole cell extracts of the IPTG-induced and non-induced transformed bacteria were prepared and analyzed by SDS-PAGE demonstrating the expression of recombinant rP17 protein *(Fig. 3A)*. The relative molecular weight of the rP17 in SDS-PAGE analysis was determined as 24 kDa. The recombinant protein was purified by Ni-NTA affinity chromatography followed by elution from polyacrylamide gel and analyzed in Western blot assay. As seen in Fig 3B, a single band of about 24 kDa recognized by monoclonal anti-HisTag antibody corresponds to the recombinant rP17 protein.

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ATGAATCACAAAGTGCATCATCATCATCATCATATCGAAGGTAGGCATATGGAGCTCGGT
 M  N  H  K  V  H  H  H  H  H  H  I  E  G  R  H  M  E  L  G
ACCCTCGAGGGATCCGAATTCATGAACCAAAGCTGTCCGAACAAGACATCCTTTAAAATC
 T  L  E  G  S  E  F  M  N  Q  S  C  P  N  K  T  S  F  K  I
GCATTCATTCAGGCCCGCTGGCACGCCGACATCGTTGACGAAGCGCGCAAAAGCTTTGTC
 A  F  I  Q  A  R  W  H  A  D  I  V  D  E  A  R  K  S  F  V
GCCGAACTGGCCGCAAAGACGGGTGGCAGCGTCGAGGTAGAGATATTCGACGTGCCGGGT
 A  E  L  A  A  K  T  G  G  S  V  E  V  E  I  F  D  V  P  G
GCATATGAAATTCCCCTTCACGCCAAGACATTGGCCAGAACCGGGCGCTATGCAGCCATC
 A  Y  E  I  P  L  H  A  K  T  L  A  R  T  G  R  Y  A  A  I
GTCGGTGCGGCCTTCGTGATCGACGGCGGCATCTATCGTCATGATTTCGTGGCGACGGCC
 V  G  A  A  F  V  I  D  G  G  I  Y  R  H  D  F  V  A  T  A
GTTATCAACGGCATGATGCAGGTGCAGCTTGAAACGGAAGTGCCGGTGCTGAGCGTCGTG
 V  I  N  G  M  M  Q  V  Q  L  E  T  E  V  P  V  L  S  V  V
CTGACGCCGCACCATTTCCATGAAAGCAAGGAGCATCACGACTTCTTCCATGCTCATTTC
 L  T  P  H  H  F  H  E  S  K  E  H  H  D  F  F  H  A  H  F
AAGGTGAAGGGCGTGGAAGCGGCCCATGCCGCCTTGCAGATCGTGGTCGACCTGCAGTCT
 K  V  K  G  V  E  A  A  H  A  A  L  Q  I  V  V  D  L  Q  S
AGATAG
 R  ***
```

Fig 2. Sequences of nucleic and amino acids of the recombinant *B. abortus* rP17 protein

The first underlined 27 amino acids at the N-terminal end correspond to 6xHisTagged fragment of pColdI vector. Stop codon TAG was indicated with ***

Şekil 2. Rekombinant *B. abortus* rP17 proteini nükleik ve amino asit dizileri

N-terminal bölgede yeralan altı çizili ilk 27 amino asit pColdI vektörüne ait 6xHis kuyruk kısmına denk düşmektedir. Stop kodon TAG *** ile gösterilmektedir

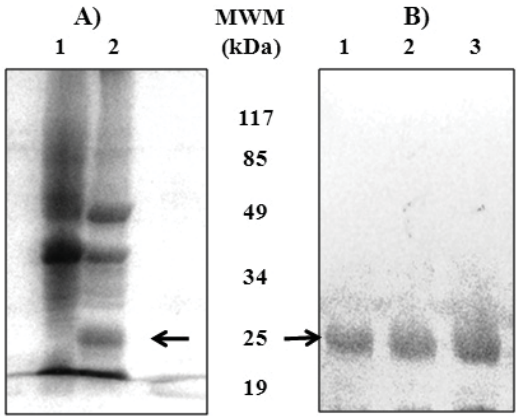

Fig 3. SDS-PAGE and Western blot analysis of the recombinant *B. abortus* rP17 protein

A) SDS-PAGE analysis of the whole cell lysate of non-induced (lane 1) and IPTG-induced bacteria (lane 2). The arrow shows expression of the rP17. MWM: Molecular weight marker.

