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Research Article

The Characteristic Analysis of Ribosomal Protein L12 in *Haemaphysalis longicornis* (Acari: Ixodidae) Ticks

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Abstract: Ribosomal protein L12 (RpL12) plays an important role in ovarian development and engorgement in vertebrates and invertebrates. However, the functional characteristics of RpL12 in ticks are not clear. Here, an open reading frame of the RpL12 gene was cloned from cDNA of *Haemaphysalis longicornis.* The sequence was analysed, and expression levels were determined in different tissues and developmental stages using qPCR. To assess the immunization and challenge of ticks, the recombinant protein rRpL12+GST was used in immunological experiments. The results showed high conservation of RpL12 among species and comparisons of the amino acid sequence from *H. longicornis.* RpL12 was approximately 60% expressed in the ovary among the examined tissues of unfed adult female *H. longicornis,* and the expression level of RpL12 in unfed ticks was significantly lower than that in *H. longicornis* at the egg and engorged stages. Western blotting showed that rabbit antiserum against *H. longicornis* adult ticks recognized RpL12, with an average egg weight of 49.94% and a 17.22% reduction in the engorged weight of adult ticks, but the mortality increased only 6.00%. These results suggest that RpL12 could be used to generate anti-tick vaccines and provide novel information on the RpL12 gene of ticks, providing a better understanding of its mechanisms in reproduction and oogenesis.

Keywords: *Haemaphysalis longicornis, Recombinant protein, Ribosomal protein, RpL12, Ticks*

Haemaphysalis longicornis (Akar: Ixodidae) Kenelerinde Ribozomal Protein L12'nin Karakteristik Analizi

Öz: Ribozomal protein L12 (RpL12), vertebralı ve vertebrasızlarda ovaryumun gelişiminde ve angorjmanında önemli bir rol oynar. Ancak kenelerde RpL12'nin fonksiyonel özellikleri net değildir. Bu çalışmada, RpL12 geninin bir açık okuma kalıbı *Haemaphysalis longicornis*'in cDNA'sından klonlandı. Sekans analizi gerçekleştirildi ve farklı dokularda ve gelişim aşamalarında ekspresyon seviyeleri qPCR ile belirlendi. İmmünizasyon ve takiben kenelerle enfestasyonu değerlendirmek için, immünolojik deneylerde rekombinant protein rRpL12+GST kullanıldı. Bulgular, kene türleri ve *H. longicornis*'e ait amino asit dizi örnekleri arasında RpL12'nin yüksek düzeyde korunduğunu gösterdi. RpL12, aç erişkin dişi *H. longicornis*'in incelenen dokuları arasında yumurtalıkta yaklaşık %60 oranında eksprese edildi. Aç kenelerdeki RpL12 ekspresyon seviyesi, *H. longicornis*'un yumurta ve doymuş formlarındakilerden önemli ölçüde düşüktü. Western blotlama, yetişkin *H. longicornis* kenelere karşı üretilen tavşan antiserumunun ortalama %49.94 yumurta ağırlığı ve doymuş kene ağırlığında %17.22'lik bir azalma ile RpL12 ile güçlü bir reaktivite sergilediğini ortaya koydu, ancak ölüm oranı %6.00 artmıştı. Bu sonuçlar, RpL12'nin, kenelerde üreme ve oogenez mekanizmalarının daha iyi anlaşılmasını sağlayarak, kene aşısı geliştirmede ve kenelerin bu geni hakkında yeni bilgiler sağlamada kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Haemaphysalis longicornis, Rekombinant protein, Ribozomal protein, RpL12, Kene*

Introduction

Ticks are ectoparasites of veterinary and medical importance with a worldwide distribution and are considered vectors of human and animal pathogens [1]. Ticks not only weaken hosts by sucking their blood but also transmit various pathogens [2]. *Haemaphysalis longicornis* Neumann, 1901 is an Acari, Ixodidae that can exchange three hosts in a

