DOI : 10.5958/2277-8934.2023.00013.9 *Vol 30 No 1, p 97-103*

SEROLOGICAL DIAGNOSIS OF *Parabronema skrjabini* INFECTION USING A RECOMBINANT ANTIGEN IN BACTRIAN CAMELS

Xindi Chen[1,2], Chenchen Feng[1,2], Jinling Wang[1,2], Yu Wang[1,2], Tengyu Wang[1,2], Chunxia Liu[3] and Wenlong Wang[1,2]

[2]Key Laboratory of Animal Disease Clinical Diagnosis and Treatment Technology,

[1]College of Veterinary Medicine, [3]College of Life Science, Inner Mongolia Agricultural University, Hohhot 010018, China

ABSTRACT

In order to find out an effective method for the *in vivo* diagnosis of parabronemosis and on the basis of results of histological analysis obtained in the laboratory, the serine threonine protein kinase (STPK) gene, an immune-related secretory gene of *P. skrjabini*, was screened. A total of 140 Bactrian camel sera samples from different regions of Inner Mongolia were screened. The results of bioinformatics analysis showed that the nucleic acid sequence of STPK gene was 579 bp, contained a complete open reading frame encoding 199 amino acids, had no signal peptide, was atypical secretory protein and had 8 antigenic epitopes. The total RNA of *P. skrjabini* was extracted and the STPK gene was obtained by RT-PCR amplification. The recombinant expression plasmid was constructed and expressed in *Escherichia coli* BL21 (DE3). A recombinant protein, rSTPK, 28 kDa in size, that was primarily expressed in inclusion bodies, was obtained. An indirect enzyme-linked immunosorbent assay (iELISA) using rSTPK as an antigen was established using recombinant protein. We observed a positive detection rate of 85.7% (120/140), indicating that rSTPK-iELISA can be used as a serological method to diagnose parabronemiasis in camels.

Key words: Camel, enzyme-linked immunosorbent assay, *Parabronema skrjabini*, recombinant antigen, serine threonine protein kinase

Parabronema skrjabini is a blood-feeding nematode that resides in the abomasum of ruminants and compartment 3 of camels. However, camels are considered the primary definitive host (Hasheminasab *et al*, 2016). *P. skrjabini* is widely distributed in Africa and Asia and it is especially common in Mongolia (Sharkhuu, 2001), Kazakhstan (Morgan *et al*, 2006), Saudi Arabia (El-Azazy, 1990), Namibia (Krecek *et al*, 1990), Turkey (Umur and Yukari, 2005) and Iran (Eslami and Nabavi, 1976). Camels infected with *P. skrjabini* experience inflammation, ulcers and bleeding of the diarrhoea, anaemia and even death. In the main camel breeding grounds of Inner Mongolia, *P. skrjabini* infections are common hence the research has primarily focused on its morphological identification, classification (Habronematidae; Hasheminasab, 2015) and transmission (Chen *et al*, 2016; Deng *et al*, 2017). Traditional nematode detection methods, such as saturated sodium chloride solution floating can be used for the diagnosis of the disease, but the detection rate is low. Polymerase chain reaction (PCR) can be used to detect a small number of parasites in the skin tissue, but this method is not suitable for the detection of *P. skrjabini* (Fischer *et al*, 1998; Morales-Hojas *et al*, 2001). In order to find out an effective diagnostic method for *P. skrjabini* the indirect ELISA detection method was established (Wang *et al*, 2022) using recombinant antigen rCPI which had good sensitivity and specificity and proved that the detection method based on recombinant antigen is feasible. The use of recombinant protein in iELISA was an effective tool for the serological diagnosis of parasitic diseases (Santos *et al*, 2019).

In this study, we developed a diagnostic method for identifying *P. skrjabini* based on the detection of serine threonine protein kinase (STPK) screened by sequencing the transcriptome of *P. skrjabini* and comparing it to common ruminant parasite genes from GenBank. The gene was cloned, expressed using a prokaryotic vector, verified antigenically and the recombinant protein was used as a diagnostic antigen to establish a serological diagnosis method.

