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Original Article

Development and Evaluation of an Indirect Capripoxvirus ELISA Based on Truncated P32 Protein Expressed in *E. coli*

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Abstract

As notifiable diseases, lumpy skin disease (LSD), sheep pox (SPP), and goat pox (GTP) are associated with a profound effect on cattle, sheep, and goat farming industries. Development of the ELISA method could effectively facilitate serodiagnosis of the infected animals. This study aimed to develop an ELISA system based on the recombinant full-length and truncated P32 protein (Tr.P32) of goat pox virus. The P32 protein was expressed in Rosetta strain of *E. coli* using pET24a^{+} vector and evaluated by SDS-PAGE and Western blotting. Then, Tr.P32 was purified by Ni-NTA affinity chromatography under denaturing conditions and used to develop a capripoxvirus-specific ELISA. Checkerboard titration and receiver-operating characteristic (ROC) analysis were used to optimize the ELISA system and determine diagnostic specificity and sensitivity, respectively. The diagnostic potential of the developed ELISA was evaluated using positive and negative control sera collected from goat, sheep, and cattle. Results showed that the expression level of full-length P32 recombinant protein was negligible, while Tr.P32, a ~ 31 kDa recombinant protein, was expressed up to 0.270-0.300 mg/200 mL of culture media. The results of checkerboard titration revealed that 675 ng/well of Tr.P32 antigen and 1:10 dilution of control sera (anti GTPV HIS and healthy goat sera) caused maximum difference in absorbance between positive and negative goat sera. The recombinant Tr.P32 showed good reactions with antibodies against GTP virus (GTPV), SPP virus (SPPV), and LSD virus (LSDV), whereas no cross-reactions with anti-Orf virus antibodies were detected. By comparing with the neutralization index (NI), cut off, diagnostic sensitivity and specificity of the developed indirect-ELISA were estimated, 0.397, 94% and 96.6%, respectively. These findings indicate that the ELISA system based on Tr.P32 protein could potentially be used in sero-surveillance of all capripoxviruses; however, further investigations are required.

Keywords: ELISA, goat pox, lumpy skin disease, P32 protein, sheep pox

Développement et Évaluation d'un ELISA Indirect du Capripoxvirus basé sur la Protéine P32 Tronquée Exprimée dans *E. coli*

Résumé: En tant que maladies à déclaration obligatoire, la dermatose nodulaire contagieuse (LSD), la variole ovine (SPP) et la variole caprine (GTP) sont associées à un effet profond sur les filières d'élevage des chèvres, des moutons et des bovins. Le développement de la méthode ELISA pourrait faciliter efficacement le sérodiagnostic des animaux infectés. Cette étude visait à développer un système ELISA basé sur la protéine P32 recombinante pleine longueur et tronquée (Tr.P32) du virus de la variole caprine. La protéine P32 a été exprimée

dans la souche Rosetta d'*E. coli* en utilisant le vecteur pET24a$^+$ et évaluée par SDS-PAGE et Western blot. Ensuite, Tr.P32 a été purifié par chromatographie d'affinité Ni-NTA dans des conditions dénaturantes et utilisé pour développer un ELISA spécifique du capripoxvirus. Le titrage en damier et l'analyse de ROC (caractéristique de fonctionnement du récepteur) ont été utilisés pour optimiser le système ELISA et déterminer la spécificité et la sensibilité du diagnostic, respectivement. Le potentiel diagnostique de l'ELISA développé a été évalué à l'aide de sérums témoins positifs et négatifs prélevés sur des chèvres, des moutons et des bovins. Les résultats ont montré que le niveau d'expression de la protéine recombinante P32 pleine longueur était négligeable, tandis que Tr.P32, une protéine recombinante de ~ 31 kDa, était exprimé jusqu'à 0.270-0.300 mg/200 ml de milieu de culture. Les résultats du titrage en damier ont révélé que 675 ng/puits d'antigène Tr.P32 et une dilution 1:10 de sérums témoins (anti GTPV HIS et sérums de chèvre sains) provoquaient une différence maximale d'absorbance entre les sérums de chèvre positifs et négatifs. Le Tr.P32 recombinant a montré de bonnes réactions avec des anticorps contre le virus GTP (GTPV), le virus SPP (SPPV) et le virus LSD (LSDV), alors qu'aucune réaction croisée avec les anticorps anti-virus d'Orf n'a été détectée. En comparant avec l'indice de neutralisation (IN), le seuil, la sensibilité diagnostique et la spécificité de l'ELISA indirect développé ont été estimées à 0.397, 94% et 96,6%, respectivement. Ces résultats indiquent que le système ELISA basé sur la protéine Tr.P32 pourrait potentiellement être utilisé dans la sérosurveillance de tous les capripoxvirus; cependant, d'autres investigations sont nécessaires.
Mots-clés: ELISA, variole caprine, maladie de la peau nodulaire, protéine P32, clavelée ovine

1. Introduction

The capripoxvirus genus, a member of the chordopoxvirinae subfamily in the Poxviridae family, is composed of lumpy skin disease virus (LSDV), goatpox virus (GTPV), and sheep pox virus (SPPV), three important pathogens that specifically infect cattle, goat, and sheep, respectively (1, 2). Lumpy skin disease (LSD) is a severe and highly contagious disease characterized by nodules in the skin, enlarged superficial lymph nodes, and occasionally death (3). However, clinical manifestations of sheep pox (SPP) and goat pox (GTP) are pyrexia and pock lesions in the skin and internal organs and are associated with a high mortality rate, particularly in young animals (1). LSD is widespread throughout Africa and has caused a severe outbreak in the Horn of Africa. Prior to 2012, only sporadic LSD outbreaks were reported in the Middle East region; meanwhile, LSD spread through Turkey and, subsequently, Iraq and Iran during recent years. Nevertheless, the global distribution of SPP and GTP covers a wider area than LSD, including northern and central Africa, across the Middle East, and the Indian subcontinent (4). Due to their potential for rapid spread and considerable economic impact in endemic regions, LSD, SPP, and GTP are categorized by the OIE as notifiable diseases (5).

Although acute cases of LSD, SPP, and GTP are usually readily diagnosed clinically, the diseases caused by low virulence strains or contagious pustular dermatitis (Orf) in sheep and goats or pseudo-LSD in cattle complicate their differential diagnosis. Laboratory testing comprising classical virological techniques used routinely in primary diagnoses (6) and more recent molecular-based methods such as amplification of viral DNA (7, 8) or quantitative real-time PCR (9) have substantially improved the capacity to detect capripoxviruses in suspected disease outbreaks.