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life cycle (three-host tick), which is a species of tick that is widely distributed in the world, including Australia and East Asia, and recently found in the US [3-6]. It is recognized as the most important vector in the worldwide human and animal transmission of bacterial and viral pathogens, such as *Rickettsia conorii*, *Theileria orientalis* and *Babesia microti* [7-9]. *H. longicornis* has wide hosts, including livestock and wild animals, such as cattle, sheep, rabbits, coyote *(Canis latrans),* eastern cottontail *(Sylvilagus floridanus),* raccoon *(Procyon lotor),* Virginia opossum *(Didelphis virginiana),* white-tailed deer *(Odocoileus virginianus),* woodchuck *(Marmota monax),* and a *Peromyscus sp.* mouse [10]. In view of the threat of the pathogen transmitted by *H. longicornis* to livestock breeding and human public health safety. Therefore, the study of *H. longicornis* is extremely important. The prevention of tick-transmitted diseases and the control of ticks remain important challenges for research [11]. The traditional method of tick control relies mainly on chemical acaricides [12], but the long-term use of insecticides leaves behind pesticide residues and causes environmental pollution [13]. Therefore, it is important to find an environmentally friendly method to kill or delouse ticks. As conserved genes, ribosomal proteins (Rps) are expressed throughout the lifecycle of each organism [14]. Among these proteins, RpL12, RpL24, RpL7/L12, Rpp0 and RpsA belong to the same family in animals. Previous studies have shown that the expression level of RpL24 is higher in the ovary than in other tissues of shrimp, which indirectly shows that RpL24 may play key roles in the reproduction of shrimp [15]. Some new evidence has shown that ribosomal proteins are transported from mitochondria to other organelles and exhibit functions leading to the failure of embryos to form germline progenitors [16]. Recently, ribosomal proteins were studied in ticks, and the results showed that these proteins have significant effects on blood feeding, moulting and reproduction and could be useful in tick control [17]. Additionally, the absence of S-27 reduced the engorgement weight and feeding ability of ticks, and the silencing of S-27 in eggs led to abnormalities in shape and hatching [18]. Ribosomal protein P0 has been demonstrated to be a multifunctional protein in the large subunit of eukaryotic ribosomes, and ticks treated with HlP0 dsRNA obtained a strikingly lower body weight, a lower engorgement rate, and higher mortality after blood sucking than the control groups [19]. Other studies have shown that a synthetic 20 amino acid peptide from the P0 sequence was effective as a vaccine against *Rhipicephalus sanguineus* tick infestations in an immunization and challenge experiment using rabbits [20]. Therefore, these ribosomal proteins could be selected as potential antigens for vaccines against ticks. It would be expected to be active against many species of ticks.

Based on the above, whether RpL12 plays a role in the anti-tick immune response is not clear in ticks. In this study, the objective was to evaluate the anti-tick immune effect of rRpL12 in *H. longicornis*.

Material and Methods

Ethical Statement

The present study was approved by the Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval no. LVRIAEC 2021-006). The tick samples were collected in strict accordance with the requirements of the Ethics Procedures and Guidelines of the People's Republic of China.

Ticks and Tissue Collection

Haemaphysalis longicornis ticks were cultured by feeding on rabbits during various developmental stages in the laboratory. All stages of the ticks were maintained at incubator temperature under a relative humidity of 80±5%. Engorged ticks were maintained separately for oviposition. All collected ticks were immediately placed in phosphate-buffered saline (PBS) and washed twice in a solution containing 0.133 M NaCl, 1.11% sodium dodecyl sulfate (SDS) and 0.0088 M ethylenediaminetetraacetic acid (EDTA). Engorged adult *H. longicornis* ticks were cut under a 20-fold dissection light microscope, and the ovary, salivary glands, midgut, and epidermis were separated [21]. Total RNA was extracted with TRIzol RNA extraction reagent (Invitrogen, China) following the manufacturer's instructions and stored at -80°C for later use.

RpL12 Cloning and Sequencing

The RNA was reverse transcribed to cDNA, and then PCR and qPCR were performed. The nucleotide sequence of RpL12 was obtained from the cDNA of *H. longicornis*, *H. punctata* and *R. sanguineus* ticks. The gene-specific primers were designed using Primer Premier 6.0 software (RpL12-No1: 5'-ATG CCT CCC AAG TTT G-3') and a universal primer (RpL12-No2: 5'-CTG TAC AAG CTT GAT CC-3') and were synthesized by TaKaRa (Dalian, China). The PCR products were purified using a TaKaRa Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa, Dalian, China), and the products were ligated into the pMD®19-T vector (TaKaRa, Dalian, China). The positive clones were sequenced with vector-specific primers (T7 and SP6) by Sangon (Shanghai, China).