B) Western blot analysis by mouse monoclonal anti-HisTag antibody. Increasing volumes of the purified rP17 protein was used (lanes 1-3)

Şekil 3. Rekombinant *B. abortus* P17 proteininin SDS-PAGE and Western blot ile analizi

A) İndüklenmemiş (hat 1) ve indüklenmiş (hat 2) bakteri total hücre lizatının SDS-PAGE analizi. Ok rP17 proteininin ekprese olduğunu göstermektedir. MWM: Molekül ağırlık markırı

B) Fare monoklonal anti-HisTag antikoru ile Western blot analizi. Saflaştırılmış rP17 proteini artan hacimlerde uygulandı (hatlar 1-3)

DISCUSSION

Serosurveillance of brucellosis is mainly based on the detection of antibodies generated against immuno-dominant antigenic molecules of *Brucella* lipopoly-

saccharide (LPS). Using LPS and O-polysaccharide as diagnostic antigen, several serological tests such as different ELISA models and FPA have been developed [2,15,20,21]. However, false-positivity due to the similarity between LPS of *Brucella* and that of other Gram-negative bacteria in particular LPS of *Yersinia enterocolitica* O:9 strains has been reported in brucellosis low prevalence areas [3,4,21,22]. To circumvent these cross-reactions, several investigations have been attempted to identify the immunoreactive proteins of *Brucella* spp. and to determine their potential as diagnostic antigens in animal or human brucellosis [7,9,10,12,21,23].

In this study, immunoreactive cytosolic P17 protein was produced as recombinant protein by cloning the *p17* gene into pColdI bacterial expression vector. Cold-shock expression vector pColdI utilizes the promoter derived from *cspA* gene and *lac* operator for controlling strictly the protein expression of the cloned gene. Translation enhancing element (TEE) including start codon, His-Tag sequence encoding 6 histidine residues, Factor Xa cleavage site and multiple-cloning site (MCS) are located at the downstream of the *cspA* promoter so that recombinant rP17 protein was expressed as His tagged fusion proteins. As seen in *Fig. 2*, rP17 fusion protein contains at the N-terminal end 27 additional amino acid residues designed to facilitate the purification of the recombinant His tagged proteins using Ni-NTA affinity column. For that reason, Ni-NTA affinity chromatography technique was used as the first step of purification of the rP17 protein. In a second step, the recombinant protein was eluted from polyacrylamide gels following SDS-PAGE to remove residual bacterial contaminants present after affinity chromatography. This two-step method resulted in obtaining of highly purified rP17 as described in a previous study for efficient purification of the recombinant PvpA protein of *Mycoplasma gallisepticum* [17].

DNA of the cloned *p17* gene and deduced amino acid sequence of the rP17 showed very high homology with those described in the literature and the DNA/Protein databases. Western blot analysis of the two-step purified rP17 showed that the recombinant protein was strongly recognized with mouse monoclonal anti-HisTag antibody (Fig 3B). In the preliminary study performed with a limited number of sera, the recognition of the rP17 was also observed in Western blot analysis. These results indicate that the immunoreactive property of the rP17 is still preserved. This observation was also supported by the fact that Hemmen et al.[7] and Letesson et al.[10] have demonstrated that recombinant P17 proteins expressed in *E.coli* were found immuno-reactive in competitive and indirect ELISA.

In conclusion, the data presented here demonstrated successful production and purification of the immuno-dominant P17 protein as recombinant protein. In our knowledge, this study is the first that described the production of the rP17 by pColdI expression system and, in the same time, demonstrated efficient purification of the recombinant protein by two-step process. Furthermore, large scale production and purification of the rP17 is carried out in our laboratory to evaluate its diagnostic performance with a large panel of well-defined sera from cattle, sheep and human collections. For this purpose, recently described qualitative and quantitative ELISA models [15] as well as field diagnostic tools based on the rapid immunofiltration assay (RIFA) [16] will be preferred to compare the diagnostic performance of *Brucella* LPS and OPS to that of the rP17. Further investigations with RIFA-based tools may allow developing multi-antigenic diagnostic format for rapid, sensitive and specific brucellosis diagnosis in the field and limited laboratory conditions.

REFERENCES

1. Corbel MJ: Brucellosis. An overview. *Emerg Infect Dis,* 3, 213-221, 1997.

2. Nielsen K: Diagnosis of brucellosis by serology. *Vet Microbiol,* 90, 447-459, 2002.

3. Kittleberger R, Hilbink F, Hansen MF, Penrose M, deLisle GW, Letesson JJ, Garin-Bastuji B, Searson J, Fossati CA, Cloeckaert A, Schurig G: Serological crossreactivity between *Brucella abortus* and *Yersinia enterocolitica* O:9: immunoblot analysis of the antibody response to *Brucella* protein antigens in bovine brucellosis. *Vet Microbiol,* 47**,** 257-270, 1995.

4. Weynants V, Tibor A, Denoel P, Saegerman C, Godfroid J, Letesson JJ: Evaluation of an ELISA using Yersinia outer membrane proteins of the involvement of *Yersinia enterocolitica* O:9 in the false positive serological responses observed in bovine brucellosis diagnostic tests. *Vet Microbiol*, 48, 101-112, 1996.

5. Zygmunt MS, Gilbert FB, Dubray G: Purification, characterization, and seroactivity of a 20-kilodalton *Brucella* protein antigen. *J Clin Microbiol,* 30**,** 2662-2667, 1992.