However, in addition to vaccination, surveillance of the disease by sensitive and specific diagnostic methods is the best way to control the incidence of disease and eradicate it in any region. Moreover, evaluating sera antibody titer against capripoxviruses would facilitate determining the level of immunity acquired in vaccinated animals. The currently available serodiagnostic assays, including the agar gel immunodiffusion (AGID) and indirect fluorescent antibody (IFAT), are not suitable for antibody detection because of the cross-reaction existing between

capripoxvirus antibodies and parapoxviruses. Moreover, Western blot and virus neutralization tests (VNT) are not appropriate for sero-surveillance studies, because these assays are labor intensive, time-consuming, and technically difficult (10-12). For example, as the immunity to capripoxvirus infection is predominantly cell-mediated and vaccinated or mildly infected animals will develop only low levels of neutralizing antibody (13), the interpretation of VNT results is difficult (14). The literature emphasizes the need to develop a highly specific, rapid, inexpensive, sensitive, and useful test such as ELISA for mass screening.

Capripoxvirus genomes consist of double-stranded DNA of about 150 kb with terminal repeat sequences at each end. There is a high degree of genome homology between different capripoxvirus isolates with 96% or greater genetic sequence identity (15, 16). The ORF 070 product of goatpox virus, also known as P32, is a vaccinia virus H3L homologue (16, 17) and immune-dominant protein in all CaPVs (7, 12). Antibody detection enzyme-linked immunosorbent assays (ELISAs), based on recombinant P32 protein, were previously developed. The developed indirect ELISA system using the P32 protein successfully detected capripox-specific antibodies in sheep (7) and bovine sera (18). However, the problems associated with the purification process and the low expression level of P32 due to poor solubility would remain a great challenge. Later, the developed indirect ELISA, which used inactivated sucrose gradient-purified SPPV as a coating antigen, was associated with satisfactory results in screening sera from all three host species. However, the viral antigen is difficult and expensive to produce in large quantities (15).

Some other structural proteins, namely ORF 095 and ORF 103, have been expressed and evaluated for diagnostic potential. Although the provided indirect ELISA system was sensitive and specific for detecting antibodies in sheep and goats following experimental infection with virulent capripoxviruses, analytical sensitivity was poor in vaccinated animals and was not able to detect antibodies against LSDV (19). Low expression level of the full-length P32 antigen was the main limitation in developing the system, mainly due to toxicity-associated hydrophobic product (7). However, the truncated P32 protein could be useful for serodiagnosis of GTPV and SPPV (20), and probably LSDV.

Therefore, the current study was designed to express both the full-length and the truncated versions of P32 protein in the prokaryotic system and evaluate their potential as an antigen in developing an indirect-ELISA system for the serodiagnosis of capripoxvirus infections in sheep, goats, and cattle.

2. Material and Methods

2.1. Virus, Cell, Vector, Host Bacteria, and Biologicals

Live attenuated goat pox vaccine (Gorgan strain) available in sheep pox and goat pox disease OIE Reference Laboratory, Razi Vaccine and Serum Research Institute (RVSRI), Karaj, Iran, was used for amplification of the P32 gene. Horseradish peroxidase (HRP) labeled rabbit anti-goat, anti-sheep, and anti-cattle IgG conjugates (Dako, Denmark) and HRP conjugated monoclonal anti-poly histidine (Sigma Aldrich, USA) were used for ELISA system development. Hyper-immune anti-GTPV serum (HIS) produced by immunization of the target animal (goat) available in the laboratory was used as the positive control.

For cloning and expression of the P32 gene, pET24a^{+} (Novagen, USA), a prokaryotic expression vector, was used along with bacterial host strains including the DH5α strain of *E. coli* cells and the Rosetta strain of *E. coli* (Novagen, USA) for initial cloning and expression, respectively.

2.2. Gene Amplification and Cloning

In order to amplify the P32 gene sequence, two primer sets were designed based on a reference gene sequence of goat pox virus/Gorgan strain available in GenBank

(Accession: KX576657.1). The restriction enzyme sites of *EcoRI* and *Sal*I were added at 5' ends of the forward and reverse primers, respectively. Primers were synthesized by Metabion International AG (Germany). Details of the primers are represented in Table 1.

Goat pox vaccine virus DNA was extracted using a commercial DNA extraction kit (Roche, Germany) according to the manufacturer's instructors. To amplify the full-length (M1-V322aa) and N&C-terminal truncated (20V-S270aa region) P32 gene sequences, polymerase chain reactions (PCRs) were performed using respective pfu and PrimeSTAR GXL DNA polymerases (Bio basic, Canada and Takara, Japan) and thermal cycles shown in Table 2. The amplified PCR products were then digested with *EcoR*I and *Sal*I enzymes (Takara, Japan) and ligated into the pET24a^{+} vector and digested with the same enzymes, using T_4 DNA ligase (Fermentas, USA). The recombinant plasmids were then transferred into the competent *E. coli* DH5α strain. The transformed cells were selected on Luria-Bertani (LB) medium agar plates containing 100 µg/ml Kanamycin. Several colonies containing full- and truncated-P32 sequences were verified by colony PCR. Thereafter, the plasmids were extracted from recombinant clones by a Miniprep plasmid isolation kit (Roche, Germany) and confirmed by restriction-enzyme digestion, followed by DNA sequencing using universal T7 primers.

Table 1. Details of primers used for amplification and cloning of full- and truncated-P32 gene sequences

Gene name	Primer sequence	Product size (bp)
Full-length P32	F: GATAGAATTCATGGCAGATATCCCATTATATG R: GAGGGTCGACAACTATATACGTAAATAAC	969
Truncated P32	F: GTGGAATTCGTTCCAGAATTAAAAAGTGGC R: GTGGTCGACAGAAAAATCAGGAAATCTATG	753

Table 2. PCR thermal cycle used for amplification of P32 full and truncated genes

P32 Gene	Cycle No.	Stage	Time	Temp. (°C)
Full	1	Initial Denaturation	3 min	94
		Denaturation	45 s	94
	×35	Annealing	30 s	60
		Extension	1 min	68
	1	Final Extension	10 min	68
Truncated	1	Initial Denaturation	4 min	95
		Denaturation	50 s	94
	×30	Annealing	30 s	63
		Extension	1 min	72
	1	Final Extension	15 min	72

2.3. Transformation of *E. coli* Rosetta Strain and Pilot Expression

The pET24a-Tr. P32 and pET24a-full-Lenght P32 recombinant plasmids were transferred into *E. coli* Rosetta strain and cultured on LB agar plates containing kanamycin (100 µg/mL) at 37°C, overnight. Single recombinant *E. coli* Rosetta colonies were cultured in LB broth (containing 100 µg/mL kanamycin) and tested for protein expression. Briefly, following the overnight culture of individual colonies at

37°C with shaking at 170 rpm, the cultures were inoculated into fresh LB broth (1:100 inoculum: medium ratio) containing kanamycin (100ug/mL). Then, they were incubated at 37°C in an orbital shaker at 200 rpm until reaching mid-log growth phase (OD600=0.6-0.9). After that, one mL of each culture was centrifuged at 6,000×g and the supernatant was discarded. The obtained cell pellet was frozen at -20°C as the zero time point sample. Different concentrations of Isopropyl β-d-1-thiogalactopyranoside (IPTG; 0.3 mM, 0.5 mM and 1 mM) were added to the remaining bacterial cultures, and incubation at 37°C was followed by vigorous shaking at 200 rpm. To determine the optimum time for maximizing P32 protein expression, 8 samples with 1-h intervals were collected and analyzed on 12% SDS-PAGE gel. The result of the full P32 protein expression was not associated with a satisfactory outcome; hence, it was not considered for further assessments.