Sequence Analysis

The open reading frames (ORFs) were amplified from *H. punctata* and *R. sanguineus*, and the PCR products were purified and ligated into the pGEM-T vector and transformed into the JM109 strain of *E. coli*. The positive clones were used for the sequencing of the nucleotide sequences of the inserts by TaKaRa (Dalian, China). The amino acid sequence of RpL12 was deduced using the Expert Protein Analysis System *(http://us.expasy.org/)*. The

similarities of the amino acid sequences were assessed with the BLAST algorithm of NCBI *(http://www.ncbi.nlm.nih.gov/BLAST/)*. The potential phosphorylation sites were predicted with the NetPhos 3.1 algorithm *(http://www.cbs.dtu.dk/services/NetPhos/)*.

qPCR Analysis of RpL12 in Different Tissues and Developmental Stages

Total cDNA was reverse transcribed from total RNA for eggs, unfed larvae, unfed nymphs, unfed adults and engorged adult ticks and from salivary glands, midguts, ovaries, epidermis and muscle dissected from engorged adult ticks. These samples were used to detect the expression levels of RpL12 in *H. longicornis* by qPCR. In the process, these tissues were thoroughly rinsed with PBS to remove haemocytes. All the samples were subjected to SYBR Green qPCR to determine the expression of RpL12. Two gene-specific primers, qRpL12-No3 (5'-AAG TGG GTG CCA CAT CTG C-3') and qRpL12-No4 (5'- ATC TTC AGC CCT TTC CAG TCC-3'), were designed to amplify a 112-bp segment of the RpL12 gene. β-actin F and R were used as internal references, and a 69-bp fragment was produced. PCR was performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China) with the temperature profile and reaction conditions recommended by the manufacturer and the ABI two-step real-time PCR system (Applied Biosystems, USA). The expression level of RpL12 was analysed by the comparative cycle threshold (CT) method. The normalized CT values (ΔCT) were determined by one-way analysis of variance, and the significance of the differences in RpL12 expression in different tissues and developmental stages was determined using Student's t test with GraphPad InStat version 4.00 (GraphPad Software, USA). Differences were considered significant if $p<0.05$ (two-tailed test).

In vitro Recombinant RpL12 Production and SDS-PAGE Analysis

Cleavage sites for the restriction enzymes BamHI and EcoRI were added to the RpL12-No1 and RpL12-No2 primers for PCR amplification. The purified PCR product was inserted into the pGEX-4T-1 vector and then expressed in *E. coli* BL21 (DE3) pLysE (Novagen, USA) competent cells. The recombinant RpL12 protein (rRpL12) was induced in 2xYT medium (20 mL) with a 1/1000 volume of isopropyl-β-D-thiogalactoside (IPTG) (at a concentration of 0.8 mM) for 8 h at 37°C with shaking at 180 rpm. rRpL12 was purified using the MagneGST™ Protein Purification System according to the manufacturer's instructions (Promega, USA). The pGEX-4T-1 empty vector was also induced as a control under the same conditions. The purified protein and GST control were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were detected/identified by staining the gel with Coomassie Brilliant Blue.

Immunization and Challenge Infestation

Six 2-month-old rabbits purchased from a tick-free area were divided into two groups of three rabbits each for immunization and challenge experiments. The rabbits belonging to the first group were immunized with rRpL12, and the rabbits of the second group served as controls. The fusion protein (1 mg) was emulsified in Freund's complete adjuvant (1:1) and subcutaneously inoculated into the experimental group of rabbits. In the protocols for rabbit immunization, the established textbook protocols suggest the second injection with antigen after 14 days and the third immune after 21 days, and the 3rd injection usually contains much less antigen to challenge infestation. For the challenge, 50 unfed adult ticks were introduced into bags that were adhered to the backs of the rabbits belonging to the first group and maintained for 30 days after the last booster injection. Various parameters, such as the feeding duration, engorged weight, degree of oviposition, and egg weight of each engorged tick, were determined to evaluate the effects of rRpL12-induced anti-tick immunity. Furthermore, the ovarian development of the fed female ticks was observed after they became engorged.