SEND REPRINT REQUEST TO WENLONG WANG, CHUNXIA LIU email: wwl.imau@163.com, lcx.imau@163.com

Materials and Methods

Parasites

Female and male *P. skrjabini* individuals were collected from the compartment 3 of Bactrian camels (*Camelus bactrianus*) in Bayannaoer, Inner Mongolia, China. The collected portions were stored in liquid nitrogen after morphological identification using microscopy, packing and marking. In total, 81 positive sera samples infected with *P. skrjabini* were collected from camels in Inner Mongolia during postmortem and nine negative serum samples not infected with *P. skrjabini* were collected from camels. Twenty four sera samples from sheep infected with *Moniezia* spp., *Haemonchus contortus, Dictyocaulus filaria, Trichuris ovis, Chabertia ovina, Nematodirus* spp., *Trichostrongylus* spp., *Oesophagostomum* spp. and *Oestrus ovis* were collected from the sheep farm in Bayannaoer City, Inner Mongolia and preserved in the laboratory.

Bioinformatics analysis of STPK gene

Bioinformatics analysis of STPK gene of *P. skrjabini* was carried out by bioinformatics software. The open reading frame was predicted by ExPASy (http://web.expasy.org/translate/) and ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) software and the signal peptide sequence was predicted by SignalIP4.1 server (https://www.ncbi.nlm.nih.gov/orffinder/) online software. SecretomeP.2.0 server (http://www.cbs.dtu.dk/services/SecretomeP/) software for prediction of atypical protein secretions, TMHMM server.v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/) software for transmembrane protein domain analysis, Target P 1.1 server (http://www.cbs.dtu.dk/services/TargetP/) software for prediction of subcellular localisation of eukaryotic proteins. BepiPred 1.0 Server (http://www.cbs.dtu.dk/services/BepiPred/) software was used to predict the antigenic epitopes.

Cloning and expression of STPK gene in Escherichia coli

P. skrjabini specimens were placed in a mortar filled with liquid nitrogen and ground thoroughly. Total RNA was extracted using the TRIzol method, followed by ethanol precipitation. The precipitate obtained was dissolved in RNase-free water and stored at −80°C.

The PCR primers were designed according to the nucleotide sequences of the STPK gene (Genbank with the following accession numbers: 2488713), which were 5′-GCC GAA TTC ATG GTT ATG AGG AAC GG-3′ and 5′-TTG CTC GAG CAC GCC TAT GCC CTG AG-3′ (italics in the primer sequence indicate the *EcoRI* and *XhoI* sites in the expression vector pET30a). The extracted total RNA was reverse-transcribed into cDNA. The cDNA was used as a template and mixed with PCR buffer, dNTP mixture and DNA polymerase for PCR amplification. PCR amplification conditions: 94°C pre-denaturation for 5min; 94°C denaturation for 30s; 57°C annealing for 30s; 72°C extension for 1.5min; 35 cycles; 72°C extension for 10min. The recombinant plasmid pET-STPK was confirmed by restriction digestion and sequencing and subsequently constructed and expressed in *E. coli* BL21 (DE3). Finally, the recombinant bacteria were completely lysed using ultrasonic cell fragmentation, the supernatant and precipitate were collected and the expression of the recombinant protein was detected by SDS-PAGE electrophoresis. The recombinant protein was purified using the Ni-NTA Sefinose™ Resin Kit and used as an antigen for enzyme-linked immunosorbent assay (ELISA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis of rSTPK

A recombinant protein rSTPK was separated *via* SDS-PAGE and transferred to polyvinylidene difluoride membranes (Jeong *et al*, 2018). The membranes were blocked using 5% skim milk. Positive serum of camel parabronemiasis (1:1000) was used as the primary antibody and rabbit anti-camel IgG labeled with horseradish peroxidase (IgG-HRP) was used as the secondary antibody (1: 5000). A negative serum control was established.

rSTPK indirect ELISA (iELISA)

