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RESEARCH

Emergence of multidrug-resistant *Haemaphysalis longicornis* populations in China

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Abstract
Many chemical acaricides have been used to mitigate the detrimental effects of *Haemaphysalis longicornis*. However, the excessive use of chemical acaricides may facilitate the development of resistance in *H. longicornis*. Larval packet tests (LPTs) were conducted on larvae aged 14–21 days collected from 10 counties in China to evaluate the effectiveness of different acaricides, including cyhalothrin, cypermethrin, deltamethrin, amitraz, and fipronil. Additionally, genomic DNA was extracted from tick samples collected from 14 counties, and partial fragments of the voltage-gated sodium channel (VGSC), beta-amino-oxidoreductase (β AOR), and gamma-aminobutyric acid (GABA)-gated chloride channel genes were amplified via PCR. According to the LPT findings, it can be inferred that ticks from all localities exhibited resistance to at least three different acaricides. Multidrug resistance to five acaricides was detected in 40% of the samples, including those from Liaoning (LYG), Hebei (HZY), Henan (HSY), and Hubei (HSG) Provinces. The resistance factors for cyhalothrin, cypermethrin, deltamethrin, amitraz, and fipronil ranged from 2.71–9.57, 1.14–10.4, 0.80–24.2, 5.2–56.97, and 4.12–41.17, respectively. Hence, the resistance levels to the different acaricides ranged from susceptible (S) to level IV. Level IV resistance was observed only for fipronil and amitraz. Despite substantial resistance, no nonsynonymous mutations in the VGSC or β AOR genes were detected. However, one novel nonsynonymous mutation (M295L) in transmembrane 2 (TM2) of the GABA-gated chloride channel gene, which may have led to conformational changes in this channel, was detected in ticks from Shandong Province (SQL). In this study, LPTs were used to evaluate the effects of various acaricides on *H. longicornis*, and the findings revealed that this tick species is resistant to many acaricides. This study provides a foundation for the development of preventative and control measures against *H. longicornis*.

Keywords *Haemaphysalis longicornis* · Acaricide · Multidrug resistance · Bioassay · Molecular assay

Extended author information available on the last page of the article

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Introduction

Haemaphysalis longicornis, a member of the Ixodidae family, primarily parasitizes livestock, including cattle, horses, sheep, goats, dogs, and other wild animals, as well as humans. *H. longicornis* is a three-host tick, and in China, it is recognized as one of the most prevalent tick species (Chen et al. 2010; Zhao et al. 2020, 2021). More than 100 tick-borne pathogen species have been reported in China, and *H. longicornis* serves as a major vector for several of them (Zhao et al. 2021). *H. longicornis* can carry and transmit more than 30 pathogens, posing risks to humans and animals (Jia et al. 2021). It can result in illnesses fatal to humans, such as Japanese spotted fever and severe fever with thrombocytopenia syndrome (SFTS). In China, SFTS is a public health issue. It has spread to 25 Chinese provinces because of the mobility of ticks and their hosts (Huang et al. 2021). Between 2010 and 2019, approximately 13,824 cases of SFTS and 713 confirmed fatalities were documented.

Chemical acaricides have been recognized as effective at controlling tick populations (Butler et al. 2021). Five chemical acaricides (cyhalothrin, cypermethrin, deltamethrin, amitraz, and fipronil) are commonly used for tick control. These five acaricides are neurotoxic. In China, these 5 acaricides are commonly used for tick control, with an annual production of several thousand tons of deltamethrin (Alonso-Díaz et al. 2013; Wen et al. 2015; Navarrete-Meneses and Pérez-Vera 2019; Kumar et al. 2021). The improper and incorrect application of these acaricides has led to an increased risk of resistance development in tick populations. However, there are few studies on tick resistance in China. Zhongbo Li tested the resistance of *Rhipicephalus microplus* to deltamethrin in Huaihua, Hunan Province, China, and reported mutations associated with resistance Li et al. (2023). Jiang Na determined the frequency of mutations in voltage-gated sodium channels (VGSCs) related to the resistance of *R. microplus* to pyrethroids (Jiang et al. 2024). The resistance of *R. microplus* to fipronil has been documented in China (Obaid et al. 2025a, b). The mutations of the VGSCs gene in *H. longicornis* have been reported previously (Obaid et al. 2025a).