Results

Here, the RpL12 protein of *H. longicornis* was analysed using the Basic Local Alignment Search Tool for proteins (BLASTp) and EditSeq. The results revealed an ORF encoding a polypeptide with 165 amino acids, including 25 strongly basic, 19 strongly acidic, 58 hydrophobic and 33 polar amino acids, and in its amino acid sequence, sites 38 and 124, which are serines, are phosphorylation sites *(Table 1, Fig. 1)*. The amino acid sequences were conserved among the ticks and Drosophila *(Fig. 2)*. The identity of RpL12 showed 98.76% for *H. longicornis* between the cultured strain and Shandong strain (KAH9366394), and the identity was approximately 90% similar with Dermacentor, Rhipicephalus and Ixodes. The identity with Drosophila was approximately 82% similar *(Table 2)*, which further confirms the conservation of RpL12 among ticks.

The expression levels of RpL12 in various tissues and developmental stages of ticks were evaluated. Total cDNA was extracted from the samples obtained at different developmental stages and from the salivary glands, midguts, epidermis, and ovaries dissected from engorged female ticks and then subjected to qPCR analysis. The results showed that RpL12 was mainly expressed in the ovary, and it was also expressed in other tissues but in relatively low abundance *(Fig. 3-a)*. Among the tested developmental stages, the expression level of RpL12 in unfed ticks was significantly lower than that in females at the engorged stage *(Fig. 3-b)*.

rRpL12 was fused with pGEX-4T-1, and the expressed rRpL12+GST fusion protein was analysed by SDS–PAGE

Table 1. *Characteristics analysed for amino acids of RpL12 with Haemaphysalis longicornis*			
Character Description	**Number**	**Character Description**	**Number**
Molecular Weight	17.67 KD	Amino Acids	165
Strongly Basic (+)	25	Strongly Acidic (-)	19
Hydrophobic Amino Acids	58	Polar Amino Acids	33
Isolectric Point	9.239	Charge at PH 7.0	6.328
Adenine (A)	24.10% (n=120)	Guanine (G)	29.52% (n=147)
Thymine (T)	17.67% (n=88)	Cytosine (C)	28.71% (n=143)
(+) represents strongly basic; (-) represents strongly acidic; (A) represents adenine; (G) represents guanine; (T) represents thymine; (C) represents cytosine; (%) indicates the percentage of bases A, T, C, and G to all bases			

atg	cct	ccc	aag	ttt	gat	ccg	acg	gaa	att	aaa	gtt	gtg	tgc	ctt	cga	gcc	gtt
M	P	P	K	F	D	P	T	E	I	K	V	V	C	L	R	A	V
ggc	ggt	gaa	gtg	ggt	gcc	aca	tct	gcc	ttg	gct	ccc	aag	att	ggt	ccc	ctt	ggt
G	G	E	V	G	A	T	S	A	L	A	P	K	I	G	P	L	G
ctg	tcg	ccg	aag	aag	gtc	ggt	gat	gac	atc	gcc	aag	gcg	acg	cag	gac	tgg	aaa
L	S	P	K	K	V	G	D	D	I	A	K	A	T	Q	D	W	K
ggg	ctg	aag	atc	acc	gtc	aag	ctc	atc	atc	caa	aac	agg	cag	gct	acc	atc	gaa
G	L	K	I	T	V	K	L	I	I	Q	N	R	Q	A	T	I	E
gtg	gtg	ccc	agc	gct	gca	tcg	ctc	atc	atc	aag	gcg	ctc	aag	gag	ccg	cca	cgc
V	V	P	S	A	A	S	L	I	I	K	A	L	K	E	P	P	R
gac	cgc	aag	aag	gtc	aag	aac	gtg	aag	cac	agc	ggg	aac	ctg	acc	ttc	gac	gag
D	R	K	K	V	K	N	V	K	H	S	G	N	L	T	F	D	E
atc	ctc	acg	atc	gca	cgg	acg	atg	cgg	gcc	cgc	tct	atg	gcg	agg	agc	ctt	tct
I	L	T	I	A	R	T	M	R	A	R	S	M	A	R	S	L	S
ggc	acc	gtc	aag	gag	atc	ctg	ggt	aca	tgc	cag	tcc	gtc	gga	tgc	act	gtc	gat
G	T	V	K	E	I	L	G	T	C	Q	S	V	G	C	T	V	D
ggc	aac	cac	cca	cac	gac	gtc	att	gac	aag	gtc	aac	agc	ggt	gac	gtc	gag	gtg
G	N	H	P	H	D	V	I	D	K	V	N	S	G	D	V	E	V
ccc	gaa	gag	taa														
P	E	E	-														