According to the chessboard test, purified rSTPK was diluted with 50 mmol/L carbonate buffer and 1, 2, 4, 8 and 16 µg was added per well. The negative and positive sera were diluted (1:25, 1:50, 1:100, 1:200 and 1:400) and the rabbit anti-camel IgG-HRP was diluted 5000 times. The reaction was carried out by adding a substrate solution containing tetramethylbenzidine chromogenic substrate and terminating the reaction with H_2SO_4. Finally, the optical density (OD) at 450 nm was measured using a mini tablet reader (Bio-Tek, USA). The optimal reaction conditions was determined according to the calculated P/N value. Based on this condition, nine camel negative sera were detected and the average value and standard deviation were calculated (cut-off value=negative serum average + 3 standard deviation). The recombinant protein rSTPK antigen

was used to detect the specificity of 24 positive sera infected with *Moniezia* spp., *Haemonchus contortus, Dictyocaulus filaria, Trichuris ovis, Chabertia ovina, Nematodirus* spp., *Trichostrongylus* spp., *Oesophagostomum* spp. and *Oestrus ovis*.

Field sera testing

The established iELISA was used to screen 140 camel sera collected from Inner Mongolia. According to the principle of statistics, when the OD450nm value of the sample to be tested is greater than the cut-off value, it is judged to be positive and if it is less than the cut-off value, it is negative. When the OD450nm of the sample to be detected was close to the critical value (the cut-off value ± 0.005) and the result of the second test was the same as that of the first test, it was determined as suspected infection of *P. skrjabini*.

Results

Bioinformatics analysis of STPK gene

The characteristics of STPK gene were analysed by ExPASy and ORFfinder online software. the results showed that the nucleic acid sequence of STPK gene was 579 bp and its molecular weight was 22.79 kDa. It contained a complete open reading frame encoding 199 amino acids, including 27 strong acid amino acids, 24 strong base amino acids, 66 hydrophobic amino acids and 43 polar amino acids and the isoelectric point was 6.208.

Using SignalP and SecretomeP software to predict the signal peptide sequence of gene-encoded protein and to analyse the atypical secretory protein, it was found that there was no signal peptide in STPK, but it was considered to be atypical secretory protein NN-Score=0.842 in the prediction of SecretomeP software (if NN-Score > 0.5, it was determined to be secretory protein) (Fig 1).

The antigenic epitopes of the gene-encoded protein were predicted by BepiPred software. The results showed that there were 8 antigenic epitopes of STPK protein: DS (aa 45~46), LLPGKDNYDQ (aa 66~75), S (aa 88), P (aa 98), LFT (100~102), SEKTED (aa 122~127), VERPKPTE (aa 144~151), DVHQAPPQGIGV (aa 182~193). It indicated that the secretory protein encoded by STPK had better antigenicity. The protein transmembrane region and protein subcellular localisation of the gene were predicted by TMHMM and TargetP software and the number of amino acids in extracellular region, transmembrane region and intracellular region was analysed. The results showed that STPK was not a transmembrane protein and the whole protein was extracellular (Fig 2).

Expression of STPK gene in E. coli

STPK was amplified with specific primers using the reverse transcription product of RNA from *P. skrjabini* as a template. The results of the agarose gel electrophoresis revealed a specific band of approximately 579 bp (Fig 3).

The recombinant plasmid pET-STPK was transformed into *E. coli* BL21 (DE3) cells. After induction, the molecular weight of rSTPK protein was 28 kDa (Fig 4).

Western blot

rSTPK was probed using camel serum infected with *P. skrjabini* (Fig 5). A specific band was observed at 28 kDa, but no band was observed upon probing with negative sera samples. This showed that rSTPK can specifically bind to IgG in the sera of camels infected with *P. skrjabini*.

Evaluation of P. skrjabini infection in camels using rSTPK ELISA

An iELISA was established for the serological diagnosis of *P. skrjabini* using rSTPK as a coating antigen. The chessboard experiment determined that the best coating concentration of rSTPK was 2 µg/well (Table 1).

