

Evaluating troponin C from *Psoroptes cuniculi* as a diagnostic antigen for a dot-ELISA assay to diagnose mite infestations in rabbits

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SUMMARY

The mite *Psoroptes cuniculi* is globally widespread and has a serious impact on commercial rabbit breeding. In China, diagnosis of *P. cuniculi* is currently based on conventional clinical methods that entail numerous disadvantages, including their failure to diagnose subclinical infections. Hence, alternative measures are required, and dot-ELISA is one of the most promising strategies. We cloned and expressed the recombinant *P. cuniculi* troponin C gene for use as a basis for novel dot-ELISA assay to detect *P. cuniculi* infections in rabbits. This amplified sequence encoded a 153 amino acid protein of 17.6 kDa and theoretical pI 4.18 without signal peptide. The recombinant troponin C of *P. cuniculi* is an outer membrane protein and may also be a new *P. cuniculi* allergen. Results of dot-ELISA test showed that this novel assay had more than 90% sensitivity but low specificity in distinguishing infections with *P. cuniculi* or *Sarcoptes scabiei*, despite very high agreement between observers (97–99%; κ values ranged from 0.95 to 0.98 for inter- and intra-observer variability test). This study showed that this novel method, at present, lacks diagnostic utility. Therefore, although simple serological assays such as dot-ELISA show great promise as diagnostic tools, we suggest that troponin C is not a suitable diagnostic antigen candidate.

Keywords dot-ELISA, *Psoroptes cuniculi*, troponin C

INTRODUCTION

In China, rabbits are reared for their fur, meat and pelts, and to be laboratory animals and pets. *Psoroptes cuniculi* is a common, globally distributed ectoparasite that can cause highly contagious ear mange in rabbits (1). *P. cuniculi* infections cause considerable weight loss, lower feed conversion rates, listlessness, anorexia, meningitis, altered immune functions and can be fatal if not treated (2, 3).

To date, diagnosis of *P. cuniculi* has been based on clinical signs, animal history and morphological identification through microscopic examination of ear scrapings. These diagnostic methods are not perfect, and animals with subclinical infestations may be misdiagnosed. Therefore, more sensitive diagnostic methods are required. The dot-enzyme-linked immunosorbent assay (dot-ELISA) is a simple, rapid and scalable process to detect antigens or antibodies while screening large numbers of samples at once (4–8). Serological diagnosis including traditional ELISA and dot-ELISA has been used to detect psoroptic sheep scab around the world (9, 10). However, this method has not yet been applied in the diagnosis of *P. cuniculi* infections in our country.

Troponin C is a protein that belongs to the troponin protein family, which consists of three separate proteins that each play an important role in regulating the interaction of actin and myosin filaments (11). Troponin C, the calcium-binding subunit of the myofibrillar thin filament, regulates excitation-contraction coupling in heart and skeletal muscles (12). In this study, we report the success of cloning and expressing the troponin C gene of *P. cuniculi*. To our knowledge, troponin C has not been exploited in the immunodiagnosis of *P. cuniculi* infection. Thus, we aimed to determine whether recombinant *P. cuniculi* troponin C may be an appropriate antigen to detect *P. cuniculi* infestation.

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Disclosures: None.

Received: 30 June 2013

Accepted for publication: 12 September 2013

MATERIALS AND METHODS

Mites and experimental animals

Psoroptes cuniculi were maintained on rabbits at the Laboratory Animal Center of Sichuan Agricultural University, China. Scrapings in the external auditory canal were collected by tweezers and warmed at 37°C in microlitre plates for 2 h. *P. cuniculi* that crawled out of the scrapings were harvested into centrifuge tubes using a soft-haired brush. Mites were preserved immediately at -80°C after harvesting. The mites included adults and nymphs of both gender and were unfed before the experiments. All rabbits (naïve female New Zealand rabbits of 1–2 kg body weight age between 2 to 3 months) were handled in strict accordance with animal protection laws of the Peoples Republic of China (A draft of an animal protection law in China was released on 18 September 2009). Serum samples of naïve, naturally and experimentally adult infected rabbits were collected. All procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals by the Animal Ethics Committee of Sichuan Agricultural University (Ya'an, China) (Approval No. 2011–028).