Fig 1. Analysed amino acid and nucleic acid sequences of the RpL12 gene. Shaded amino acids represent phosphorylation sites

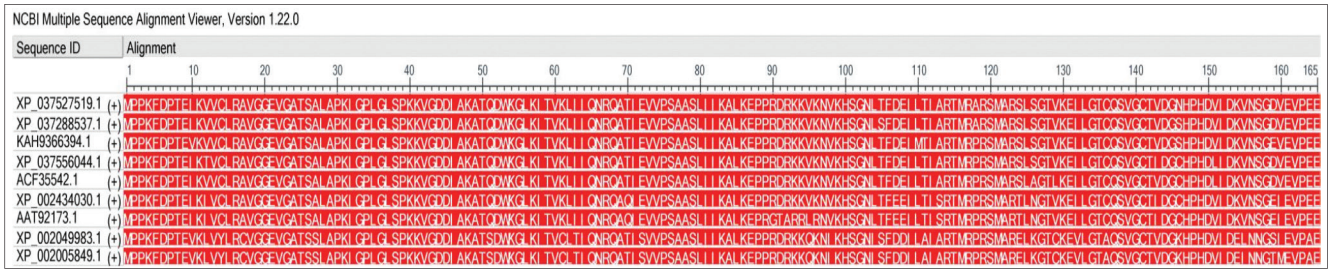

Fig 2. Multiple sequence alignments of RpL12 amino acid sequences in major arthropods (NCBI Multiple Sequence Alignment Viewer, Version 1.22.0)

on a 12% w/v polyacrylamide gel. The molecular weight of the fusion protein was observed to be approximately 44 kDa, close to its calculated MW (43.68 kDa). Western blotting revealed that rabbit anti-*H. longicornis* adult serum exhibited strong reactivity with rRpL12+GST proteins, whereas negative rabbit serum did not react with the fusion proteins *(Fig. 4)*.

The effects of rRpL12 on the examined physiological and behavioural parameters of tick feeding are shown in *Table 3*. No apparent differences were observed in the duration of feeding or percentage of mortality, whereas significant differences ($0.01<P<0.05$) in the spawning rate (62.45 vs. 80.12, respectively) and oviposition rate (77.32 vs. 82.68, respectively) were observed between

Table 2. RpL12 amino acid sequence alignment from ticks and Drosophila					
Species	**Accession NO.**	**Identity (%)**	**Species**	**Accession NO.**	**Identity (%)**
H. longicornis	No accession	Query	*Dermacentor silvarum*	XP_037556044	97.43
H. longicornis	KAH9366394	98.76	*Rhipicephalus sanguineus*	XP_037527519	96.97
Ixodes scapularis	XP_002434030	94.55	*Ixodes pacificus*	AAT92173	89.70
Dermacentor variabilis	ACF35542	97.58	*Rhipicephalus microplus*	XP_037288537	96.36
Drosophila mojavensis	XP_002005849	82.42	*Drosophila busckii*	XP_017838361	82.42
Drosophila navojoa	XP_017966042	82.42	*H. longicornis* (4D8 gene)	DQ159972	-