The recombinant antigen rSTPK was used to detect 9 negative sera samples from camels uninfected with *P. skrjabini*. The average OD450nm value of negative serum screened by rSTPK was 0.229 and the standard deviation was 0.025. Therefore, the cut-off value was determined to be 0.304. The 24 sera samples used for the specificity test did not exhibit any cross-reactivity with other parasitic-infected sera samples.

Table 1. Chessboard method to determine the best dilution conditions for ELISA, based on the OD value of the positive and negative serum.

Conjugate dilution	Antigen (µg/mL)				
	16	8	4	2	1
1:25	4.38	4.45	4.79	5.11	5.28
1:50	4.74	4.91	5.43	7.23	6.60
1:100	5.24	5.85	5.72	6.41	5.91
1:200	4.92	5.45	5.70	5.97	5.87
1:400	5.03	5.81	6.10	6.03	4.88

Field serum test results

We used iELISA to test 140 camel serum samples, of which 120 were positive (85.7%). Twenty

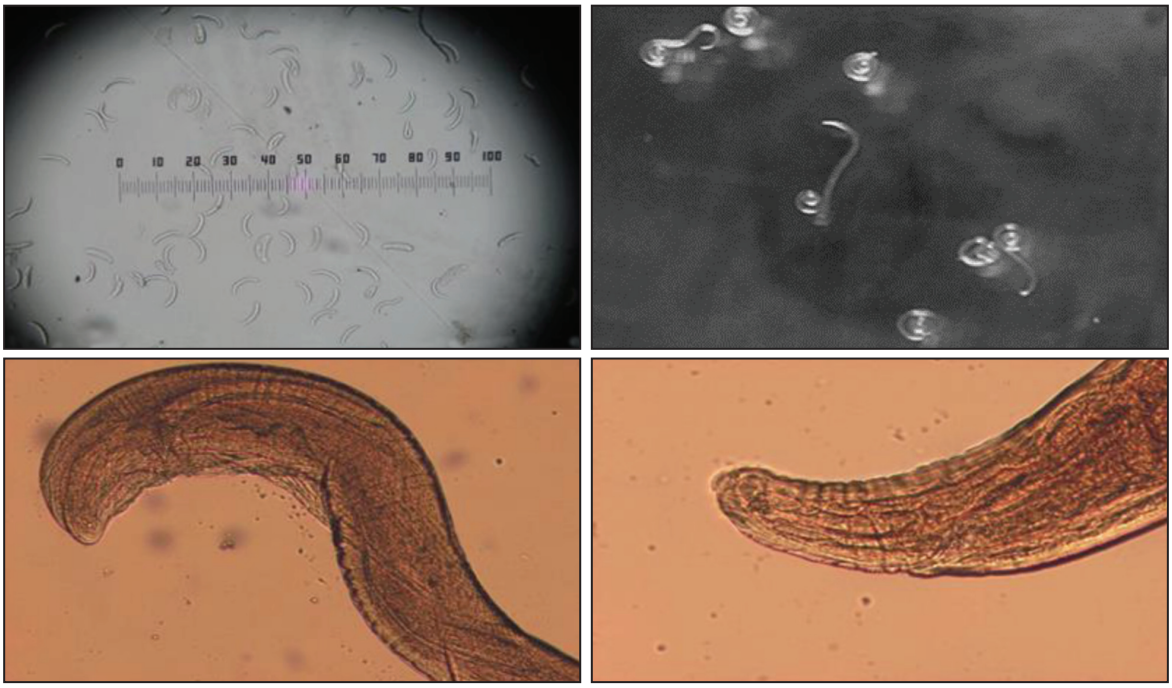
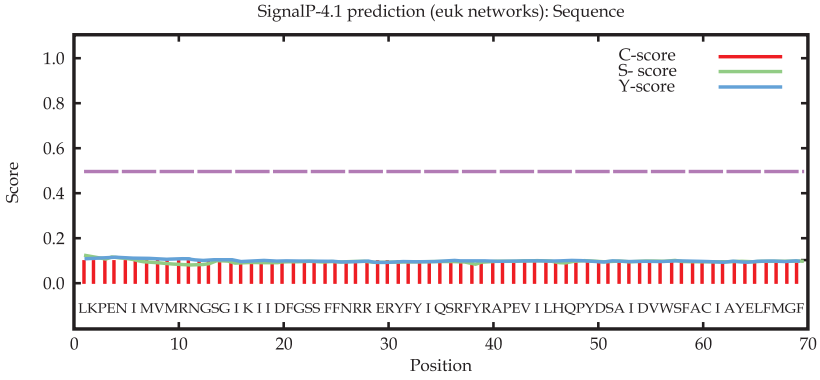


Fig 1. Morphology of *P. skrjabini*: egg, larva, adult (Left, female; Right, male).



