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Proteomic and Immunological Identification of Antigens from Ticks with Artificial Antiserum

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Authors' Contribution
JL and KZ designed the study. QC, YW and XW collected the samples. SL, LL, BL, KM and AL performed the experimental work.KZ analysed the data and wrote the manuscript.

Key words
2-DE-WB, Antigen, Artificial antiserum, Tick

ABSTRACT

We aimed to screen novel tick antigens using two-dimensional gel electrophoresis (2-DE) followed by western blot analysis (WB) with artificial antiserum. A total of 105 *Haemaphysalis longicornis* (*H. longicornis*) ticks were selected to prepare protein samples. Some of them were used for 2-DE, and the others were used to immunize mice to collect tick antiserum for WB. Then, 2-DE followed by WB and MALDI-TOF was carried out to screen and identify tick antigens. We identified 19 protein spots representing 12 ORFs: 4 from ticks and 8 from cattle. Among the the 4 ORFs, Tropomyosin, Elongation factor 1-alpha (EF-1α) and Protein Disulfide Isomerase-2 (PDI-2) have been reported as antigens and even vaccine candidate proteins in ticks or other pathogens but not in *H. longicornis*. Few reports have described dual specificity phosphatase 3 (DUSP-3) in parasites as an antigen. We successfully screened four potential antigens and found that DUSP-3 is a potential novel antigen for ticks.

INTRODUCTION

Ticks are blood-feeding ectoparasites with a worldwide distribution and transmit bacterial, parasitic and viral pathogens that cause diseases in humans and animals. Tick vaccines offer the advantages of being cost-effective and environmentally friendly alternatives to other tick control measures, with the dual effects of reducing tick infestation and preventing ticks from transmitting pathogens (Contreras *et al*., 2016). The success of tick vaccines depends on the detection, identification, and functional investigation of antigenic proteins that target specific proteins that play pivotal roles in tick life processes, such as blood feeding and digestion processes (Kröber *et al*., 2007). 2-DE followed by WB and MALDI-TOF is an effective technique for antigenic protein separation, detection and identification, and antiserum plays a key role in the binding of antigenic proteins.

Usually, to generate antiserum, live ticks are allowed to bite the host, and the antiserum is collected for detection of tick antigenic proteins. During blood sucking, saliva is secreted into the host by the ticks. The saliva interacts with the immune system of the host, which generates specific antibodies targeting tick saliva. In WB, the antigens are screened with specific antibodies that bind the antigens. This approach is limited because antigens from saliva are limited in number; thus, so is the antiserum from the host bitten by the ticks. However, candidate vaccine antigens can be obtained from sources other than saliva; for example, Bm86 is a tick gut glycoprotein (Rand *et al*., 1989). Thus, if the antigens used to immunize the host are from the whole tick body, the antiserum from the host will represent a more comprehensive range of proteins than those from the saliva.

In this study, we extracted proteins from whole ticks, mixed them with adjuvant, and immunized mice to prepare antisera. We then screened tick antigenic proteins using 2-DE-WB and MALDI-TOF.

MATERIALS AND METHODS

Tick collection

Ticks were collected from two local cattle grazing on a mountain near the city of Pingdingshan. The ticks were identified to the species level by morphology (Murrell *et al*., 2001). The ticks were frozen in liquid nitrogen and immediately stored at –80°C until use.

A total of 105 ticks were collected from two cattle

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and contained a mix of fed, unfed, and partially fed adults and nymphs. All ticks were identified as *H. longicornis*. Protein samples from whole tick bodies were extracted, some for 2-DE and 2-DE-WB and the rest for immunizing mice.

Protein sample preparation

All the ticks we collected were washed with precooled PBS containing penicillin/streptomycin. Then, the ticks were placed in a mortar with liquid nitrogen for grinding. The ticks were ground into powder without obvious particles while liquid nitrogen was added continuously to the mortar. Then, the powder was dissolved in 800 µl of ALK lysis buffer (containing 2 M thiourea, 2% CHAPS, 7 M urea, 10 mM DTT, 1 mM PMSF, and 20 mM Tris) and sonicated (ultrasonic treatment 5 s, interval 10 s, power 120 w, 99 cycles, performed in ice water until clear and bright). Then, the lysates were centrifuged at 15,000 rpm for 30 minutes at 4°C. Then, 250 µl of the supernatant and 1 ml of precooled acetone (4°C) were mixed and stored at –20°C for one night to precipitate proteins. Finally, the precipitated proteins were centrifuged at 20,000 rpm for 30 minutes at 4°C, collected, and placed on clean filter paper for natural acetone volatilization. Then, the proteins were dissolved in ALK lysis buffer and quantitated using a BCA Protein Assay Kit (Thermo Fisher Scientific Pierce). Finally, the protein samples were stored at –80°C until 2-DE and antiserum preparation.