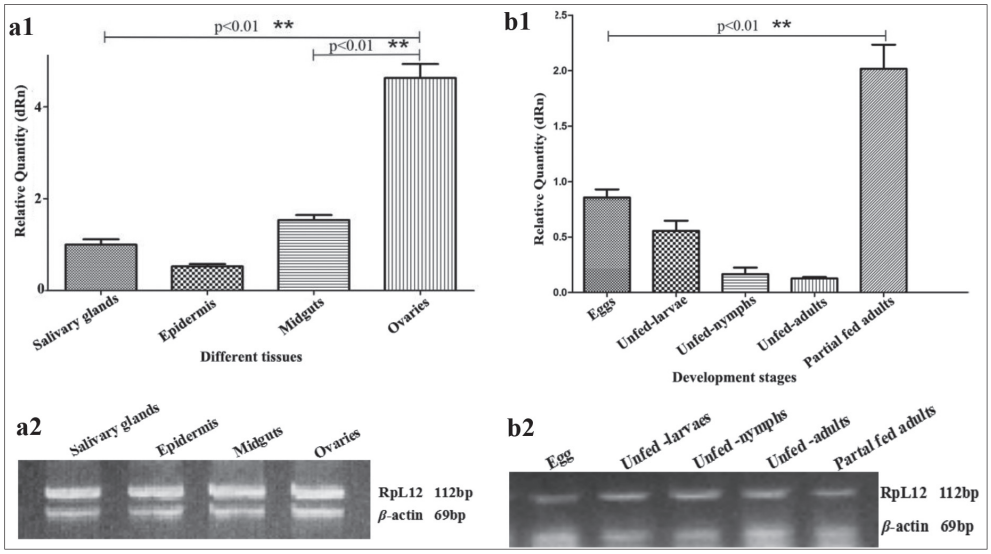

Fig 3. Expression of RpL12 in various tissues and at different developmental stages of ticks. **a1**- Column chart showing the expression level of RpL12 in different tissues of *Haemaphysalis longicornis*, **a2**- The corresponding PCR products were analysed in agarose gels, **b1**- Expression level of RpL12 at different developmental stages of *Haemaphysalis longicornis*, **b2**- The corresponding PCR products of different developmental stages. The results are shown as the means ± SEMss from three separate repeats. Asterisks indicate significant differences identified from the comparison of the various tissues and developmental stages **P<0.01 (Student's t test)

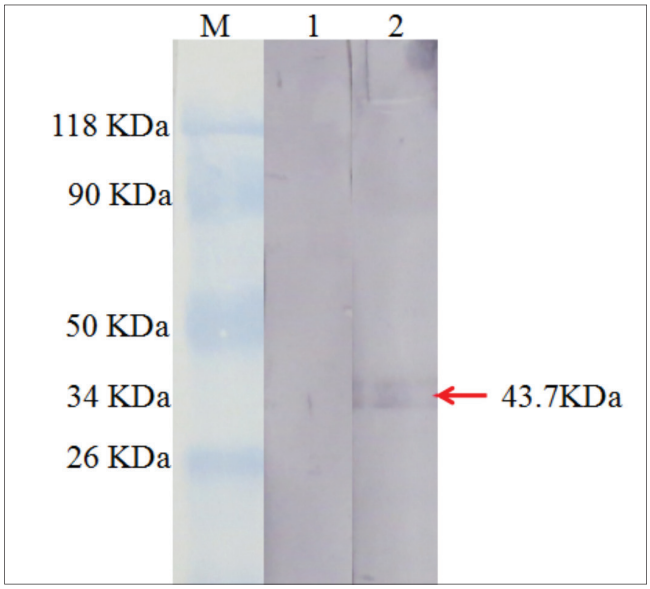

Fig 4. Western blot analysis of rRpL12. Rabbit-negative serum from *Haemaphysalis longicornis* adults was used as the primary antibody. M: Protein molecular weight marker; Lane 1, GST tag protein product with negative serum; lane 2, pGEX-4T-1+ RpL12-expressing product reacted with rabbit anti-*H. longicornis* adult serum

the vaccinated and control groups. Engorged female ticks were dissected, and their ovaries and midguts were examined. The evaluation of ovarian morphology showed that the ovary length of the experimental group was less than that of the control group. Although the density of nodules was high in the experimental group, this density was not conducive to the acquisition of nutrients for eggs, thus affecting the development of eggs. Therefore, in this study, ovarian development was significantly affected in ticks feeding on rabbits that were immunized with RpL12. In contrast, the morphology of the midgut was unchanged in the control group *(Fig. 5)*.

Discussion

L12, a ribosomal protein found in eukaryotes and archaea, is located near a translation factor-binding site on the surface of the large ribosomal subunit. It plays a role in the kinetics of peptide synthesis and might be involved in the interactions between the proteins L14 and L3 through other factors [22]. Another study showed that ribosomal proteins have some functions in animal reproduction

Table 3. *Effect of vaccination with rRpL12 on tick feeding*

Parameter	Immunization Treatment		Percentage (%)
	rRpL12	PBS	
Duration of feeding (days)	10-13	11-14	-
Engorged weight, mean (mg)[a]	260±10*	294±8	17.22 (down)
Mortality (%)[b]	15±2.3	13±1.7	6.00 (up)
Oviposition rate (%)	77.32	82.68	5.36 (down)
Average egg weight (mg)	20.26±4.2**	28.56 ± 3.6	49.94 (down)
Spawning rate (%)	62.45*	80.12	17.67 (down)