#	Measure		Position	Value	Cutoff	signal peptide?
	max.	C	51	0.108		
	max.	Y	11	0.108		
	max.	S	1	0.125		
	mean	S	1–10	0.106		
		D	1–10	0.107	0.450	NO

Name=Sequence SP= 'NO' D=0.107 D–cutoff=0.450 Networks=SignalP–noTM

Fig 1. The analysis of signal peptide sequence of STPK gene.

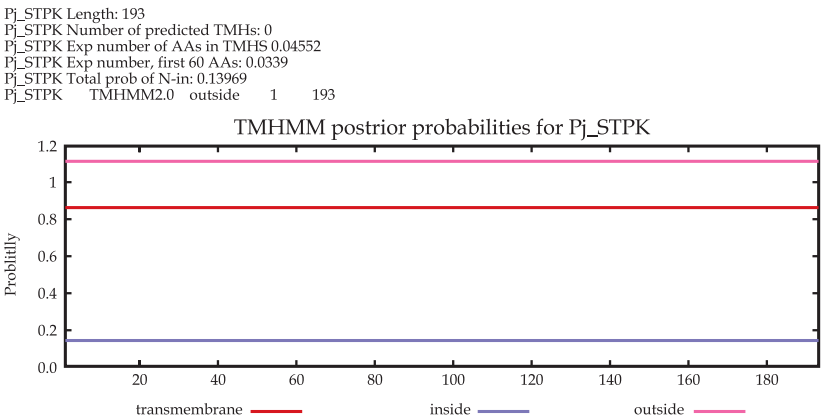


Fig 2. The analysis of STPK transmembrane domain.

samples tested negative, of which 4 were suspected of being infected with *P. skrjabini*. The results showed that the camels in Inner Mongolia were seriously infected with *P. skrjabini*.

Discussion

The diagnosis of this nematode disease primarily depends on the postmortem of diseased animals, but this method cannot provide a reference for the *in vivo* detection of the disease. The improved CTAB+SiO_2 adsorption method was used to extract DNA from artificially simulated positive faeces and established a method for *in vivo* diagnosis of the disease *via* PCR (Zheng, 2015). However, the detection rate of *P. skrjabini* eggs in infected animal faeces was low, making it difficult for practical applications. The serological method established with recombinant antigen by our research team in the early stage was highly feasible.

With the continuous application of high-throughput sequencing technology in parasite research,

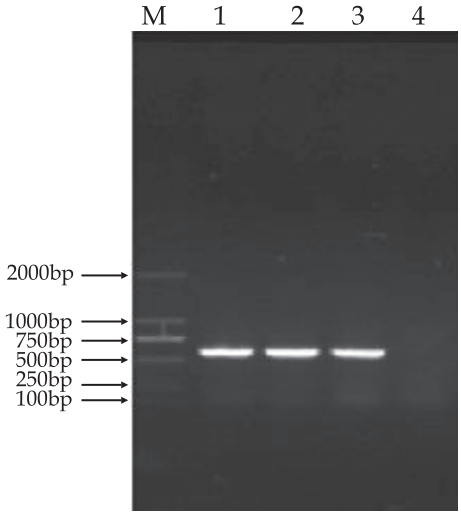


Fig 3. Electrophoresis following the reverse transcription polymerase chain reaction for STPK. M: DNA standard DL2000; line 1-3: STPK; line 4: negative control.