Target site resistance is often linked to nonsynonymous mutations in VGSCs (domains II and III) (Foil et al. 2004). Nonsynonymous mutations in VGSCs in the exon sequence of the domain-II S4-5 junction region (T170C, G184C, and C190A) are associated with resistance in *R. microplus* (Williamson 1996; Morgan et al. 2009; Stone et al. 2014). The mutation in the β adrenergic octopamine receptor (β AOR) gene is primarily responsible for the development of resistance of ticks to amitraz (De Rouck et al. 2023). The resistance of *R. microplus* to amitraz is related to the β AOR mutation A181T (I61F) (Corley et al. 2013). Fipronil has been extensively utilized to manage ticks, which could lead to resistance in tick species such as *R. microplus*, *Rhipicephalus sanguineus* (*R. sanguineus*), and *Hyalomma anatolicum* (*H. anatolicum*) (Casida and Durkin 2015; Shyma et al. 2015; Taylor Wells et al. 2015; Becker et al. 2019). Gamma-aminobutyric acid (GABA) is a target of fipronil and dieldrin (Casida and Durkin 2015; Taylor Wells et al. 2015). Previously, a nonsynonymous mutation in the GABA-gated chloride channel gene (T290L) associated with dieldrin resistance was detected in *R. microplus* (Hope et al. 2010). Moreover, five nonsynonymous mutations in the GABA-gated chloride channel gene (A286S/L, S281T, V317I, T328A, and A329S) associated with fipronil resistance were detected in *R. microplus* (Castro et al. 2019).

This study focused on the resistance of *H. longicornis* to multiple acaricides, acaricide resistance in 10 counties was examined, and partial gene sequences from ticks from 14

counties were amplified to assess the current status of acaricide resistance among *H. longicornis* in China.

Materials and methods

Reference susceptible tick strain (CHLS-1)

A sensitive strain of *H. longicornis* (CHLS-1) was obtained from the Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (LVRI, CAAS), China. For the past 27 years, it has been maintained in an environment with a temperature of 28 °C ± 1 °C and a relative humidity of 75–90%, with an average light exposure time of 8–10 h. Fresh rabbit blood should be regularly supplied to ensure sufficient nutrition (Li et al. 2007). Notably, during the maintenance period, there was no contact with any acaricides. Through LPT experiments, the sensitivity of the tick strain to cyhalothrin, cypermethrin, deltamethrin, diazinon, DDT, ivermectin, amitraz, and fipronil was validated (Diao et al. 2025). The diagnostic doses (DDs) and median lethal dose (LC_{50}) of the sensitive strain CHLS-1 have been established (Diao et al. 2025).

Collection of samples

From 2019–2024, samples of *H. longicornis* were collected from 14 counties in 10 provinces (Table 1). The collection sites are unorganized farms, and ticks are collected from sheep raised by farmers, sheep sheds, and grasslands where the sheep graze. The collected engorged female ticks and unfed female ticks were placed in dry bottles that were sealed with white cotton cloth to ensure air and moisture circulation and taken to the parasitic laboratory of the department. The identity of the ticks was determined via a digital microscope (VHX-5000; KEYENCE, Japan) on the basis of their morphological characteristics (Chen et al. 2010). Engorged female ticks were directly placed in glass tubes, while unfed female ticks were reared on rabbits until engorgement, after which they were collected, washed thoroughly and placed in glass tubes. The blood sucking time was 10–21 days (FAO 2004). The rabbits were fed in the laboratory at 28 °C and 85 ± 5% relative humidity. Three to five engorged female ticks were placed in a glass tube that was covered with cotton cloth and placed in an incubator until the eggs hatched. Larvae obtained after 14 to 21 days of complete hatching were used for larval packet tests (LPTs). All coordinates were obtained with a global positioning system and stored and processed with Microsoft® Excel® in MSO2019; the map was drawn in ArcGIS (Fig. 1).