6. Zygmunt MS, Cloeckaert A, Dubray G: *Brucella melitensis* cell envelope protein and lipopolysaccharide epitopes involved in humoral immune responses of naturally and experimentally infected sheep. *J Clin Microbiol*, 32, 2514-2522, 1994.

7. Hemmen F, Weynants V, Scarcez T, Letesson JJ, Saman E: Cloning and sequence analysis of a newly identified *Brucella abortus* gene and serological evaluation of the 17-kilodalton antigen that it encodes. *Clin Diagn Lab Immunol*, 2, 263-267, 1995.

8. Lindler LE, Hadfield TL, Tall BD, Snellings NJ, Rubin FA, Van De Verg LL, Hoover D, Warren RL: Cloning of a *Brucella melitensis* group 3 antigen gene encoding Omp28, a protein recognized by the humoral immune response during human brucellosis. *Infect Immun,* 64, 2490-2499, 1996.

9. Rossetti OL, Arese AI, Boschiroli ML, Cravero SL: Cloning of *Brucella abortus* gene and characterization of expressed 26-kilodalton periplasmic protein: potential use for diagnosis. *J Clin*

Microbiol, 34, 165-169, 1996.

10. Letesson JJ, Tibor A, Eynde G van, Wansard V, Weynants V, Denoel P, Saman E: Humoral immune responses of *Brucella*-infected cattle, sheep, and goats to eight purified recombinant *Brucella* proteins in an indirect enzyme-linked immunosorbent assay. *Clin Diagn Lab Immunol*, 4, 556-564, 1997.

11. Goldbaum FA, Velikovsky CA, Baldi PC, Mortl S, Bacher A, Fossati CA: The 18-kDa cytoplasmic protein of Brucella species-an antigen useful for diagnosis-is a lumazine synthase. *J Med Microbiol*, 48, 833-839, 1999.

12. Cloeckaert A, Baucheron S, Vizcaino N, Zygmunt MS: Use of recombinant BP26 protein in serological diagnosis of *Brucella melitensis* infection in sheep. *Clin Diagn Lab Immunol*, 8, 772-775, 2001.

13. Connolly JP, **Comerci D, Alefantis TG, Walz A, Quan M, Chafin R, Grewal P, Mujer CV, Ugald RA, DelVecchio VG**: Proteomic analysis of *Brucella abortus* cell envelope and identification of immunogenic candidate proteins for vaccine development. *Proteomics*, 6, 3767-3780, 2006.

14. Kumar S, Tuteja U, Kumar A, Batra HV: Expression and purification of the 26 kDa periplasmic protein of *Brucella abortus*: A reagent for the diagnosis of bovine brucellosis. *Biotechnol Appl Biochem*, 49, 213-218, 2008.

15. Genç O, Büyüktanır Ö, Yurdusev N: Development of qualitative and quantitative ELISA models for bovine brucellosis diagnosis. *Kafkas Univ Vet Fak Derg*, 16, 287-291, 2010.

16. Yurdusev N, Genç O, Büyüktanır Ö: Anti-*Brucella* antikor tanı kitleri. *Turkish Patent Institut (TPE)*, Patent no: TR 2006 06661 B, 1-21, 2010.

17. Büyüktanır Ö, Yıldırım T, Yakıcıer C, Genç O, Yurdusev N: A recombinant PvpA protein-based diagnostic prototype for rapid screening of chicken *Mycoplasma gallisepticum* infections. *Vet Microbiol*, 129, 139-149, 2008.

18. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London), 227, 680-685, 1970.

19. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA*, 76, 4350-4354, 1979.

20. Saegerman C, De Waele L, Gilson D, Godfroid J, Thiange P, Michel P, Limbourg B, Vo TK, Limet J, Letesson JJ, Berkvens D: Evaluation of three serum i-ELISAs using monoclonal antibodies and protein G as peroxidase conjugate for the diagnosis of bovine brucellosis. *Vet Microbiol*, 100, 91-105, 2004.

21. Muñoz PM, Marín CM, Monreal D, González D, Garin-Bastuji B, Díaz R, Mainar-Jaime RC, Moriyón I, Blasco JM: Efficacy of several serological tests and antigens for diagnosis of bovine brucellosis in the presence of false-positive serological results due to *Yersinia enterocolitica* O:9. *Clin Diagn Lab Immunol*, 12, 141-151, 2005.

22. Baldi PC, Giambartolomei GH, Goldbaum FA, Abdón LF, Velikovsky CA, Kittelberger R, Fossati CA: Humoral immune response against lipopolysaccharide and cytoplasmic proteins of *Brucella abortus* in cattle vaccinated with *B. abortus* S19 or experimentally infected with *Yersinia enterocolitica* serotype O:9. *Clin Diagn Lab Immunol*, 3, 472-476, 1996.

23. Cloeckaert A, Kerkhofs P, Limet JN: Antibody response to *Brucella* outer membrane proteins in bovine brucellosis: immunoblot analysis and competitive enzyme-linked immunosorbent assay using monoclonal antibodies. *J Clin Microbiol*, 30, 3168-3174, 1992.