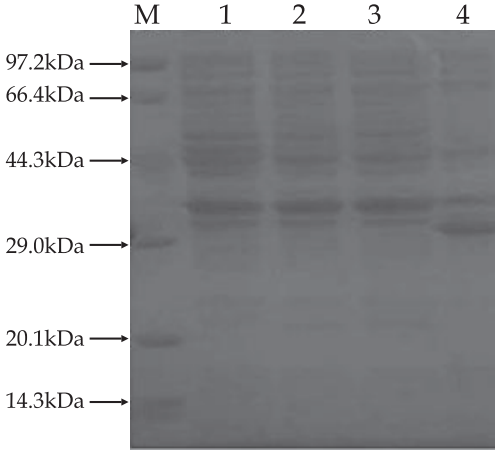


Fig 4. Electrophoresis following the reverse transcription polymerase chain reaction for STPK. M: molecular protein marker; Line 1: BL21 (DE3) empty bacteria; Line 2: Before induction of BL21 (pET30a); 3: After induction of BL21 (pET30a); 4: After induction of BL21 (pET-STPK).

many scholars began to use multi-omics sequencing analysis technology to carry out more in-depth basic research in immune regulation and immune protection of parasites and identified many specific diagnostic antigens and vaccine candidate genes (Blazie *et al*, 2015; Hewitson *et al*, 2011; Laing *et al*, 2013; Zheng *et al*, 2011). A comparative analysis of the secretory gene and protein data predicted in the transcriptome and proteome studies revealed that 76.6% of the secretory proteins were consistent with the transcriptome secretory gene data (Feng *et al*, 2017), indicating that these genes are secreted at the mRNA and protein level and can be used as candidate genes for immune-related gene screening of *P. skrjabini*. The secretory antigen expressed on the body

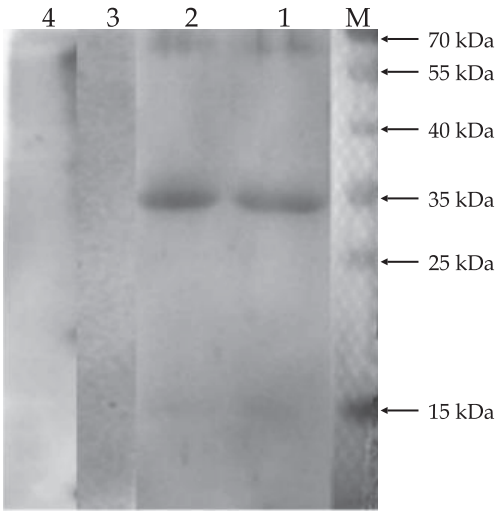


Fig 5. Western blotting analysis of recombinant rSTPK. M: Molecular protein marker; Line 1-2: Positive serum test; Line 3-4: Negative serum test.

surface of *Schistosoma mansoni* can directly interact with the host immune system and thus influence the host immune response. It was reported that serine / threonine protein kinase can be expressed in the body surface of *Schistosoma mansoni* as a TGF- β receptor (Forrester *et al*, 2004), but its biological characteristics and functions were unknown, therefore, STPK was selected as a diagnostic candidate gene to provide a reference for the diagnosis and control of *P. skrjabini*. Bioinformatic analysis showed that STPK gene was a specific gene of *P. skrjabini* and was an atypical secretory protein without a signal peptide sequence. The identification of the transmembrane region and subcellular localisation analysis showed that STPK was not a transmembrane protein; instead, the whole protein was located in the extracellular region. The protein was predicted to have eight antigenic epitopes, which indicates sound antigenicity. The selected STPK gene conforms to the characteristics of candidate genes for immunological diagnosis and immune prevention.

Toxoplasma gondii secretory protein particle antigens (GRA1 and GRA7) was used for recombinant expression and their diagnostic value by iELISA assay was assessed with a great significance (Wang *et al*, 2014). Teimoori *et al* (2015) used a recombinant antigen of the cysteine protease of *Opisthorchis viverrini* from Thailand for the serological diagnosis of *Fascioliasis hepatica*. The iELISA results showed that the sensitivity and specificity of this diagnostic antigen were 62.1% and 84.05%, respectively. In this study, STPK was cloned into the plasmid pET30a and expressed in *E. coli*. Western blotting showed

that the rSTPK protein could stimulate host-specific antibodies. Using recombinant protein rSTPK as an antigen, an iELISA detection method for the diagnosis of *P. skrjabini* infection was established. This method exhibits good specificity. A total of 140 clinical sera samples were screened and the positivity rate was 85.7%. Therefore, the detection method established in this study can provide a reference for the prevention and treatment of camel parabronemiosis and can be used for early diagnosis.

