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# Comparative proteomic and bioinformatic analysis of *Theileria luwenshuni* and *Theileria uilenbergi*

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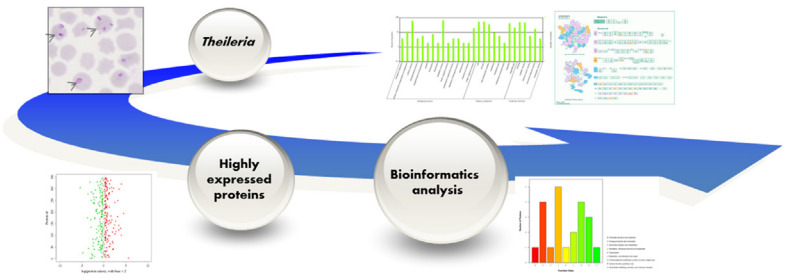
## HIGHLIGHTS

- Identified 72 differentially expressed proteins between two ovine *Theileria*.
- All proteins were classified into categories by functions and relationship.
- Analyzed several differentially expressed enzymes related to virulence and invasion.

## GRAPHICAL ABSTRACT

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## ABSTRACT

*Theileria* is an obligatory intraerythrocytic protozoan parasite that causes economic losses to the cattle, sheep and goats industry. However, very little information is available on the genomes, transcriptomes, and proteomes of the ovine parasites, *Theileria luwenshuni* and *Theileria uilenbergi*. Differences in protein expression between these species were investigated to better understand their biology. Parasites were digested with trypsin, and the resulting peptides labeled with isobaric tags for relative and absolute quantification, followed by LC-MS/MS. More than 670 proteins, classified into categories primarily related to cellular process (29.78%), metabolic process (28.80%), localization (5.22%) and biological regulation (5.00%), were identified. Seventy-one proteins were differentially expressed; *T. luwenshuni* had 39 proteins more highly expressed than in *T. uilenbergi*, whereas *T. uilenbergi* had 32 that were more highly expressed. Several proteins related to parasite virulence and invasion (cysteine proteinase, histone deacetylase, pyruvate kinase, small nuclear ribonucleoprotein and orotate phosphoribosyltransferase) were differentially expressed. Real-time quantitative PCR validated protein expression changes at the transcript level. This is the first report on protein expression for the two most economically important *Theileria* species in China, and our findings may provide novel opportunities for ovine and caprine theileriosis control.



## 1. Introduction

The infection of sheep and goats by the tick-borne parasites of the genus *Theileria* can cause enormous economic losses in

*Abbreviations:* itraq, isobaric tags for relative and absolute quantification; SCX, strong cation exchange; IAM, iodoacetamide.

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disease-affected regions (Mehlhorn and Schein, 1985). These parasites have a common life cycle in which sporozoites invade the mononuclear cells of the host, where they become trophozoites and multinucleate schizonts via asexual reproduction. This process stimulates host cell proliferation, allowing further multiplication of the parasite. The local lymph nodes are first infected. Schizonts are then disseminated throughout the lymphoid tissues, before they differentiate into merozoites. The merozoites enter the erythrocytes and form piroplasms, which are infective to ticks and capable of sexual reproduction. Sexual reproduction occurs within the nymph and larval stages of the tick, and the final infective stage is present within the tick's salivary glands. It is then transmitted to the mammalian host when its blood is sucked by the tick. Our study focuses on the merozoite stage of the parasite, when it is present in the red blood cells.

Theileriosis was first reported in sheep and goats in Sichuan Province, China, by Yang et al., in 1958. Since then, this parasitic infection has been reported in several Chinese provinces, including Qinghai, Gansu, Liaoning, Inner Mongolia, Shanxi, and Ningxia. Recently, *Theileria* spp. were also reported in Xinjiang (Li et al., 2009) and Hubei Provinces (Ge et al., 2011), so the damage caused by this organism occurs throughout the country.

*Theileria* is a single-celled eukaryote, so proteomic studies are relatively straightforward. The genomes of two bovine *Theileria* species, *Theileria annulata* and *Theileria parva*, have been sequenced and transcriptomic and proteomic data for these species are accumulating rapidly, which facilitates the proteomic identification and characterization of proteins using existing databases (Gardner et al., 2005; Pain et al., 2005). In contrast, little information is available on the ovine and caprine *Theileria* species.

Among the ovine *Theileria* parasites, *Theileria lestoquardi*, *Theileria luwenshuni*, and *Theileria uilenbergi* are considered highly pathogenic (Schnittger et al., 2003; Yin et al., 2007), whereas other species, such as *Theileria ovis*, *Theileria recondita*, and *Theileria separata*, are considered benign, causing only subclinical infections in small ruminants. Only *T. uilenbergi*, *T. luwenshuni*, and *T. ovis* have been reported in China (Li et al., 2010, Li et al., 2009). The various *Theileria* species differ in their biological characteristics, pathogenicity, and vectors. Therefore, studying the protein expression of different *Theileria* species may reveal important aspects of ovine theileriosis, which could contribute to disease prevention, treatment, and control.