**Significance (0.01<P<0.05) was calculated using Student's t test; [a] Dead ticks were excluded; the values are expressed as the means ± standard deviations; [b] The mortality and oviposition rates of the ticks after the feeding period were statistically analysed*

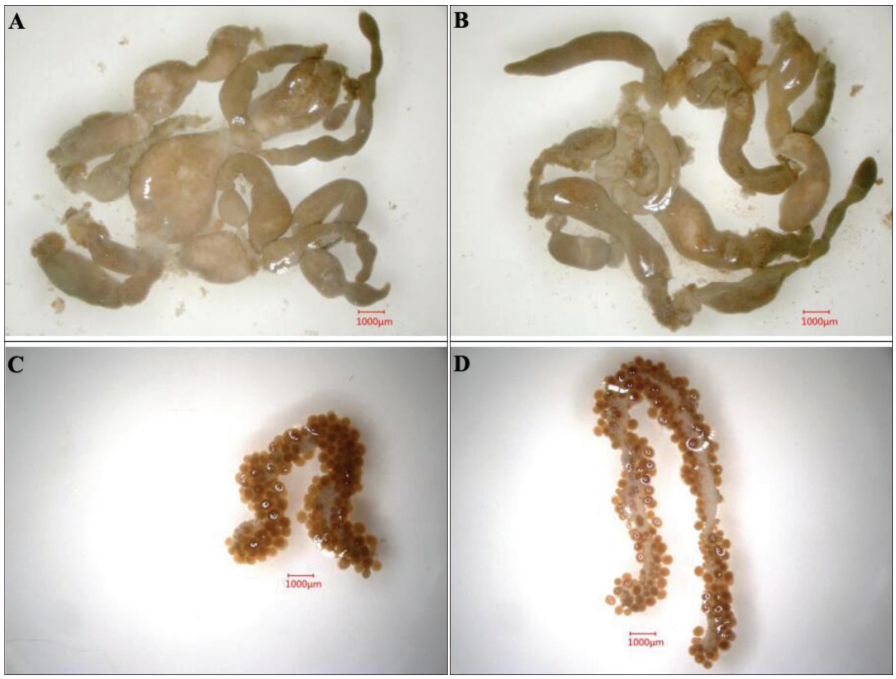

Fig 5. rRpL12 inhibition affects ovarian development. Engorged ticks were dissected to obtain their ovaries and midgut, and morphological development was compared between the experimental and control groups. **A** indicates the midguts of the immune group; **B** indicates the midguts of the control group; **C** indicates the ovaries of the immune group; **D** indicates the ovaries of the control group. In addition, the ruler was 1000 μm

and oogenesis [23,24]. Therefore, we were interested in the gene features of RpL12 in different tissues and at various developmental stages that might be related to physiological functions in ticks.

Here, the ORF sequence of RpL12 was cloned from *H. longicornis* ticks. The analysis of the pI of RpL12 showed that it is a strongly basic ribosomal protein with a pI of 9.239. The RpL12 nucleic acid sequence showed that the A+T content was much higher than the C+G content, indicating that the gene had high activity. It may play an important role in animal immunity *(Table 1)*. Two typical phosphorylation sites were predicted in RpL12 *(Fig. 1)*. Phosphorylation and dephosphorylation play a fundamental role in directly regulating various aspects of protein function, for example, cell growth, differentiation, apoptosis and cell signalling, under healthy conditions [25]. Multiple sequence alignment of amino acids from different species showed that RpL12 was highly conserved *(Fig. 2)*. The conserved features make it possible for the vaccine to be broad-spectrum.