In this study, STPK was identified as a candidate antigen gene which was cloned into pET30a and recombinant rSTPK was used as an antigen for the serological diagnosis of camels infected with *P. skrjabini*. rSTPK was also used as the antigen to establish an iELISA method for diagnosing parabronemiosis. The results showed that the method had high specificity, suggesting that rSTPK is a potential serological marker for detecting *P. skrjabini* in camels.

Acknowledgements

We would like to thank the parasite research team from the College of Veterinary Medicine, Inner Mongolia Agricultural University.

Ethics statement

Our research was conducted strictly in accordance with the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China and the protocol was reviewed and approved by the Research Ethics Committee of Inner Mongolia Agricultural University, Hohhot, Inner Mongolia, China. Permission was obtained from a local official slaughterhouse before specimen collection.

References

Blazie SM, Babb C, Wilky H, Rawls A, Park JG and Mangone M. Comparative RNA-Seq analysis reveals pervasive tissue-specific alternative polyadenylation in *Caenorhabditis elegans* intestine and muscles. BMC Biology. 2015; 13:4.

Chen LJ, Shi HL, Yang LR, Yang XY, Deng Q, Li B, Li YY. Molecular Identification of *Parabronema skrjabini* larvae in different instars larvae of horn flies. Chinese Journal of Veterinary Medicine 2016; 52(2):15-21(in Chinese).

Deng Q, Yang LR, Yang XY, Wang R, Li B, Zhang W and Yang B. Morphological study on camel parabronemosis vectors of horn flies *Haematobia irritans* and *H. titillans* (Diptera: Muscidae). Chinese Journal of Veterinary Science. 2017; 37(6):1077–1083 (in Chinese).

Eslami AH and Nabavi L. Species of gastro-intestinal nematodes of sheep from Iran. Bulletin de la Societe de pathologie exotique et de ses filiales. 1976; 69(1):92–95.

El-Azazy OM. Absence of hypobiosis in abomasal nematodes of sheep and goats in Egypt. Veterinary Parasitology. 1990; 37(1):55-60.

Feng CC, Wang WL and Hu HBTE. Sequencing and analysis of the transcriptome of *Parabronema skrjabini* in camel. Chinese Journal of Veterinary Science. 2017; 37(4):671-675 (in Chinese).

Fischer P, Büttner DW, Bamuhiiga J and Williams SA. Detection of the filarial parasite *Mansonella streptocerca* in skin biopsies by a nested polymerase chain reaction-based assay. The American Journal of Tropical Medicine and Hygiene. 1998; 58(6):816-820.

Forrester Sean G, Warfel Paul W and Pearce Edward J. Tegumental expression of a novel type II receptor serine/threonine kinase (SmRK2) in *Schistosoma mansoni*. Molecular and Biochemical Parasitology. 2004; 136(2):149-156. https://doi.org/10.1016/j.molbiopara.2004.03.007.

Hewitson JP, Harcus Y, Murray J, van Agtmaal M, Filbey KJ, Grainger JR, Bridgett S, Blaxter ML, Ashton PD, Ashford DA, Curwen RS, Wilson RA, Dowle AA and Maizels RM. Proteomic analysis of secretory products from the model gastrointestinal nematode *Heligmosomoides polygyrus* reveals dominance of venom allergen-like (VAL) proteins. Journal of Proteomics. 2011; 74(9):1573-1594.

Hasheminasab SS. Molecular characterisation of the first internal transcribed spacer of rDNA of *Parabronema skrjabini* for the first time in sheep. Annals of Parasitology. 2015; 61(4):241-246.