Gene cloning and analysis

Total RNA extraction from mites was carried out with an RNA isolation kit (Watson, Parsippany, NJ, USA), and the cDNA was synthesized using a cDNA synthesis kit (Fermentas, Shenzhen, Guangzhou, China) according to the manufacturers instructions. The troponin C gene of *P. cuniculi* was amplified with primers designed according to the published expressed sequence tag (EST) sequence of *Sarcoptes scabiei* [forward primer: 5'-CCAAAATGTCGGTCGAA-3'; reverse primer: 5'-TTAAGTCCCAGTCATCAGTCCC-3']. The PCR procedure was as follows: 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 53°C for 45 s, 72°C for 1 min. Samples were incubated for a further 10 min at 72°C then held at 4°C. The QIAquick Gel Extraction Kit (Qiagen Inc., Dusseldorf, Germany) was used to recover and purify the amplified fragments separated by agarose gel electrophoresis. Then, the PCR products were subcloned into a pMD 19-T vector (TaKaRa Bio CO. Ltd., Dalian, Liaoning, China) and sequenced by Invitrogen (Beijing, China). The plasmid was transferred into *Escherichia coli* strain DH5 α (Fermentas).

Expression and purification of recombinant troponin C

The cDNA was amplified using the primers with restriction sites [forward primer: 5'-CGGGATCC AAATGT-TACGAAAAGCTTTCG-3'; reverse primer: 5'-CGCTC

GAG TTAGTCACCAGTCATCATTCC-3']. The PCR product was subcloned into the *Bam*H I and *Xho* I restriction sites of the pET-32a(+) vector (Novagen, Irene, Centurion, South Africa) using T4 DNA ligase under standard conditions and transformed into *E. Coli* DH5 α cells. The construction was verified by sequencing the insert from both junctions and was inserted into *E. coli* BL21 (DE3; Novagen) for expression. The bacteria were transformed overnight and transferred into LB medium supplemented with ampicillin (50 μ g/mL). After shaking and incubating at 37°C, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Growth was stopped 4 h after induction. Troponin C was purified according to the methods described in Chen *et al.* (13).

Detection of recombinant troponin C

The protein samples were adjusted to the composition of the electrophoresis sample buffer, boiled for 5 min and subsequently detected by SDS-PAGE according to the methods described by Sambrook and Russell (14). The gels stained with Coomassie blue were transferred to a PVDF membrane (Millipore, Shanghai, China) for 1 h in the electrophoretic transfer cell (Bio-Rad, Shanghai, China). TBST (40 mM Tris-HCl, 0.5 M NaCl, 0.1% Tween-20, pH 7.4) containing 5% skimmed milk was used to block the blotting membrane at 4°C for 2 h. The membrane was incubated overnight at room temperature in rabbit antiserum (diluted 1 : 200 (v/v) with 1% skimmed milk-TBST). The membrane was washed with TBST three times and subsequently incubated for 2 h with 1 : 1000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (AMRESCO, Solon, OH, USA). Then, the diaminobenzidine (DAB) substrate (Sigma, St. Louis, MO, USA) was used for 3–5 min to visualize the recombinant troponin C.

Dot-ELISA diagnoses

PVDF membrane 0.22 μ m (Millipore) was cut into strips and soaked in PBS for 1 h then allowed to air dry for 2 h at 37°C. Strips annotated with P1, P2 and P3 were primed with dilutions of 4, 8 and 16 μ g purified antigen/mL, respectively. And those annotated with S, T, E and N were primed with samples from *S. scabiei*-, *Taenia pisiformis*-, *Eimeria stiedae*-infected and uninfected (negative) rabbits. Individual dilutions of the recombinant troponin C were applied directly onto PVDF membranes in different lines, which then dried for 2 h at 37°C. The membranes were blocked with PBS (0.1 M phosphate-buffered saline, pH 7.4) containing 0.3% Tween-20 and 5% skimmed milk for

30 min and washed three times with PBST (PBS with 0.3% Tween-20) and twice with PBS for 5 min each. Serum samples were applied at 1 : 100 dilutions and incubated with the test strips for 1 h at 37°C. Then, the strips were washed once with PBST and PBS for 5 min each. The membranes were incubated for 30 min together with HRP-conjugated goat anti-rabbit IgG (AMRESCO). After three additional washing steps of 5 min each, the membranes were stained for 2 min with DAB substrate (Sigma). Staining was stopped by washing in distilled water.