Generation of tick antisera in mice

Freund's adjuvant is mineral oil that is emulsified together with a solution of an antigen to form a water-in-oil emulsion. The water-in-oil emulsion continuously releases the antigen and stimulates the immune response, resulting in enhanced cellular and humoral antibody responses. Complete Freund's Adjuvant (CFA) contains whole or pulverized heat-killed mycobacteria, while Incomplete Freund's Adjuvant (IFA) does not. Although the use of CFA can result in severe side effects, and thus alternatives to CFA should be used whenever possible, no comparable alternatives are known to exist (Jackson and Fox, 1995). To generate high-titer tick antisera in mice, proteins from whole ticks mixed with CFA for the initial immunization and IFA for a subsequent immunization and booster immunization were used to immunize mice by subcutaneous injection at multiple sites. Seven-week-old female BALB/c mice (SPF) were purchased from Sibeifu Animals Biotechnology Co., Ltd. (Beijing, China). The mice were bred in the Experimental Animal Room at the Chinese Centre for Disease Control and Prevention, Beijing. The mouse feeding was performed in individual ventilated cages (IVCs); the conditions were a temperature of 20-23°C, humidity of 40-70%, illumination of 15-20 lx, air change rate of ≥15 T/h, and light/dark cycle of 10/14 h. The mice were free to drink and eat. The drinking water was prepared by UV irradiation after reverse osmosis filtration. Irradiation-sterilized Co60 mouse feed was purchased from Beijing Keaoxieli Feed Co., Ltd.

After five days of adaptive feeding, when the mice were 8 weeks old and approximately 22 g in weight, the initial immunization began. There was a two-week interval between the initial and the subsequent immunization and between the subsequent immunization and the booster immunization. Equal amounts of adjuvant were mixed with whole tick protein samples, and the mixtures were formulated immediately before application. Subcutaneous injection was performed at five sites. For initial immunization of each mouse, 100 µg of whole tick proteins and 100 µL of CFA were mixed, and the CFA dose per site was 20 µL. Our protocol was in conformity with the Guidelines for the Use of Adjuvants in Research. The detailed parameters of the immunization program are shown in Table I. The blank controls were immunized using an equal amount of sterilized physiological saline. Four days after the last subsequent immunization, blood was collected and the serum precipitated naturally at room temperature, and the IgG concentration was tested with an IgG ELISA kit (Biolab, Beijing China, cat. no. 23060077).

Two-dimensional gel electrophoresis (2-DE)

After the whole tick proteins were prepared, the proteins were separated by 2-DE first, and the methods were similar to those described previously (Zhou *et al*., 2013). Briefly, each whole tick protein sample (1500 µg) was mixed with rehydration buffer (containing 2 M thiourea, 7 M urea, 2 µL of IPG buffer, 10 µL of 1 M DTT and 4% CHAPS). The volume of the mixture was 450 µL. Then, the mixture was loaded onto an IPG strip (Immobiline 17 cm, pH 4-7, from Bio-Rad, USA) without introducing air bubbles, and isoelectric point focusing was carried out by an Ettan IPGphor 3 apparatus (GE Healthcare, Sweden). The total VH was approximately 110,000. Then, the strip was equilibrated for reduction of the proteins in 8 mL of SDS equilibration buffer (with 50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 160 µL of 1 M DTT and 0.002% bromophenol blue), and then the proteins were separated by SDS-PAGE (13%). 2-DE was performed in duplicate three times. Three of the gels were dyed with Coomassie brilliant blue R-250 for spot excision, whereas the other gels were used for WB.