Larval packet test (LPT) for acaricide resistance monitoring

LPT was performed according to the guidelines of the Food and Agriculture Organization (FAO 2004) with slight modifications. Standard cyhalothrin (CAS NO. 68,085–85-8), cypermethrin (CAS NO. 52,315–07–8), deltamethrin (CAS NO. 52,918–63-5), amitraz (CAS NO. 33,089–61-1), and fipronil (CAS NO. 120,068–37-3) were used. The stock solu-

Table 1 Sampling information and experimental purposes for the different field populations

Strain	County	City	Province	Sampling date	Test
GLL	Li	Longnan	Gansu	May 2023	SNP
GLX	Xihe	Longnan	Gansu	May 2023	LPT/SNP
GQN	Ning	Qingyang	Gansu	July 2019	LPT/SNP
GTZ	Zhangjiachuan	Tianshui	Gansu	May 2023	SNP
HSY	Yongcheng	Shangqiu	Henan	August 2022	LPT/SNP
HZY	Wei	Zhangjiakou	Hebei	May 2022	LPT/SNP
HHM	Macheng	Huanggang	Hubei	June 2024	LPT/SNP
HSG	Guangshui	Suizhou	Hubei	June 2024	LPT/SNP
JYH	Helong	Yanji	Jilin	June 2022	LPT/SNP
LYG	Gaizhou	Yingkou	Liaoning	May 2022	LPT/SNP
NGJ	Jingyuan	Guyuan	Ningxia	May 2019	LPT/SNP
SLL	Lin	Lvliang	Shanxi	May 2022	LPT/SNP
SQL	Laixi	Qingdao	Shandong	August 2024	SNP
ZTH	Huangyan	Taizhou	Zhejiang	May 2022	SNP

Note: SNP, single-nucleotide polymorphism; LPT, larval packet test

tions were prepared with acetone, and the working solutions were prepared with distilled water; the concentrations of the working solutions are shown in Supplementary Table 1. The working solution of amitraz was soaked on a 4.5 cm × 6.5 cm nylon cloth, and the working solutions of other acaricides were soaked on 4 cm × 6 cm filter paper. These nylon cloths and filter papers were dried in a fume hood for 2 h, and the moisture was dried to make an acaricide membrane. Afterwards, the film was folded in half along the long end, both ends were sealed with metal clips, approximately 100 larvae were placed at the opening, and the sample was sealed with a third metal clip. For each concentration of acaricide, the experiments were conducted in triplicate, and the samples were placed together in a glass jar. After 24 h (48 h with amitraz), the packaging was removed, and the number of tick deaths was recorded. Microsoft® Excel® in MSO 2019 (Microsoft Corporation) was used for statistical analysis. The LC_{50} was determined via IBM SPSS Statistics 26 software. The resistance factor (RF) was obtained by dividing the LC_{50} for the field population by the LC_{50} for the sensitive strain. Resistance levels were assigned as described in a study conducted in India as follows: level S: RF < 1.4; level I: RF = 1.5 ~ 5; level II: RF = 5.1 ~ 25; level III: RF = 26 ~ 40; and level IV: RF > 41(Ghosh et al. 2017).

Fig. 1 Sampling information for the different field populations

Extraction of genomic DNA and amplification of target genes

DNA extraction was performed using adult ticks that were depleted after spawning. Each tick was individually placed in a 1.5 mL centrifuge tube and washed with phosphate-buffered saline (PBS). The washed ticks were then homogenized using a tissue homogenizer (QIAGEN GmbH TissueLyser 2). A QIAamp DNA Mini kit (Germany QIAGEN, LOT-175018422, cat. no. 51306) was used to extract DNA according to the instructions. The primers and PCR conditions used are shown in Supplementary Table 3 for amplifying partial fragments of the VGSC, β AOR, and GABA-gated chloride channel genes. The reaction volume of the PCR was 25 μL, comprising 5.7 μL of PCR-grade water, 12.52 μL of 2 × Phanta Max Buffer, 0.5 μL of dNTP mixture, 1.5 μL of forward primer, 1.5 μL of reverse primer, 2.5 μL of genomic DNA, and 0.8 μL of Phanta Max Super-Fidelity DNA Polymerase were prepared separately for the amplification of each gene. The amplification products were observed via agarose gel electrophoresis (2%) under ultraviolet radiation with a fully automatic gel imaging system (ProteinSimple, AlphaImager HP). The obtained amplified products were sent to Tsingke Biotechnology Company for bidirectional sequencing. The homologous sequences were downloaded from GenBank for comparative alignment of the obtained sequences via BioEdit, and comparative graphs were generated via GeneDoc.