We tested 85 sera from infested rabbits, and a further 85 negative control sera were obtained from naïve (uninfected) rabbits. To test cross-reactivity, we also assayed serum samples obtained from 6 rabbits infested with *E. stiedae*, 6 with *T. pisiformis* and 12 with *S. scabiei*. PDFV membrane strips were read by two independent observers on the same day in a double-blind experiment. Results were read on the basis of colour variation in the PVDF membranes: brown dots indicated a positive result, while no dots indicated a negative result. After 2 days, the strips were re-read by both observers. Test sensitivity was calculated as the following: sensitivity = ELISA positive/true positive $\times$ 100%. Test specificity was calculated as the following: specificity = ELISA negative/true negative $\times$ 100% (7). Percentage agreement (PA) = same reading/total samples $\times$ 100%. Parallel tests were executed three times to exclude the possible interference of external accidental factors.

Statistical analysis

DNA sequences fragment and alignments were analysed by NCBI BLAST and DNAMAN. Prediction of the recombinant protein structure was performed with the online protein prediction tool, EXPASY (http://us.expasy.org/tools/pi_tool.html). The TMHMM server version 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) was used to predict the transmembrane domain. Inter- and intra-observer variability was assessed by calculating the κ value (a widely used measure of observer agreement which accounts for the effect of chance) (15). A FASTA alignment was run, using the Structural Database of Allergenic Proteins (SDAP) (<http://fermi.utmb.edu/SDAP/>), to predict whether the recombinant *P. cuniculi* troponin C had any cross-reactivity with published allergens.

RESULTS

Gene cloning and sequence analysis

A DNA fragment (Accession number: KC693034) of 462 nucleotides length was amplified from the total RNA of *P. cuniculi* through RT-PCR. The gene showed relatively

high degrees of homology to *Tyrophagus putrescentiae*, *Aphonopelma* sp. SH-2011, *Lycosa singoriensis* and *Apis mellifera* sequences (76.75%, 72.72%, 71.19% and 66.87% homology, respectively). Results showed that this sequence encoded a 153 amino acid protein of 17.6 kDa and theoretical pI of 4.18 without a signal peptide. Transmembrane prediction indicated it is an outer membrane protein. The protein showed 93.46%, 84.97% and 82.35% amino acid identity to *T. putrescentiae*, *Aphonopelma* and *L. singoriensis*, respectively (Figure 1).

Expression, purification and cross-reactivity of recombinant troponin C

Recombinant *P. cuniculi* troponin C was successfully expressed in a pET-32a vector. It was found in the inclusion body of *E. coli* BL21 (DE3) and estimated by SDS-PAGE to be approximately 38 kDa, with a 109 amino acid thioredoxin 1-fusion partner of 20 kDa (Figure 2). Results demonstrated that the expression peak occurred 4 h after the introduction of IPTG. A FASTA alignment predicted that *P. cuniculi* troponin C had cross-reactivity with 34 allergenic proteins (seven of them, ordered by the highest bit score, are listed in Table 1.). The most homologous protein in the SDAP to the present recombinant protein was Tyr p 24 (*Tyrophagus putrescentiae* troponin C). Western blot analysis of the recombinant troponin C showed that sera from infected and uninfected rabbits separated clearly into a positive lane and a naïve lane (Figure 3). Hence, SDS-PAGE and Western blot detection results both indicated that the expressed product was *P. cuniculi* troponin C.

Dot-ELISA diagnosis

Results showed that assay sensitivity was 94.1% (80/85) and 96.5% (82/85) for the two observers, while assay specificity was 98.8% (84/85) and 97.6% (83/85), respectively (Table 2). The quantity of antigen in the dots had little effect on the sensitivity of the assay (Figure 4). Agreement between the first readings of the two observers was 97.4%, with a corresponding κ value of 0.95 ($P < 0.01$; Table 2). In the second reading, results showed that assay sensitivity was 95.3% (81/85) and 96.5% (82/85) for the two observers, while assay specificity was 100% (85/85) and 98.8% (84/85), respectively (Table 3). Agreement between observers was 98.0%, with a corresponding κ value of 0.96 ($P < 0.01$; Table 3). The percentage agreement between the initial and second readings by each observer was 99.0% ($\kappa = 0.98$; $P < 0.01$) and 98.4% ($\kappa = 0.97$; $P < 0.01$), respectively. In the cross-reaction assay, only one of the six serum samples from rabbits infected with