The expression level of RpL12 was determined in different developmental stages and various tissues of *H. longicornis* ticks *(Fig. 3-a,b)*. The results indicated that RpL12 is required for feeding and ovary development in ticks. Although RpL12 is expressed in the salivary glands and midgut, its expression level is lower than that in the ovaries. Arguably, this could mean that there is no secondary function of RpL12 in these two tissues for blood digestion. In addition, RpL12 expression is relatively high in the egg stage and decreases during starvation along with tick development and maturity. The results also confirmed that the expression of RpL12 showed no relationship with unfed status in ticks. Again, the function of RpL12 has an impact on ovarian development. With the progression of tick development and increases in tick weight, the expression level of RpL12 decreased. However, the expression of RpL12 increased immediately after the unfed ticks were fed. It was also found that the expression level of RpL12 was higher in the ovary, indicating that feeding on blood by adult ticks plays an important role in

the maturation of ovarian cells; the putative physiological effect of L12 is in accordance with that of RpL12 of the same family [26]. These results have also been demonstrated in *Drosophila melanogaster*. Ribosomal proteins are associated with ribosomal subunits after fertilization of *Drosophila melanogaster* embryos. This event substantially precedes the blastoderm stage of embryonic development. At the preblastoderm stage, embryos synthesize many and possibly all of the r-proteins, and these subsets are incorporated into ribosomes. After blastoderm formation, all of the newly synthesized r-proteins along with newly synthesized rRNA are incorporated into ribosomal subunits [27]. This contributes to the normal development of the ovaries in arthropods.

Ribosomal proteins have also been shown by several studies to participate in the innate immune response. One of the prominent examples is RpL13A, which was reported to engage the interferon-γ (IFN-γ)-mediated inflammatory response by selectively modulating gene expression [28]. The inflammatory response is a double-edged sword that kills both pathogens and host cells. Identification of RpL13A as a negative regulator of inflammatory proteins suggests that this Rps could be a repressor of inflammatory signalling. Another example of Rps involvement in immune signalling is RpS3, which selectively modulates NF-κB target gene expression. NF-κB was originally identified to regulate genes crucial for the immune response but was later shown to also regulate genes implicated in cell survival or proliferation [29]. Together, these lines of evidence indicate that some Rps play diverse roles in the host immune response by either boosting immune signalling or facilitating pathogen production under different circumstances [30]. To investigate the immune effects of RpL12 on ticks, this putative function will be confirmed by monitoring the anti-tick immune effect of rRpL12. Based on the immune reaction between antisera against unfed adults and rRpL12 determined by Western blotting, the recombinant protein was well recognized by rabbit anti-*H. longicornis* tick serum. The cross-reactivity of rRpL12 from different tick species was analysed, and the results showed strong immunoreactivity *(Fig. 4)*. Because the RpL12 gene is highly conserved in arthropods, the protein might be developed into a universal immunogen. The results of the rRpL12 vaccination experiment showed that the physiological behaviour of the ticks had a significant effect in the rabbit model, and this finding was obtained for the effects on the duration of feeding, mortality, and average egg weight per tick. However, a significant effect was found on the engorged weight and the oviposition and spawning rates of the ticks, particularly the oviposition rate, which was decreased *(Table 3)*. As a ribosomal protein, RpL12 is a suitable candidate vaccine for antisera against rRpL12 that recognizes the target site because the expression of RpL12 *in vivo* was neutralized by antiserum against rRpL12. Another possible explanation is that RpL12 plays structural and functional roles in the eggs of ticks and is thus not essential for their survival and blood feeding. Morphological analysis showed that rRpL12 affected ovary development, which was a key reason for the observed decrease in the spawning rate (62.45% in the experimental group and 80.12% in the control group) observed in the immunization experiment. The evaluation of ovarian morphology indicated that the development of the ovaries was significantly inhibited in the immune group *(Fig. 5)*. These findings suggested that in the process of egg maturation in the ovary, abnormal ovarian development was induced by rRpL12, and egg development was consequently affected; therefore, the oviposition rate was also significantly decreased *(Table 3)*.

In conclusion, RpL12 is a conserved gene of *H. longicornis*. The expression level of the gene was significantly increased during tick feeding and in ovaries, while its expression levels were different in other developmental stages and among tissues. These findings demonstrated that RpL12 has important functions in feeding and ovarian development mortality and egg weight, although it has no effect on feeding time. Therefore, it might induce a strong protective effect against *H. longicornis* in rabbits. However, the processes of feeding and ovary development in ticks should be further investigated in further studies.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available to the corresponding authors.

Ethical Statement

The present study was approved by the Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval no. LVRIAEC 2021-006).

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

XRL and GYL: the hypothesis of this study; JL, GYL and XRL: work management, article writing; JL, WGL and QYR: experimental procedure follow-up; XKS and RFY: literature review, review of results, final decision.

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