2.4. Expression Scaling up and Purification of the Tr.P32 Protein

A single recombinant *E. coli* Rosetta colony was inoculated into 3 mL LB Kan^+ broth and cultured with the above-mentioned protocol. Thereafter, 2 mL of the overnight cultured bacteria was inoculated into 200 mL of fresh LB Kan^+ to grow and reach mid-log phase under vigorous shaking and at 37°C. Then, 1m M of IPTG was added and incubation was continued up to 6 h as the optimum time.

The recombinant Tr.P32 protein was purified using Ni-NTA resin (Qiagen) under denaturation conditions, according to the manufacturer's instructions. Briefly, 200 mL of an IPTG-induced culture was centrifuged (5000×g, 20 min) at 4°C, and the cell pellet was suspended in 7 mL of the lysis buffer (50Mm NaH2PO4+ 300mMNaCl) containing 1 mg/mL lysozyme and 30 µL of PMSF (100 mM) at 4°C for 45 min. The lysed cells were then sonicated (10 s per each 6 cycles, and 10 s intervals between cycles) at 100% pulse amplitude and centrifuged at 10,000×g for 30 min at 4°C. Supernatant was removed and the pellet (insoluble proteins) was dissolved in 3.5 mL of 8 M urea buffer (pH=8) by constant agitation for 30–60 min at 4°C. After centrifugation at 10,000×g for 30 min at 4°C, supernatant was collected, filtered through a 0.45 µm disposable syringe filter, and transferred to a fresh tube as crude cell lysate. Thereafter, 20 µL of the lysate was stored at -20°C for SDS-PAGE analysis, and the remaining lysate was added to pre-equilibrated 50% Ni-NTA resin (4:1 ratio, V/V) and mixed under constant agitation for 60 min at 20-25 °C. The lysate–Ni-NTA mixture was then loaded into a column with a capped outlet. After a few minutes, the outlet was opened and the flow-through was collected. The column was washed two times with 4.0 mL of washing buffer (8 M Urea buffer pH=6.3). Protein elution was conducted in two successive steps: 1) elution with 0.5 mL elution buffer (8M Urea buffer pH=5.9) four times; and 2) elution with 0.5 ml of another elution buffer (8M urea buffer pH=4.5). The eight collected fractions were stored at -20°C for SDS-PAGE analysis. Concentrations of the eluted proteins were determined using the Bradford method and bovine serum albumin (Sigma, USA) as a standard.

2.5. SDS-PAGE and Western Blotting

The harvested samples (pre- and post-induction, mock vector, and eluted fractions) were analyzed for expression of the expected recombinant proteins in 12% SDS-PAGE gel using a standard protein molecular weight marker. The proteins separated in SDSPAGE were transferred to a nitrocellulose membrane and detection was carried out using two different antibodies: a monoclonal anti-poly histidine horseradish peroxidase (HRPO) conjugate (diluted 1:1500) and an anti-GTPV hyper-immune serum (as the primary antibody, diluted 1:250) followed by a rabbit anti-goat IgG\HRPO conjugate (as the secondary antibody, diluted 1:15,000) (Figure 3). The blots were developed using 3, 3′ diaminobenzidine tetrahydrochloride (DAB) substrate (21).

2.6. Serum Samples

A total of 151 sera samples comprising 65 negative and 86 positive sera (including 35, 52, and 62 samples from cattle, goat, and sheep, respectively) were collected for analysis by ELISA and neutralization index (NI). The golden standard for diagnosis of the negative or positive sera was NI (OIE, 2017). Negative sera were collected from animals (goats, sheep, and cattle) not previously exposed to capripoxvirus. Positive sera were obtained from animals vaccinated with goat pox or sheep pox vaccines and animals naturally or experimentally infected with SPPV, GTPV, or LSDV. The collected samples were screened initially for antibody titer against capripoxvirus by NI, using the GTPV Gorgan and SPPV RM 65 strains as described in the OIE manual (5). The sera were considered as positive when the difference between titer (log10) of test serum and negative control was ≥1.5 (5).

2.7. Optimization of Indirect-ELISA using Recombinant Tr.P32 Protein

A checkerboard titration (CBT) method was carried out to optimize the working concentration of Tr.P32 antigen and serum dilution. Briefly, a 96-well microtiter ELISA plate was divided into two sets of 48 wells (6×8 wells), and each of 6 columns was coated (100 µL/well) with a different concentration of the antigen prepared from two-fold serial dilutions of the purified recombinant Tr.P32. The antigen dilutions ranging from 1350 to 42 ng/well were prepared in ELISA coating buffer (carbonate/bicarbonate buffer, pH 9.6) and plates were coated overnight at 4°C. Thereafter, plates were washed five times with PBS containing 0.05 % Tween20 (PBST) and then blocked with 200 µL/well of 1% bovine serum albumin in PBST overnight at 4°C. The positive and negative control sera were then serially diluted in dilution buffer (PBST containing 0.25 % bovine serum albumin and 5% skim milk) and added (100 µL/well) to each half of the 96-well microplate, in rows A to H, from 1:10 to 1:1280 dilutions, respectively. Following incubation at 37°C for 1 h and washing five times, 100 µL of anti-goat IgG HRPO conjugate, diluted at 1:20000, was added to each well and incubated for 1 h at 37°C. After washing the plate as described above, 100 µL of a commercial ready-to-use solution of 3, 3', 5, 5'-tetramethylbenzidine-H_2O_2 (Hyphen, French) was added to each well, and the plate was incubated in a dark place at room temperature for 7-10 min. Then, the reaction was stopped by the addition of an equal volume of 1 M H_2SO_4 and the optical densities (ODs) of the wells were measured at 450 nm. The antigen and serum dilutions with maximum OD difference, calculated by subtracting positive and negative sera samples, were selected as the optimal working concentration.