Results

Ticks

The results of the morphological analysis revealed that all the field populations were *H. longicornis*. In addition to the ticks of the sensitive strain, 71,421 larvae were used for LPTs, and the target genes were amplified from DNA from 68 adult ticks. The numbers of larvae used for the analysis of resistance to cyhalothrin, cypermethrin, deltamethrin, amitraz, and fipronil were 13,864, 14,085, 14,386, 14,724, and 14,362, respectively.

LPT results

LPT results for the sensitive strain from earlier experiments were used. The resistance of the field populations of *H. longicornis* collected across 10 counties to 5 acaricides was assessed (Table 2, Supplementary Table 2–6). The slope varied between 1.24 and 3.26, indicating a high level of homogeneity in the data (Supplementary Table 2–6). All field populations exhibited resistance to cypermethrin and amitraz, with resistance rates of 60%, 80%,

Table 2 Resistance levels of the different field populations to cyhalothrin, cypermethrin, deltamethrin, amitraz, and fipronil

Insecticide	Result	LYG	JYH	SLL	NGJ	GLX	GQN	HZY	HSY	HHM	HSG
Cyhalothrin	LC50	0.023	0.207	0.029	0.030	0.019	0.032	0.049	0.020	0.065	0.049
	95%CI	0.020–0.027	0.180–0.238	0.025–0.033	0.026–0.034	0.016–0.022	0.028–0.037	0.043–0.056	0.018–0.024	0.042–0.098	0.037–0.048
	RR50	3.29	29.57	4.14	4.29	2.71	4.57	7.00	2.86	9.29	7.00
	RL	I	III	I	I	I	I	II	I	II	II
Cypermethrin	LC50	0.023	0.252	0.025	0.027	0.034	0.024	0.033	0.173	0.144	0.208
	95%CI	0.020–0.027	0.217–0.291	0.022–0.028	0.023–0.030	0.029–0.039	0.021–0.028	0.029–0.038	0.153–0.199	0.128–0.164	0.175–0.245
	RR50	3.29	12.60	1.25	1.35	1.70	1.14	1.65	8.65	7.20	10.40
	RL	I	II	S	S	S	S	I	II	II	II
Deltamethrin	LC50	0.022	0.004	0.018	0.023	0.008	0.024	0.036	0.121	0.006	0.024
	95%CI	0.019–0.025	0.003–0.004	0.016–0.021	0.020–0.026	0.007–0.009	0.021–0.027	0.032–0.042	0.102–0.142	0.006–0.007	0.021–0.028
	RR50	4.40	0.80	3.60	4.60	1.60	4.73	7.20	24.20	1.20	4.80
	RL	I	S	I	I	I	I	II	II	S	I
Amitraz	LC50	0.117	0.084	0.076	0.099	0.052	0.076	0.045	0.570	0.322	0.333
	95%CI	0.100–0.137	0.073–0.097	0.067–0.087	0.086–0.113	0.041–0.065	0.067–0.087	0.039–0.052	0.501–0.651	0.276–0.375	0.287–0.385
	RR50	11.70	8.40	7.60	9.90	5.20	7.60	4.50	56.97	32.20	33.30
	RL	II	II	II	II	II	II	I	IV	III	III
Fipronil	LC50	0.026	0.009	0.008	0.010	0.011	0.013	0.017	0.018	0.054	0.080
	95%CI	0.023–0.030	0.008–0.011	0.007–0.009	0.008–0.011	0.009–0.012	0.011–0.015	0.014–0.021	0.015–0.020	0.027–0.107	0.071–0.090
	RR50	13.38	4.63	4.12	5.15	5.20	6.69	8.75	9.26	27.79	41.17
	RL	II	I	I	II	II	II	II	II	III	IV