Two-dimensional gel electrophoresis immunoblotting (2-DE-WB)

After the tick antisera were prepared in mice, the

other gels were used for WB. The methods were similar to those described previously (Zhao *et al*., 2015). In brief, the proteins in the gel were transferred to nitrocellulose membranes. Then, the membrane was blocked in TBST (TBS buffer containing 1% Tween-20 and 5% skimmed milk). Then, for antigen-antibody (primary antibody) binding, the membrane was incubated with tick antiserum diluted 1:250 (v/v) at 4°C overnight. Then, the membrane was washed with TBST buffer. For antibody-anti-antibody binding, the membrane was incubated with secondary antibody (biotinylated goat anti-mouse IgG antibody) for two hours. The membrane was washed with TBST. Finally, an ECL Western Blot kit (Beijing CoWin Biotech, Beijing, China) was used to detect antigens on the membrane. The positive spots on the 2-DE gels were then excised and subjected to MALDI-TOF analysis according to the results of WB.

Identification of antigenic proteins (MALDI-TOF/MS)

The protein spots corresponding to mouse antisera identified were excised manually from the 2-DE gels. The methods of MALDI-TOF were similar to those described previously (Pazouki *et al*., 2009). In brief, the protein spots we excised were cleaned with Milli-Q water twice and decolorized with 50% methanol until they were colorless. After dehydration, trypsin digestion, and peptide extraction, the peptides were dissolved in resolving solution. Then, the samples were repeatedly aspirated and spotted, dried at room temperature, and analyzed by MALDI-TOF-MS on a mass spectrometer (Bruker Dalton, Germany). FlexAnalysis (Bruker Dalton) software was used to filter the signal baseline peak and distinguish the signal peak. BioTools (Bruker Dalton) software was used to search for peptides and proteins in the Universal Protein databases.

RESULTS

Mouse tick antisera

During the whole process of immunization, the mice were in good condition. The injection sites of mice were without skin ulceration, local abscesses or tissue sloughing, and the mice were without (obvious) pain and distress. After three immunizations, mouse blood was collected. The serum was precipitated naturally at room temperature, and the concentration of IgG antibody was tested. The IgG concentration was, on average, 893.75 μg/ml for the mice immunized with tick whole-body proteins, which was far higher than the average of 226 μg/ml for those immunized with equal amounts of sterilized physiological saline.

Comparative proteomic analysis by 2-DE and 2-DE-WB

Protein spots were distributed throughout the 2-DE gel (Fig. 1a), with a concentration of spots at PI 7 and a smeared band at 70 kDa. 2-DE and WB revealed many protein spots clustered at >40 kDa in the gel. Ultimately, nineteen spots were screened after 2-DE, WB and 2-DE matching (Fig. 1a, 1b).

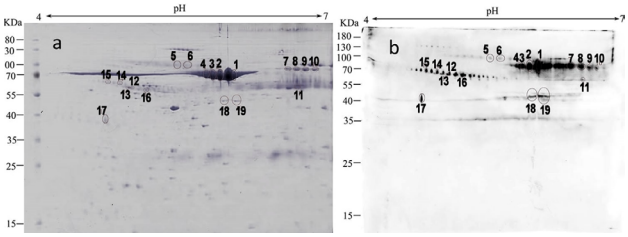

Fig. 1. Identification of antigenic proteins from the whole body of the tick *H. longicornis* (Acari: Ixodidae) (a) 2-DE gel stained for whole tick proteins; (b) Spots selected for MALDI-TOF/MS analysis. Note: The circles in the figure show the antigenic proteins sent for MALDI-TOF analysis.

Identification of antigenic proteins from H. longicornis

We found nineteen protein spots that were subjected to MALDI-TOF analysis. A protein with several matched peptides (score >50) the established peptide mass fingerprint database was considered significant (Ma *et al*., 2009). In the peptide mass fingerprint analysis, the mass results of protein spots 1, 12, 13, 17-19 showed the highest correlations with proteins from Ixodoidea, with peptide sequence coverages of 15-64% and sequence scores of 72-896. In addition, protein spots 1-12 and 14-19 showed the highest correlations with proteins from the host cattle, with peptide sequence coverages of 3-76% and sequence scores of 64-4161 (Table II).