2.8. Evaluation of Tr.P32 Protein based Indirect-ELISA

To evaluate the diagnostic potential of Tr.P32 protein, the collected positive and negative sera samples were tested using the optimized indirect ELISA. The cut-off point and diagnostic sensitivity and specificity of the ELISA system were determined by comparing the obtained results with NI as the gold standard for capripox serology, using the interactive dot diagram of MedCalc software. To evaluate the cross-reactivity of the ELISA system with antibodies against the Orf virus, 12 sera samples collected from animals naturally or experimentally infected with the Orf virus were examined.

2.9. Statistical Analysis

The capripoxvirus neutralization index and ELISA measured on the positive and negative sera of goat, sheep, and cattle were compared by the chi-square method and the Genmod procedure of SAS 9.4 software using the logit link function. In each species, data was expressed as percentage of negative or positive sera to the total evaluated samples. A *p-value* <0.05 was considered as a significant difference between the experimental groups. In addition, the sensitivity and specificity diagnostic of the developed ELISA was measured by receiver-operating characteristic (ROC) analysis using MedCalc 19.4.0 software.

3. Results

3.1. Gene Amplification and Cloning

PCR products, amplified by the designated primers were electrophoresed on 1% agarose gel (Figure 1). As expected, PCRs resulted in amplification of the respective DNA bands of 753bp and 969 bp for Tr.P32 and full-length P32 genes, respectively.

Figure 2 indicates the results of full-length P32 and Tr.P32 gene cloning. As shown, the colony PCR conducted on positive colonies confirmed successful cloning of the 753bp and 969 bp DNA bands in the pET24a+ plasmid (Figure 2, lanes 1 and 2). In addition, the restriction enzyme digestion of recombinant plasmid also indicated successful insertion of the full-length P32 and Tr.P32 genes (Figure 2. lanes 4, 5, and 6). Sequence analysis of recombinant plasmids revealed that the cloned genes had no mutation and were inserted in a correct frame and orientation (the sequence of full length P32 gene is available in gene bank with accession number MK948083.1).

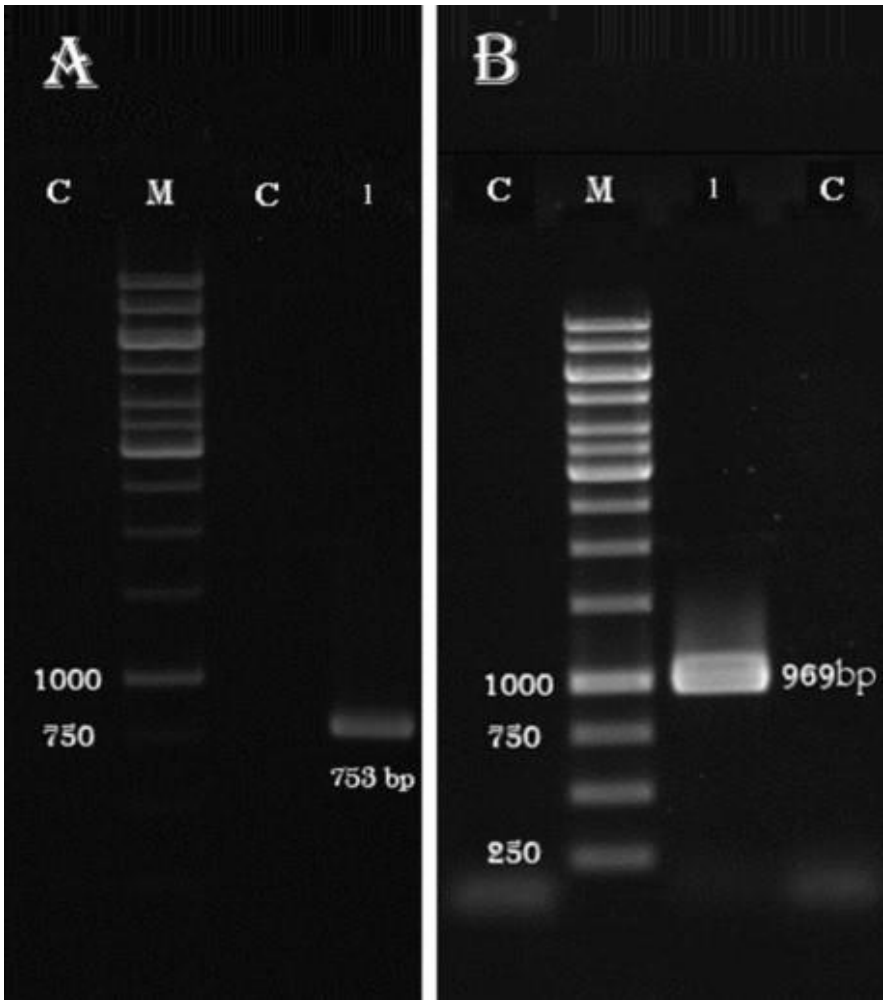

Figure 1. Agarose gel electrophoresis showing PCR amplified truncated (753 bp; panel A) and full-length P32 (969 bp; panel B) genes. Lane C, negative control; Lane M, 1Kb DNA ladder; Lane 1, amplified gene.