Recent advances in instrumentation, reagents, and techniques for high-throughput sequencing are making it possible to simultaneously identify and compare different parasite species, life stages, and treatment-related changes at the level of protein expression (Aebersold and Mann, 2003). These techniques rely mainly on two dimensional gel electrophoresis or liquid chromatography (LC) to separate proteins or peptides by charge, mass, or other chemical properties, followed by identification using mass spectrometry (MS) (Schwacke et al., 2009). Though two dimensional echocardiography (2DE) is a powerful tool for high-resolution detection of intact and modified proteins, protein detection by 2DE is biased towards hydrophilic proteins (Wittmann-Liebold et al., 2006) of high abundance (Rabilloud et al., 2010). Recently, isobaric tags for relative and absolute quantitation (iTRAQ) has been developed as a technique for quantitative proteomics (Ross et al., 2004).

In the present study, iTRAQ was used to determine changes in protein expression between two *Theileria* species, *T. luwenshuni* and *T. uilenbergi*. The aims of the study were to identify differentially expressed proteins between the two species, compare the protein expression patterns obtained, and identify proteins involved in parasite virulence and invasion.

## 2. Materials and methods

### 2.1. Sheep infections

All the experiments involving *Theileria* challenge in sheep were approved by the animal ethics committee of Lanzhou Veterinary Research Institute (No. LVRIAEC2013-00) and followed both the national guidelines for the use of animals in scientific research and the standard protocol described by the OIE. Six *Theileria* strains were used in this study. Three strains were *T. uilenbergi* and the other three were *T. luwenshuni*. Specific-pathogen-free sheep were purchased from Jingtai, which is an area of China free *Theileria* and other protozoa. Smears of ear vein blood, PCR (Yin et al., 2008) and ELISA (Liu et al., 2014) were examined prior to setting up the experimental infections. Only *Theileria*-negative sheep were used. Parasite stocks were maintained in liquid nitrogen and were grown by inoculating these stocks into experimental sheep under laboratory conditions. To examine *Theileria* infections in sheep, microscopic examination and PCR were performed every 2 days starting 2 weeks after inoculation, until the parasitemia reached high levels in the sheep. *Theileria* species were detected using species-specific primer sets for *T. luwenshuni* and *T. uilenbergi* (Yin et al., 2008).

### 2.2. Parasite purification and protein extraction

The merozoites of six strains representing the two *Theileria* species were purified. Parasites were harvested when the parasitemia was highest. Erythrocytes that contained parasites were lysed by glycerol-enhanced osmotic shock, as described by Figueroa et al. (1990) (Figueroa et al., 1990). Merozoites were purified from lysates by differential centrifugation, and poorly lysed erythrocytes and white blood cells were removed by centrifugation at 700 × g for 10 min. Supernatants were collected into 50 mL tubes and the free merozoites were harvested by centrifugation at 3500 × g for 15 min.

Parasites were suspended in lysis buffer (7 M Urea, 2 M thiourea, 4% CHAPS, 40 mM Tris–HCl, pH 8.5, 1 mM PMSF, 2 mM EDTA) and sonicated on ice. Proteins were reduced with 10 mM DTT (final concentration) at 56 °C for 1 h and then alkylated with 55 mM IAM (final concentration) for 1 h in the dark. The reduced and alkylated protein mixtures were precipitated by adding a 4 × volume of chilled acetone at −20 °C overnight. After 30,000 × g centrifugation for 30 min at 4 °C, the pellets were dissolved in 0.5 M TEAB (Applied Biosystems, Milan, Italy) and sonicated on ice. After centrifuging at 30,000 × g for 30 min at 4 °C, an aliquot of the supernatant was taken for protein concentration determination using a Thermo Scientific Nanodrop 2000 spectrophotometer (Thermo Fisher, Waltham, USA). Supernatant proteins were kept at −80 °C until further analysis.

### 2.3. iTRAQ sample preparation

A critical step for iTRAQ success is trypsin digestion. Hence, use of a trypsin of mass spectrometric-grade quality is necessary. Total protein (100 μg) was removed from each sample and digested with Trypsin Gold (Promega, Madison, WI, USA) at a ratio of protein to trypsin (30: 1) at 37 °C for 16 h. After digestion, peptides were dried by vacuum centrifugation, reconstituted in 0.5 M TEAB (Applied Biosystems) and then labeled six samples according to the manufacturer's protocol for the 8-plex iTRAQ reagent (Applied Biosystems). Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24 μL of isopropanol. The protein digests obtained from the six strains, namely, *T. uilenbergi* Lintan strain, *T. uilenbergi* Longde strain, and *T. uilenbergi* Yongjing strain, and *T. luwenshuni* Lintan strain, *T. luwenshuni* Weiyuan strain, and *T. luwenshuni*