DISCUSSION

Currently, vector control by immunological approaches is the most important strategy for the prevention and control of major infectious diseases throughout the world. However, the success of vaccines depends on the specific proteins we screen, specifically whether these proteins play key roles in pathogenic processes or not (Kröber *et al*., 2007). 2-DE followed by WB and MALDI-TOF was an effective technique for antigenic protein separation, detection and identification. In this technique, antigens were bound by specific antibodies from antisera and identified. To identify antigens other than those from only tick saliva, we generated antisera by using whole tick body proteins to immunize mice. Ultimately, we selected 19 protein spots and identified 12 antigenic proteins: four antigenic proteins from ticks and eight from cattle blood.

Table I. Immunization program.

Immunization program	Interval	Dose (μg/mouse)	Adjuvant	Route
Initial immunization	5 days after adaptive feeding	100	CFA	Back, subcutaneous
Subsequent immunization	2 weeks after initial immunization	100	IFA	Back, subcutaneous
Booster immunization	2 weeks after subsequent immunization	80	IFA	Back, subcutaneous
Blood collection	Four days after booster immunization	-----	-----	-----

CFA means Complete Freund's Adjuvant, and IFA means Incomplete Freund's Adjuvant. Dose means the whole tick protein dose in units of μg/mouse.

Table II. Summary of antigenic proteins identified in the whole bodies of *H. longicornis* by MALDI-TOF.

	Spot no.	Protein ID (GenBank accession no.)	Description	Protein score	Sequence coverage (%)	No. of peptides P<0.05	Calculated MW/PI
Tick	1	JAP76203.1	Dual-specificity phosphatase 3	72	42%	10	29732/5.98
	12	BAF63671.1	Protein disulfide isomerase-2	127	15%	6	57270/4.94
	13	BAF63671.1	Protein disulfide isomerase-2	284	48%	24	57270/4.91
	17	AAN05633.1	Tropomyosin	632	64%	25	32853/4.69
	18	EEC13271.1	Elongation factor 1-alpha	896	17%	5	51048/9.18
	19	JAA71972.1	Elongation factor 1-alpha	525	16%	3	51048/9.18
Bovine	1	ALBU_BOVIN	Serum albumin	593	48%	24	71244/5.98
	2	ALBU_BOVIN	Serum albumin	563	42%	25	71244/5.84
	3	ALBU_BOVIN	Serum albumin	599	45%	25	71244/5.76
	4	ALBU_BOVIN	Serum albumin	510	28%	15	71244/5.69
	5	G5E513_BOVIN	Uncharacterized protein	174	17%	7	50623/5.40
	6	G5E5T5_BOVIN	Uncharacterized protein	142	21%	10	43013/5.50
	7	TRFE_BOVIN	Serotransferrin	526	40%	26	79870/6.55
	8	TRFE_BOVIN	Serotransferrin	639	42%	24	79870/6.64
	9	TRFE_BOVIN	Serotransferrin	609	36%	24	79870/6.73
	10	G3X6N3_BOVIN	Serotransferrin	588	37%	21	79783/6.83
	11	ALBU_BOVIN	Serum albumin	64	5%	5	71244/6.65
	12	ITIH4_BOVIN	Inter-alpha-trypsin inhibitor heavy chain H4	285	6%	6	101620/4.94
	14	SPA31_BOVIN	Serpin A3-1	182	22%	9	46322/4.82
	15	SPA32_BOVIN	Serpin A3-2	157	12%	5	46322/4.71
	16	ITIH4_BOVIN	Inter-alpha-trypsin inhibitor heavy chain H4	514	10%	9	101620/5.08
	17	E1BKT9_BOVIN	Uncharacterized protein	66	3%	12	334572/4.69
	18	ALBU_BOVIN	Serum albumin	4161	76%	45	71244/5.82
	19	ALBU_BOVIN	Serum albumin	3893	72%	39	71244/5.82

The tropomyosin protein is a salivary protein that has actin regulator activity and plays an important role in allergic reactions against parasites (Ranjbar *et al*., 2014). This protein has previously been reported to be an antigenic protein in ectoparasites such as *Sarcoptes scabiei* and the ticks *Hyalomma anatolicum* and *Boophilus annulatus* (Nabian *et al*., 2013; Manjunathachar *et al*., 2017; Morgan *et al*., 2016). This is the first time that tropomyosin has

been identified as an antigenic protein from *H. longicornis*.