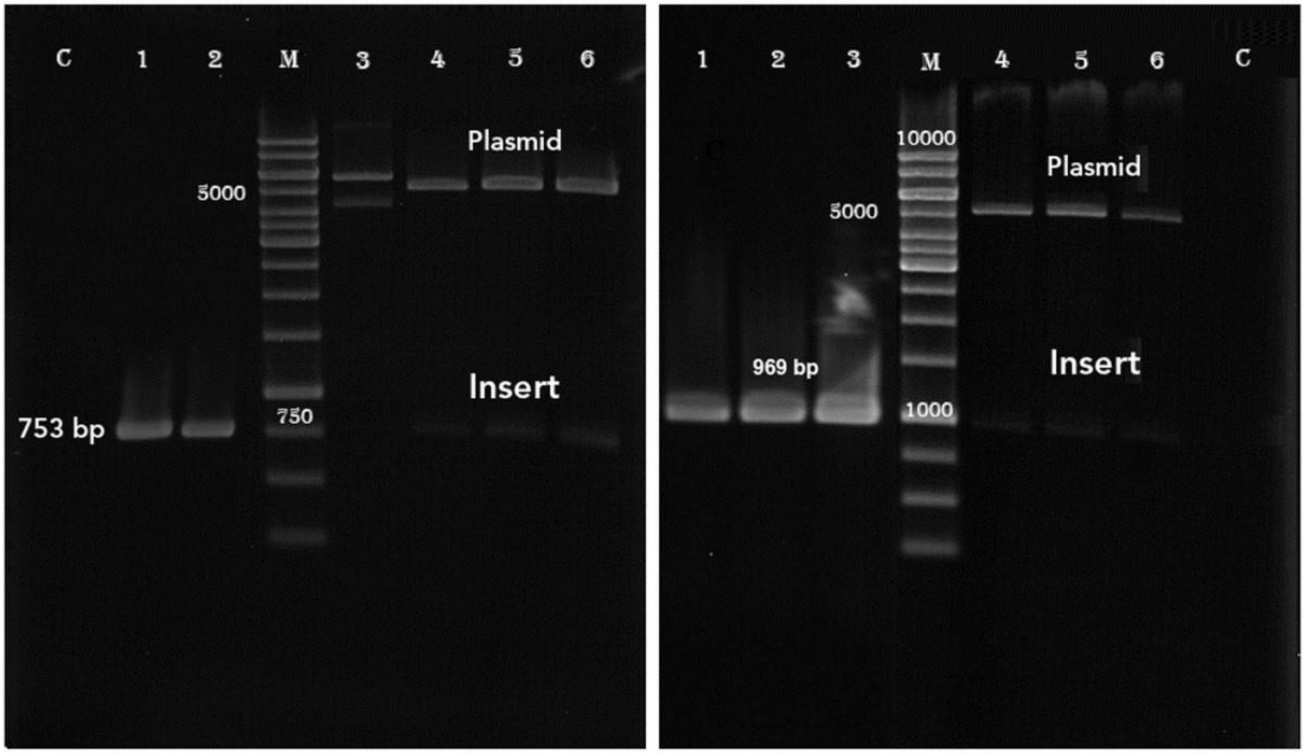

Figure 2. Agarose gel electrophoresis showing colony PCR and restriction enzyme digestion of recombinant plasmid of truncated **(Panel A)** and full-length **(Panel B)** P32 genes.
Panel A: Lane C, negative control; Lane M, molecular weight marker; Lanes 1 and 2, results of colony PCR; Lane 3, following RE; Lanes 4, 5, and 6, release of truncated P32 inserts following RE of pET24a$^+$ plasmid. **Panel B:** Lane C, negative control; Lane M, molecular weight marker; Lanes 1 and 2, results of colony PCR; Lane 3, positive control; Lanes 4, 5, and 6, release of full-length P32 inserts following RE of pET24a$^+$ plasmid.

3.2. Pilot Expression and Characterization of Protein

The expression level of recombinant full-length p32 in the Rosetta strain of *E. coli* was negligible. However, compared to the controls (mock vector and uninduced recombinant *E. coli* Rosetta cell bacteria), a visible protein band with a molecular weight (~ 31 kDa) corresponding to that expected for Tr.P32 was present in bacteria transformed with pET24a- Tr.P32 plasmid, after induction with IPTG (Figure 3, Panel A). Therefore, the subsequent process of scaling up the recombinant protein expression was continued only on Tr.P32. The results of SDS-PAGE revealed that the optimal time and concentration of IPTG for the expression of recombinant Tr.P32 protein were 6 h and 1 mM IPTG, respectively. The identity of recombinant Tr.P32 was confirmed by Western blot. Results showed an intensive brown color reaction observed with a protein size corresponding to ~31kDa, while there was no color reaction with any of protein from uninduced culture and controls (Figure 3, Panels B and C).

3.3. Scale up Expression and Purification of Tr.P32 Protein

Results of Tr.P32 protein scaled up using 200 mL of transformed *E. coli* Rosetta followed up by purification are shown in Figure 4, Panel A. The purified recombinant protein was analyzed on SDS-PAGE and subsequently confirmed by Western blotting (Figure 4, Panels B and C). The concentration of recombinant protein estimated by the Bradford method was 0.270-0.300 mg/200 mL of culture media.

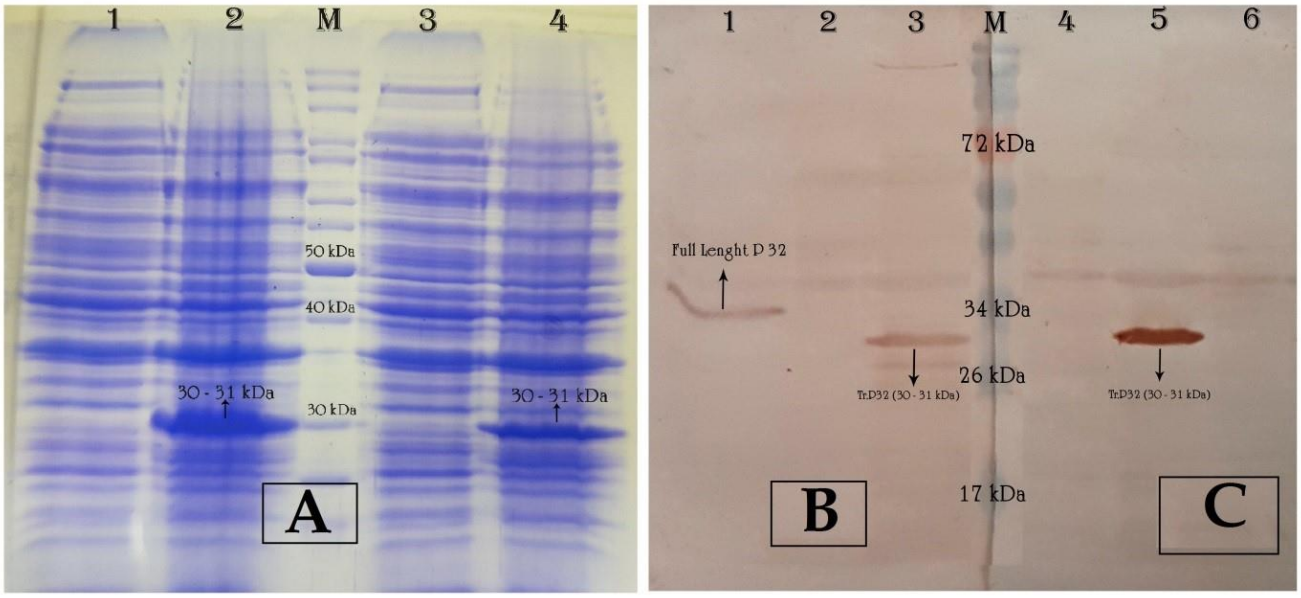

Figure 3. Panel A, Identification of the expressed Tr.P32 by SDS-PAGE; Lane 1, uninduced recombinant Rosetta cell; Lane 2, 6 h PI of Tr.P32 protein; Lane M, protein marker; Lane 3, mock-transformation with pET24a+ vector; Lane 4, 4 h PI of Tr.P32 protein; **Panel B**, specific reactivity of P32 with anti-GTPV HIS; Lane 1, full-length P32 protein; Lane 2, uninduced recombinant Rosetta cell; Lane 3, truncated P32 protein; Lane M, pre-stained protein marker. **Panel C**, specific reactivity of P32 with HRPO conjugated monoclonal anti-poly histidine; Lane 4, full-length P32 protein; Lane 5, truncated P32 protein; Lane 6, uninduced recombinant Rosetta cell.