Hasheminasab SS, Jalousian F and Meshgi B. Molecular and morphological characterisation of *Parabronema skrjabini* of sheep and goats at 3 different geographical zones in Iran. Annals of Parasitology. 2016; 62(1):55-61.

Jeong DE, Lee Y and Lee SV. Western Blot Analysis of *C. elegans* proteins. Methods in Molecular Biology. 2018; 1742:213-225.

Krecek RC, Boomker J, Penzhorn BL and Scheepers L. Internal parasites of giraffes (*Giraffa camelopardalis angolensis*) from Etosha National Park, Namibia. Journal of Wildlife Diseases. 1990; 26(3):395-397.

Laing R, Kikuchi T, Martinelli A, Tsai IJ, Beech RN, Redman E, Holroyd N, Bartley DJ, Beasley H, Britton C, Curran D, Devaney E, Gilabert A, Hunt M, Jackson F, Johnston SL, Kryukov I, Li K, Morrison AA, Reid AJ, Sargison N, Saunders GI, Wasmuth JD, Wolstenholme A, Berriman M, Gilleard JS and Cotton JA. The genome and transcriptome of *Haemonchus contortus*, a key model parasite for drug and vaccine discovery. Genome Biology. 2013; 14(8):R88.

Morales-Hojas R, Post RJ, Shelley AJ, Maia-Herzog M, Coscarón S and Cheke RA. Characterisation of nuclear ribosomal DNA sequences from *Onchocerca volvulus* and *Mansonella ozzardi* (Nematoda: Filarioidea) and development of a PCR-based method for their detection in skin biopsies. International Journal for Parasitology. 2001; 31(2):169-177.

Morgan ER, Torgerson PR, Shaikenov BS, Usenbayev AE, Moore AB, Medley GF and Milner-Gulland EJ. Agricultural restructuring and gastrointestinal parasitism in domestic

ruminants on the rangelands of Kazakhstan. Veterinary Parasitology. 2006; 139(1-3): 180-191.

Sharkhuu T. Helminths of goats in Mongolia. Veterinary Parasitology. 2001; 101(2):161-169.

Santos LMD, Donassolo RA, Berne ME, Leite FPL, Avila LFDC, Scaini CJ, Moreira ÂN and Conceição FR. The serodiagnostic potential of recombinant proteins TES-30 and TES-120 in an indirect ELISA in the diagnosis of toxocariasis in cattle, horses and sheep. PLoS One. 2019; 14(3):e0213830.

Teimoori S, Arimatsu Y, Laha T, Kaewkes S, Sereerak P, Tangkawattana S, Brindley PJ and Sripa B. Immunodiagnosis of opisthorchiasis using parasite cathepsin F. Parasitology Research. 2015; 114(12):4571-4578.

Umur BA and Yukari. Seasonal activity of gastro-intestinal nematodes in goats in Burdur Region, Turkey. Turkish Journal of Veterinary and Animal Ences. 2005; 29(2):441-448.

Wang Z, Ge W, Huang SY, Li J, Zhu XQ, Liu Q. Evaluation of recombinant granule antigens GRA1 and GRA7 for serodiagnosis of *Toxoplasma gondii* infection in dogs. BMC Veterinary Research. 2014; 10:158.

Wang Y, Feng CC, Liu CX, Li JY and Wang WL. Effectiveness evaluation of recombinant antigen rCPI for iELISA detection of camel parabronemiosis. Journal of Camel Practice and Research. 2022; 28(3):277-282.

Zheng M, Hu K, Liu W, Hu X, Hu F, Huang L, Wang P, Hu Y, Huang Y, Li W, Liang C, Yin X, He Q and Yu X. Proteomic analysis of excretory secretory products from *Clonorchis sinensis* adult worms: molecular characterisation and serological reactivity of a excretory-secretory antigen-fructose-1, 6-bisphosphatase. Parasitology Research. 2011; 109(3):737-744.

Zheng WQ. Epidemiological investigation and establishment of prenatal PCR for diagnosis of Parasoft nematode disease of Camellia. Inner Mongolia Agricultural University, 2015; (in Chinese).