Protein disulfide isomerases (PDIs) and their homologs play essential roles in the oxidative folding and chaperone-mediated control of proteins in the secretory pathway (Benham, 2012). PDIs from *H. longicornis* expressed primarily in the salivary gland are involved in blood feeding, viability and oocyte development (Liao *et al*., 2008). Previous studies have shown that PDI is a candidate antigen for vaccines in endoparasites such as *Toxoplasma gondii*, *Leishmania donovani*, *Besnoitia besnoiti*, and even *Amblyomma americanum* ticks (Radulović *et al*., 2014; Ma *et al*., 2009; Wang *et al*., 2013; Marcelino *et al*., 2011). This is the first time that PDI-2 has been identified as an antigenic protein in *H. longicornis*.

Elongation factor 1-alpha (EF-1α), a GTP-binding protein, is an essential component of the eukaryotic translational apparatus that catalyzes the binding of aminoacyl-transfer RNAs to ribosomes (Tatsuka *et al*., 1992). It has been found that this protein is a protective antigen or vaccine candidate antigen of several endoparasites, such as *Echinococcus granulosus*, *Cryptosporidium parvum*, and *Toxoplasma gondii* (Margutti *et al*., 1999; Matsubayashi *et al*., 2013; Wang *et al*., 2017, Wang *et al*., 2015), but not ectoparasites. In this study, EF-lα was identified for the first time as an antigenic protein in *H. longicornis*.

Dual specificity phosphatases (DUSPs) form a subclass of protein tyrosine phosphatases that can hydrolyze the phosphate ester bond on both a tyrosine and a threonine or serine residue on the same protein (Lyon *et al*., 2002). DUSPs dephosphorylate threonine and tyrosine residues on mitogen-activated protein kinases (MAPKs) and hence are referred to as MAPK phosphatases (MKPs) (Jeffrey *et al*., 2007). DUSPs negatively regulate the musculature and development of the whole body in *Caenorhabditis elegans* (Hu, 2006). Few have been reported to be antigens. In this study, DUSP-3 was identified as an antigenic protein in *H. longicornis* for the first time.

There are some areas in our research that need further improvement. For example, IPG strips of pH 4-7 were selected in 2-DE, which caused the proteins out of the pH range of 4-7 to be missed. In addition, proteins with low abundance were likely lost during protein sample preparation. Even if these missed proteins in 2-DE are able to induce the host immune response and generate antibodies, they would not have been detected in WB. However, the antigenic proteins we identified were all in the pH range of 4-7 and had relatively high abundance. In future research, expanding the range of the IPG strip pH and reducing the loss of proteins with low abundance will improve the identification of valuable antigenic proteins.

In addition, some proteins from the host were identified, which is not what we intended. In this research, the fed and partially fed ticks contained considerable amounts of undigested blood from the host cattle. In the procedure of antiserum generation, the protein samples made from ticks contained undigested blood. These proteins from undigested blood interacted with the mouse immune system and prompted specific antibody generation. Using 2-DE-WB-MALDI-TOF, antigenic proteins from the host cattle blood were identified, including three inhibitors (inter-alpha-trypsin inhibitor heavy chain H4α, Serpin A3-1 and Serpin A3-2), two serum proteins (serum albumin and Serotransferrin), and three proteins with unknown functions (E1BKT9_BOVIN, G5E5T5_BOVIN and G5E513_BOVIN).

CONCLUSIONS

2-DE followed by WB and MALDI-TOF is a valuable technique for antigen separation and identification. In this study, we screened tick antigens by using antisera from mice immunized with whole tick body proteins, antisera not from animals bitten by ticks. Three antigens we screened have been reported in other tick species or pathogens. Only the screened antigen DUSP-3 has not previously been reported as an antigen. Our results suggest that antisera from animals immunized by proteins extracted from whole ticks are feasible for screening antigens using 2-DE followed by WB and MALDI-TOF.

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Ethics approval and consent to participate

All procedures involving animals in our research were approved and overseen by the Chinese Center for Disease Control and Prevention Institutional Animal Care and Use Committee (No. EAWE-2017-012).

Statement of conflict of interest

The authors have declared no conflict of interest.

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