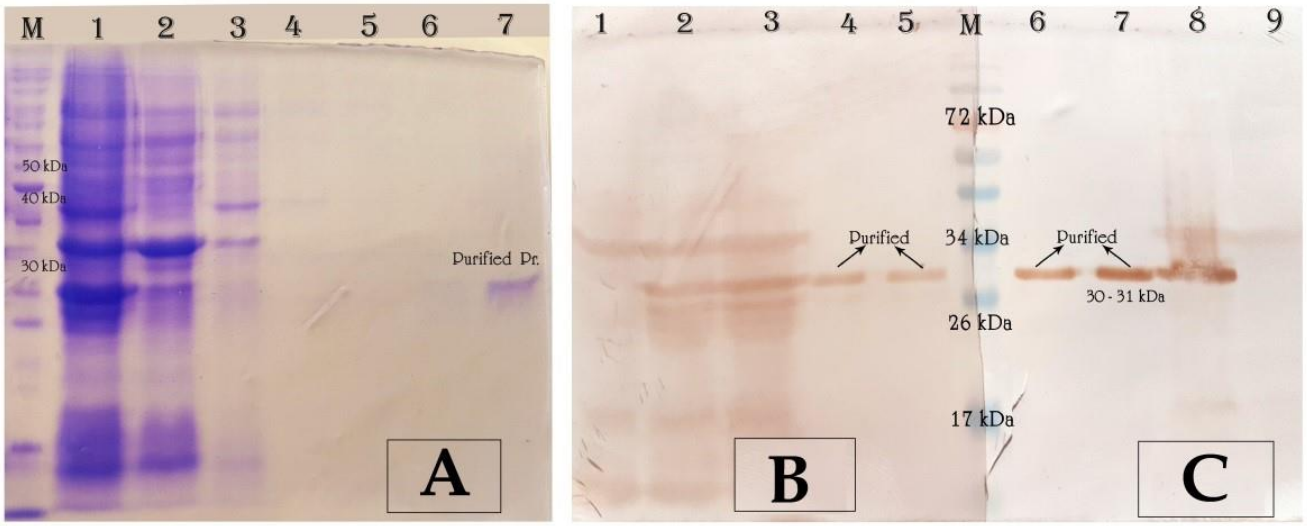

Figure 4. SDS-PAGE analyses showing purified Tr.P32 protein using Ni-NTA resin under denaturing conditions. **Panel A**, Lane M, protein marker; Lane 1, over-expression (8M urea lysis); Lane 2, flow through; Lane 3, washing (8 M urea buffer, pH=6.3); Lanes 4 and 5, elute (8 M urea, pH=5.9); Lanes 6 and 7, elute (8 M Urea, pH=4.5). **Panel B**, Specific reactivity of Tr.P32 with anti-GTPV HIS. Lane 1, mock-transformation with pET24a+ vector; Lane 2 and 3, unpurified Tr.P32 protein; Lanes 4 and 5, purified Tr.P32 protein; Lane M, pre-stained protein marker. **Panel C**, specific reactivity of Tr.P32 with conjugated monoclonal anti-poly histidine; Lanes 6 and 7, purified Tr.P32 protein; Lane 8, unpurified Tr.P32 protein; Lane 9, mock-transformation with pET24a+ vector.

3.4. Neutralization Index

With the exception of five positive samples collected from experimentally or naturally infected and vaccinated animals, all sera samples which were considered as positive had a virus neutralization index of ≥1.5 Log10. However, all sera collected from non-infected animals were assessed as negative by neutralization index. There was no significant difference between the percentages of negative or positive samples assessed by ELISA or NI method in each animal species (Table 3).

Table 3. Comparison of capripoxvirus neutralization index and ELISA measured on positive and negative sera of goat, sheep, and cattle

Animal	Nature of sera	Neutralization index		ELISA	
		NI value	Positive (No./total)	OD	Positive (No./total)
Goat	Negative (Healthy)	0-1	0% (0/25)	0.091-0.384	0% (0/25)
	Post-vaccination	0.5-2.5	83.3% (5/6)	0.354-0.961	83.3% (5/6)
	Infected[1]	1-3	86.3% (19/22)	0.354-1.602	81.81% (18/22)
Sheep	Negative (Healthy)	0-0.5	0% (0/25)	0.171-0.397	0% (0/0)
	Post-vaccination	0.7- 2.2	88% (22/25)	0.469-1.155	100% (25/25)
	Infected	0.7-3.2	92.3% (12/13)	0.345-1.731	92.3% (12/13)
Cattle	Negative (Healthy)	0-0.2	0% (0/15)	0.080-0.187	0% (0/15)
	Post-vaccination[2]	1.7-2.7	100% (8/8)	0.601-0.715	100% (8/8)
	Infected	07-2.7	83.3% (10/12)	0.417-0.971	100% (12/12)

3.5. Optimization of Tr.P32 Protein-Based Indirect-ELISA

The results of checkerboard titration revealed that 675 ng of Tr.P32 antigen/well and 1:10 dilution of control sera (anti-GTPV HIS and healthy goat sera) caused the maximum difference in absorbance between positive and negative goat sera (Figure 5).

3.6. Evaluation of Tr.P32 Protein-Based Indirect-ELISA

The diagnostic sensitivity and specificity of the Tr.P32 protein-based indirect ELISA were 94% and 96.6%, respectively. Using MedCalc software, the cutoff value was calculated to be 0.397 (Figure 6A). The recombinant Tr.P32 protein showed good reactions with antibodies against GTPV, SPPV, and LSDV, whereas no cross-reactions with anti-Orf virus antibody was detected (Figure 6B). The results of NI and the developed ELISA were generally in agreement. The ROC curve analysis of Tr.P32-ELISA results for negative and positive test sera from sheep, goats, and cattle are shown in Fig. 7. The ROC curve analysis was expressed in terms of area under curve (AUC= 0.991) for the Tr.P32-ELISA test for known negative and positive test sera against LSDV, SPPV, and GTPV (Figure 7).