P. <i>Cuniculi</i>	MSVEELTKEQVQMLRKAQDMFDREKKGHIHTNMVSTILRTLQGTFEKDLQQLITEIDAD
A. <i>Mellifera</i>	-----MFHQFLRKAFDSFDREKSGSIPTDMVADILRLMGQPFNKKILDELIEVDAD
A. <i>Pisum</i>	--MEDLPPEQISVLRKAQDAFDRERSGSIPTDMVADILRLMGQPFNKKILDELIEVDAD
Aphonopelma	-MVEELSKEQVEMLRKAQDMFDREKKGSIHTNMVSTILRTLQGTFESESELKELIIEIDQD
L. <i>Singoriensis</i>	-MVEELSKEQVEMLRKAQDMFDREKKGSIHTNMVSTILRTLQGTFESESELKELIIEIDQD
M. <i>Occidentalis</i>	--MDDLTKQVSMRLKAQDMFDRDKKGFHTNMVSAIILRTLQGTFEKDLKDLISEIDQD
T. <i>Putrescentiae</i>	MSVEELSKEQVQMLRKAQDMFDRDKKGYIHTNMVSTILRTLQGTFEENDLQQLIIEIDAD
	*.**** *... * * * * . * * * * *
P. <i>Cuniculi</i>	GSGELEFDEFLLTLARFLVEEDAETMQEELREAFRMYDKEGNGYIPTSLREILRASDDK
A. <i>Mellifera</i>	KSGRLEFEFVTLAAKFIVEEDAELKELREAFRLYDKEGNGYIPTCLREILRELDQD
A. <i>Pisum</i> .pro	KSGRLEFEFVTLAAKFIVEEDDEAMQELREAFRLYDKEGNGYIPTCLREILRELDQD
Aphonopelma	GSGELEFDEFLLTLARFLVEEDSEAMQEELREAFRMYDKEGNGYINVSRLREILRALDDK
L. <i>Singoriensis</i>	GSGELEFDEFLLTLARFLVEEDSEAMQEELREAFRMYDKEGNGYINVSRLREILRALDDK
M. <i>Occidentalis</i>	GSGELEFDEFVALAARFLVEEDSEAMQEELREAFRLYDKEGNGYINVSRLREILRALDDA
T. <i>Putrescentiae</i>	GSGELEFDEFLLTLARFLVEEDTEAMQEELREAFRMYDKEGNGYIPTSLREILRALDDK
	** *...*. *... *... *... *... *... *... *... *... *
P. <i>Cuniculi</i>	LTEDELDEMAEIDTDGSGTVDFDEFMGMGTGT
A. <i>Mellifera</i>	LTDEELDIMIEEIDSDGSGTVDFDEFMEMMTGE
A. <i>Pisum</i>	LTNEELDMIDEIDSDGSGTVDFDEFMEMMTGE
Aphonopelma	LTEDELDEMAEIDTDGSGTVDFDEFMEMMTGD
L. <i>Singoriensis</i>	LTEDELDEMAEIDTDGSGTVDFDEFMEMMTGD
M. <i>Occidentalis</i>	LTEDELDEMAEIDTDGSGTVDFDEFMEMMTGD
T. <i>Putrescentiae</i>	LTEDELDEMAEIDTDGSGTVDFDEFMEMMTGD
	** . *... *... *... *... *... *... *... *... *

Figure 1 Comparison of recombinant *Psoroptes cuniculi* troponin C with the amino acid sequences of troponin C or predicted troponin C-like of other species. Accession numbers were as follows: *Apis mellifera* troponin C type I: NP_001014430, *Acyrtosiphon pisum* troponin C-like: NP_001155712, *Aphonopelma* sp. SH-2011: ADY76981, *Lycosa singoriensis*: ABX75382, *Metaseiulus occidentalis* troponin C-like (predicted): XP_003743084, *Tyrophagus putrescentiae*: ACL36923.

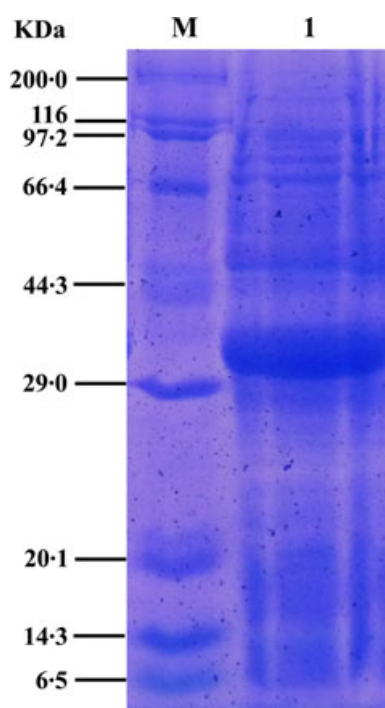


Figure 2 Coomassie-stained gel examined by SDS-PAGE analysis of the recombinant troponin C of *Psoroptes cuniculi*. Lane 1: purified recombinant proteins; lane M: protein marker.