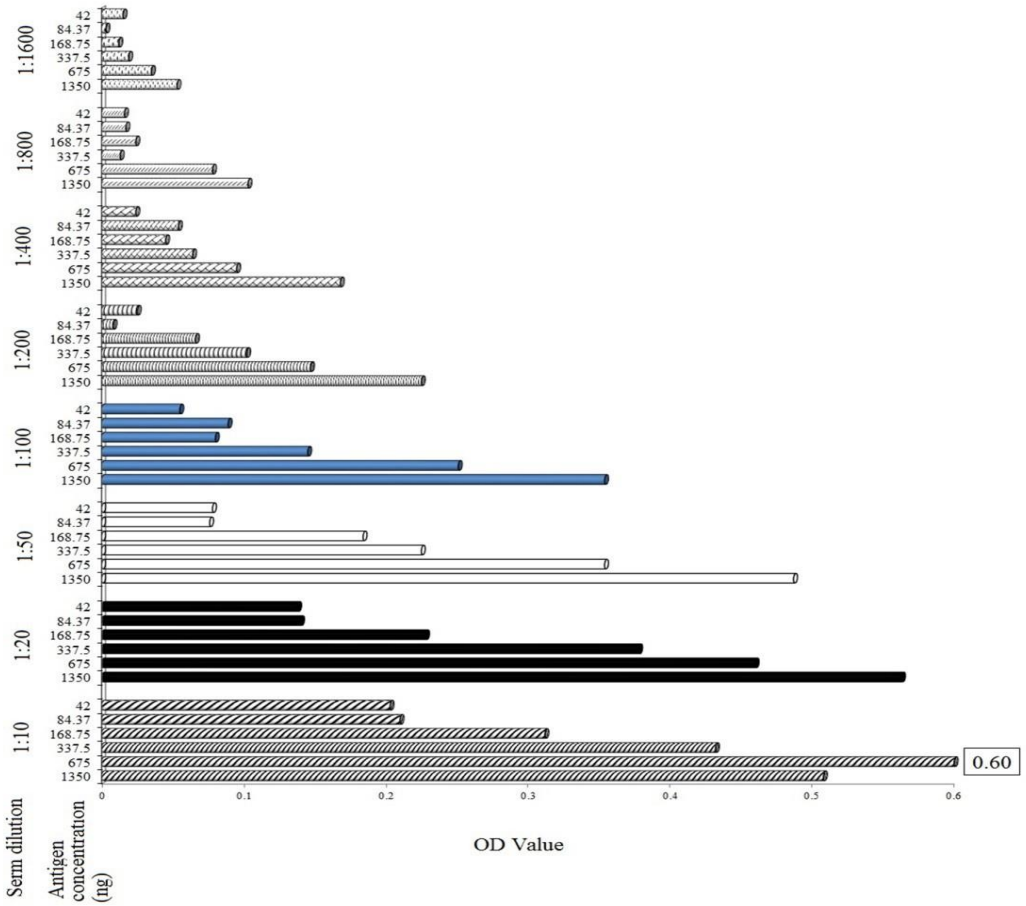

Figure 5. Checkerboard titration to optimize Tr.P32 protein concentration and serum dilution

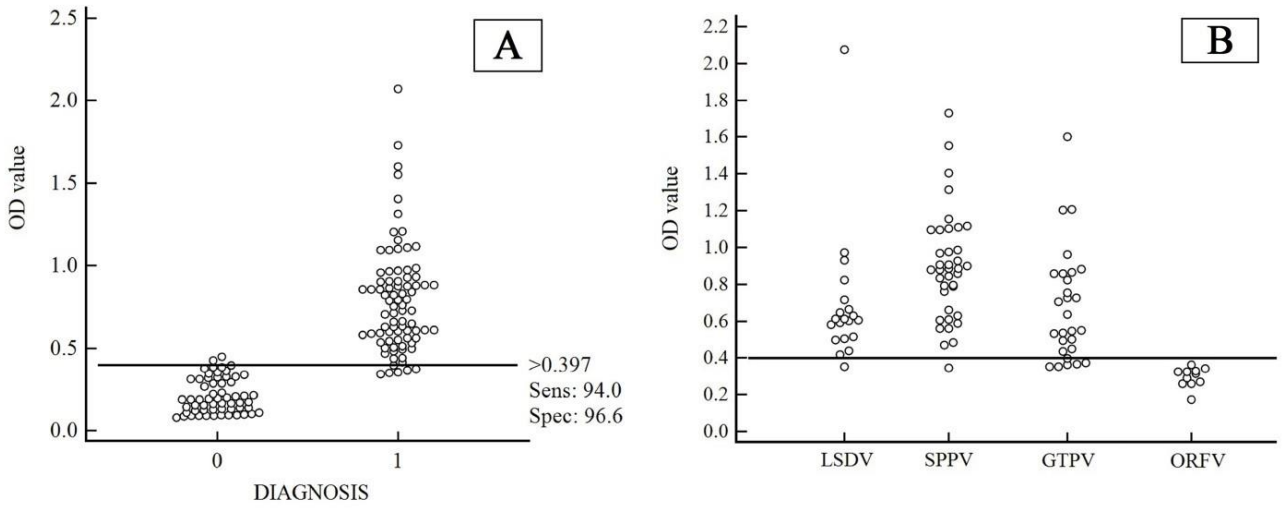

Figure 6. Interactive dot diagram of ROC analysis of Tr.P32 protein reactions in ELISA system vs. NI method. **Panel A**, diagnostic specificity (% DSpec) and diagnostic sensitivity (% DSens) with cut off values. **Panel B**, reaction evaluation of Tr.P32 protein in ELISA system against LSDV, SPPV, GTPV, and Orfv anti-sera, separately. OD = optical density.

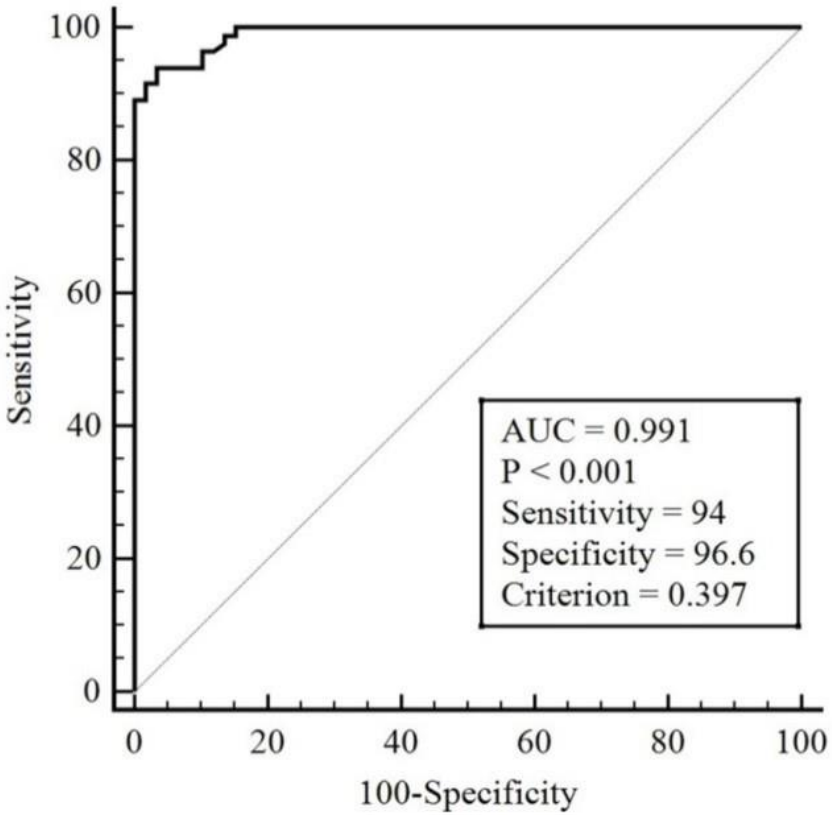

Figure 7. Receiver-operating characteristic (ROC) analysis of Tr.P32

4. Discussion

Lumpy skin disease, sheep pox, and goat pox are notifiable diseases associated with profound effects on cattle, sheep, and goat farming industries in endemic regions (4). ELISA is one of the most feasible assays being used in sero-epidemiologic investigations of capripoxvirus infections. Therefore, this study was conducted to develop an ELISA system based on the recombinant P32 protein.

Previously, an attempt was made to develop an ELISA for capripoxvirus infections using the inactivated virus as antigen in the detection of capripoxvirus-specific antibodies (15). However, the inactivated virus-based ELISA is not an ideal assay, as it is expensive and difficult to produce the large quantities of whole virus antigen that would be needed for serological surveillance (15). In the present study and previous research (7, 12), the P32 protein of capripoxviruses, an immune-dominant protein eliciting neutralizing antibodies in host species, was expressed and used to develop an ELISA system. The results of the present study indicated that the expression of full-length P32 sequence (969 bp) in *E. coli* was negligible and the expressed protein was recognized by a goat polyclonal antibody against goat pox virus. However, as revealed by Western blotting, the expressed protein had a lower molecular weight (~33-34 kDa) compared to its sequence, and it did not react with an anti-His tag HRPO-labeled monoclonal antibody, expected to recognize the His tag sequence at C-terminus part of the protein. The absence or low levels of expression of full-length P32 protein in *E. coli* have been previously reported by Heine and Stevens (7). The reaction of the

expressed full-length p32 protein with anti-GTPV polyclonal antibodies and its lack of reaction with HRPO-conjugated anti-His antibodies could be associated with a proteolytic removal of the transmembrane domain of the protein harboring the His tag sequence (7, 18). In agreement with this finding, a previous investigation on the expression of the H3 protein of the vaccinia virus also showed that it was expressed without its transmembrane region (22, 23). Meanwhile, the truncated P32 gene (encoding 20V-S270 AAs), lacking N- and C-terminal hydrophobic regions, was successfully expressed in *E. coli.* with the expected molecular weight (~31 kDa), including the His tag sequence. The expressed protein was analyzed in SDS-PAGE and confirmed by Western blot or sequencing of the pET24a+ expression vectors. Consistent with the present results, truncated P32 protein has been expressed in a prokaryotic system, and the purified protein was evaluated for detection of antibodies against GTPV and SPPV. The previously expressed truncated P32 gene was relatively longer than that expressed in this study, and the efficiency of the developed ELISA-based truncated P32 protein against LSDV was not evaluated, while it is still questionable (20).

Solubility analysis revealed that overexpressed Tr.P32 protein was insoluble in *E. coli* and was found largely in pelleted materials after centrifugation of sonicated bacterial lysate. Therefore, attempts to solubilize the protein by 8M urea were made. Although a previous study on the purification of P32 showed a difficulty in solubilizing full-length P32 protein in 8M urea or guanidine hydrochloride (24), the present results showed that after the removal of hydrophobic regions, the truncated P32 protein was easily solubilized and purified by Ni-NTA affinity chromatography. The yield of the expressed protein from 1000 ml culture of transformed *E. coli* in LB broth was approximately 1.35 to 1.5 mg of the purified protein, which is sufficient to coat 20 to 23 ELISA plates.

Diagnostic specificity and sensitivity were calculated as 96.6% and 94%, respectively, as compared to NI. By ROC analysis, AUC was estimated to be 0.991, showing a high specificity and sensitivity of the developed ELISA. Venkatesan and Teli (20) used Tr.P32 with a slight difference to develop a sheep pox and goat pox ELISA system and also reported 98.7% and 98.1% for specificity and sensitivity, respectively. However, in the present study, in addition to the sheep pox and goat pox positive sera, the LSD positive sera were applied to set up the ELISA system. Meanwhile, determination of species specific cut off could also be valuable.

To evaluate the cross-reactivity of the ELSA system based on Tr.P32, the positive Orf sera were tested; no cross-reactivity was detected, indicating that the developed ELISA system is applicable for the differential diagnosis of ORFV and CaPV infections; the IFAT, AGID, and CIE have been shown to have cross-reactions with parapox viruses such as the Orf virus. This result was supported by the findings of Gnanavel Venkatesan and Teli (20) who reported the ELISA based on Tr.P32 has no cross-reactivity with antibodies against the Orf virus. One of the concerns reported for the ELISA system based on virion core proteins is their inability or poor performance on detecting positive sera of capripox-vaccinated animals. However, the present results revealed that the developed ELISA has acceptable diagnostic activity in the detection of anti-SPPV and GTPV in vaccinated animals. It is worth noting that the intervals between vaccination time and sera collection may affect the results of analytical sensitivity and specificity of the test (25). However, the present study is the first report of the diagnostic potential of Tr.P32 for detecting antibodies against LSDV in bovine sera. In regions such as Middle East countries where LSD is newly introduced, the indirect Tr.P32-ELISA could be suitable for sero-epidemiological investigations and vaccination programs; further investigations are warranted.

5. Conclusion

In this study, the Tr.P32 protein, as an immunodominant protein of capripoxviruses, was expressed and purified to develop an ELISA system for use in sero-surveillance of sheep pox, goat pox, and lumpy skin diseases. The results showed that truncated P32 was successfully expressed in *E. coli*, but the expression of full-length P32 protein was negligible, and the protein was most likely cleaved by host proteolysis enzymes. The ELISA based on the Tr.P32 protein showed acceptable sensitivity and specificity not only for sero-surveillance of SPPV and GTPV, but also for LSD. The developed system showed a good reactivity with the sera of vaccinated animals as well as experimentally and naturally infected animals. However, the efficiency of the developed ELISA based on Tr.P32 should be evaluated during various post-infection or vaccination intervals by analytical analysis.

Authors' Contribution

Study concept and design: H. R. V. and M. H. E.
Acquisition of data: M. H. E.
Analysis and interpretation of data: M.R. S. and M. H. E.
Drafting of the manuscript: M. L.
Critical revision of the manuscript for important intellectual content: H. K.
Statistical analysis: M.R. S. and M. H. E.
Administrative, technical, and material support: RVSRI

Ethics

All procedures performed in studies involving animals were in accordance with the ethical standards of the Razi Vaccine and Serum Research Institute under the project number of (No. 2-970641-047-18-18).

Conflict of Interest

The authors declare that they have no conflict of interest.

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