E. stiedae was read as positive by both observers. In addition, just 1 serum sample from rabbits infected with *T. pisiformis* was read as positive by observer 1, while none were read as positive by observer 2. However, both observers identified a large number of positive samples among

the rabbits infected with *S. scabiei* leading to a positive percentage of 75% (9/12) and 83.3% (10/12). Therefore, specificity for *P. cuniculi* was extremely low.

DISCUSSION

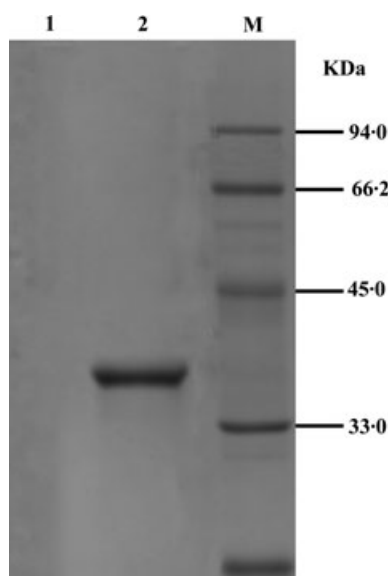
In the present study, we demonstrated the successful cloning and prokaryotic expression of recombinant *P. cuniculi* troponin C for use in a novel dot-ELISA assay to diagnose *P. cuniculi* infection in rabbits.

Standardizing the allergenic components of allergen extracts is difficult. Hence, purified recombinant allergen is becoming increasingly used for diagnosis as a substitute for whole mite extracts (16). Crude *Psoroptes* antigens have been used in serodiagnosis to detect *Psoroptes* infections (9, 17). In recent years, however, there has been a trend towards using recombinant protein antigens as the basis for ELISA assays as these were reported to be capable of detecting subclinically infested animals (10, 18). Moreover, conventional serological tests are too time-consuming and cumbersome for use in the field. Hence, the dot-ELISA was developed for screening for antibodies (6). In this study, we demonstrated very high sensitivity, but low specificity of our novel dot-ELISA assay. The high sensitivity was consistent with the results of previous studies by Nunn and Burgess (10, 18). This indicates that recombinant troponin C might have great potential as a diagnostic antigen for detecting *P. cuniculi* infections, even in subclinically infested animals, as long as the problem of cross-reactivity with *S. scabiei* can be solved, which will be addressed in our future research.

Table 1 Predicted cross-reactivity between recombinant *Psoroptes cuniculi* troponin C and known allergens in Structural Database of Allergenic Proteins (SDAP)

No	Allergen	GeneBank accession number	Sequence length	Bit score	E score
1	Tyr p 24.0101	ACL36923	153	178.5	5.7e-47
2	Bla g 6.0301	ABB89298	154	137.0	1.8e-34
3	Per a 6.0101	Q1M0Y3	151	135.8	3.9e-34
4	Bla g 6.0101	ABB89296	151	135.8	3.9e-34
5	Bla g 6.0201	ABB89297	151	131.6	7.4e-33
6	Hom a 6.0101	P29291	150	115.8	4.1e-28
7	Pen m 6.0101	ADV17344	150	108.8	5.4e-26

The bit score is equivalent to the bit score reported by BLAST. A 1 bit increase in score corresponds to a twofold reduction in expectation, and a 10-bit increase implies 1000-fold lower expectation. Sequences with *E* values <0.01 are almost always homologous.

**Figure 3** Western blot analysis of recombinant troponin C of *Psoroptes cuniculi*. Lane 1: negative control; lane 2: purified recombinant proteins; M: protein marker.

The results of our cross-reactivity assays indicated that the novel assay easily distinguished *P. cuniculi* infection from *E. stiedae* and *T. pisiformis* infection, but could not distinguish between a psoroptic and a sarcoptic infection. This could be explained by the fact that troponin C protein sequences of the two mite species could be very similar and share high homology, which is a reasonable expectation. Indeed, we amplified the *P. cuniculi* troponin C gene using primers that were developed according to the published EST sequence of *S. scabiei*, which shared 93% identity with the *P. cuniculi* troponin C gene. Therefore, the amino acid sequences, and even the advanced structures of the troponin C proteins of the two kinds of mite, are likely to show remarkable similarity.

During the evaluation of new technologies and assays, it is important to assess the effect of interpretation of the assay results by different observers. In the present study, inter- and intra-observer variability was remarkably small (κ -values ranged from 0.81 to 1.0, indicating very high agreement between observers). Thus, diagnostic judgments of the two observers were highly consistent, and the results appeared to be very reliable.

We found that the putative protein showed high degrees of homology to troponin C previously isolated from *T. putrescentiae*, *Aphonopelma* and *L. singoriensis* (19, 20). Troponin C from *T. putrescentiae* was demonstrated to be an allergen with calcium-dependent IgE reactivity (21). The results of this study showed that the present recombinant protein may also be a new *P. cuniculi* allergen.

To conclude, our results showed that the use of recombinant troponin C in a dot-ELISA lacks diagnostic utility because of low specificity even though the sensitivity was extremely high. Therefore, alternative antigens need to be explored in the future. We believe that the potential of dot-ELISA in *P. cuniculi* serodiagnosis is still considerable – especially for diagnosis of chronic or subclinical infections – as long as the problem of

Table 2 Results of the initial set of readings by observers

Serums	Observer 1 number			Observer 2 number			PP (%)	PA (%)	κ value
	+	–	PP (%)	+	–	PP (%)			
Positive (<i>n</i> = 85)	80	5	94.1	82	3	96.5	96.5	0.74	
Negative (<i>n</i> = 85)	1	84	1.2	2	83	2.4	98.8	0.66	
<i>E. stiedae</i> (<i>n</i> = 6)	1	5	16.7	1	5	16.7	100	1.0	
<i>T. pisiformis</i> (<i>n</i> = 6)	1	5	16.7	0	6	0	83.3	0	
<i>S. scabiei</i> (<i>n</i> = 12)	9	3	75	10	2	83.3	91.6	0.75	
Total (<i>n</i> = 194)	92	102	47.4	95	99	49.0	97.4	0.95	

PP, percentage positive; PA, percentage agreement.

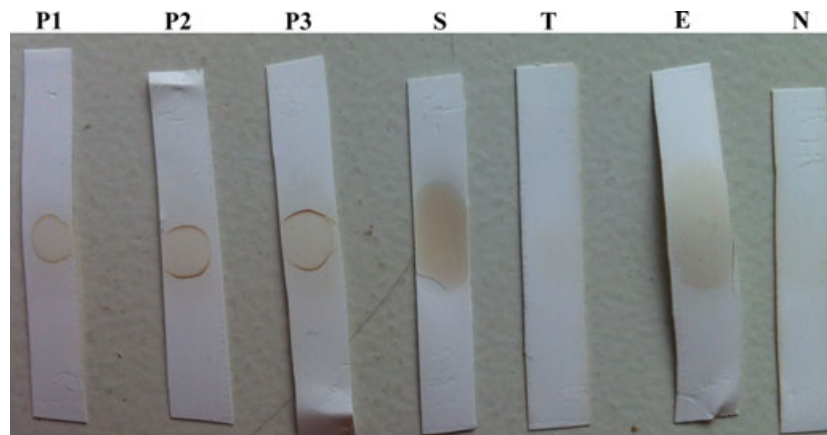


Figure 4 Photographs of Dot-ELISA results for sera. Strips annotated with P1, P2 and P3 were primed with dilutions of 4, 8 and 16 µg purified antigen/mL, respectively. P1–P3: positive; S: *S. scabiei*; T: *T. pisiformis*; E: *E. stiedae*; N: Negative.

Table 3 Results of the second set of readings by observers

Serums	Observer 1 number		PP (%)	Observer 2 number		PP (%)	PA (%)	κ value
	+	–		+	–			
Positive (<i>n</i> = 85)	81	4	95.3	82	3	96.5	98.8	0.85
Negative (<i>n</i> = 85)	0	85	0	1	84	1.2	98.8	0
<i>E. stiedae</i> (<i>n</i> = 6)	1	5	16.7	1	5	16.7	100	1.0
<i>T. pisiformis</i> (<i>n</i> = 6)	1	5	16.7	0	6	0	83.3	0
<i>S. scabiei</i> (<i>n</i> = 12)	7	5	58.3	8	4	66.7	91.6	0.82
Total (<i>n</i> = 194)	90	104	46.4	92	102	47.4	98.0	0.96

PP, percentage positive; PA, percentage agreement.

distinguishing *P. cuniculi* from *S. scabiei* infection can be solved. Dot-ELISA is a promising way forward for diagnostic testing in the field and in situations where technical expertise and expensive instruments are restricted or absent.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Programme for Changjiang Scholars and Innovative Research Team in University (PCSIRT) (No. IRT0848).

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