Note: RF_{50} = LC50 of the field population ÷ LC50 of the sensitive strain; RL represents the resistance level; level S: RF < 1.4; level I: RF = 1.5 ~ 5; level II: RF = 5.1 ~ 25; level III: RF = 26 ~ 40; and level IV: RF > 41

and 90% to cypermethrin, deltamethrin, and fipronil, respectively (Table 2, Supplementary Table 2–6).

The degree of resistance to each acaricide varied significantly. The RFs of the field population for cyhalothrin varied from 2.71 to 29.57. The resistance level of most field populations to cyhalothrin was level I (60%), with only the JYH population exhibiting level III resistance. The resistance levels of the HZY, HHM and HSG populations are shown in Supplementary Table 2. The RFs of the field population for cypermethrin were between 1.14 and 12.6. Cypermethrin resistance was generally low, with four populations demonstrating sensitivity; the highest RF recorded was 12.60 for the JYH population. *H. longicornis* field populations from Central China and Northeast China presented high levels of cypermethrin resistance, as shown in Supplementary Table 4. The RFs of the field population for deltamethrin ranged from 0.80 to 24.2. Eighty percent of the ticks from the field population were resistant to deltamethrin, but the majority of these ticks (60%) exhibited low resistance, categorized as level I, as shown in Supplementary Table 5. All the ticks in the field population were resistant to amitraz, with 90% exhibiting level II or higher resistance and the highest resistance level being level IV (RF_{50} of 56.97). The resistance levels of the HSY, HHM, and HSG populations from Central China were IV, III, and III, respectively, as shown in Supplementary Table 6. The RFs of the field populations for fipronil ranged from 4.12 to 41.17. Among the 10 populations, the HSG population presented the highest resistance (level IV), as shown in Supplementary Table 7.

Resistance to multiple acaricides was prevalent in the field populations examined in this study. The frequency of resistance to multiple acaricides in the field population is shown in Table 3. All the populations developed resistance to three or more acaricides. Fifty percent of the strains exhibited multidrug resistance to 4 chemical acaricides, and the SLL, NGJ, GLX, and GQN populations exhibited resistance to amitraz, cyhalothrin, deltamethrin, and fipronil. Additionally, 40% of the ticks in the population demonstrated resistance to five acaricides, including cross-resistance to three type II pyrethroids (cyhalothrin, cypermethrin, and deltamethrin), as shown in Table 3.

Target gene amplification

Voltage-gated sodium channel gene mutations

VGSC domain II (IIS4-5) of *H. longicornis* populations in targeted regions of China was amplified. No mutations associated with resistance to *R. microplus* (T170C, G184C and

Table 3 Resistance levels of different field populations from different locations to multiple insecticides

Abbreviations for insecticides	Resistance frequency (%)/sampling location (N)	Multidrug resistance level
AMI; CYH; CYP	10%/JYH	resistance to 3 acaricides
AMI; CYH; DEL; FIP	40%/SLL; NGJ; GLX; GQN	resistance to 4 acaricides
AMI; CYH; CYP; FIP	10%/HHM	resistance to 4 acaricides
AMI; CYH; CYP; DEL; FIP	40%/LYG; HZY; HSY;HSG	resistance to 5 acaricides

Note: AMI: amitraz; CYH: cyhalothrin; CYP: cypermethrin; DEL: deltamethrin; FIP: fipronil

C190A) were identified. However, two new synonymous mutations (G240C and G255C) were detected in the SLL population, as shown in Fig. 2.

β AOR gene mutations

In this study, the β AOR gene sequences of *H. longicornis* populations across various regions of China were amplified. As shown in Fig. 3, through genetic analysis, 8 synonymous mutations that were previously detected in *R. microplus* and *R. sanguineus* (T186C, G189C, A213G, G216C, G225A, G228C, G258C, and C231A) were found (Koh-Tan et al. 2016).

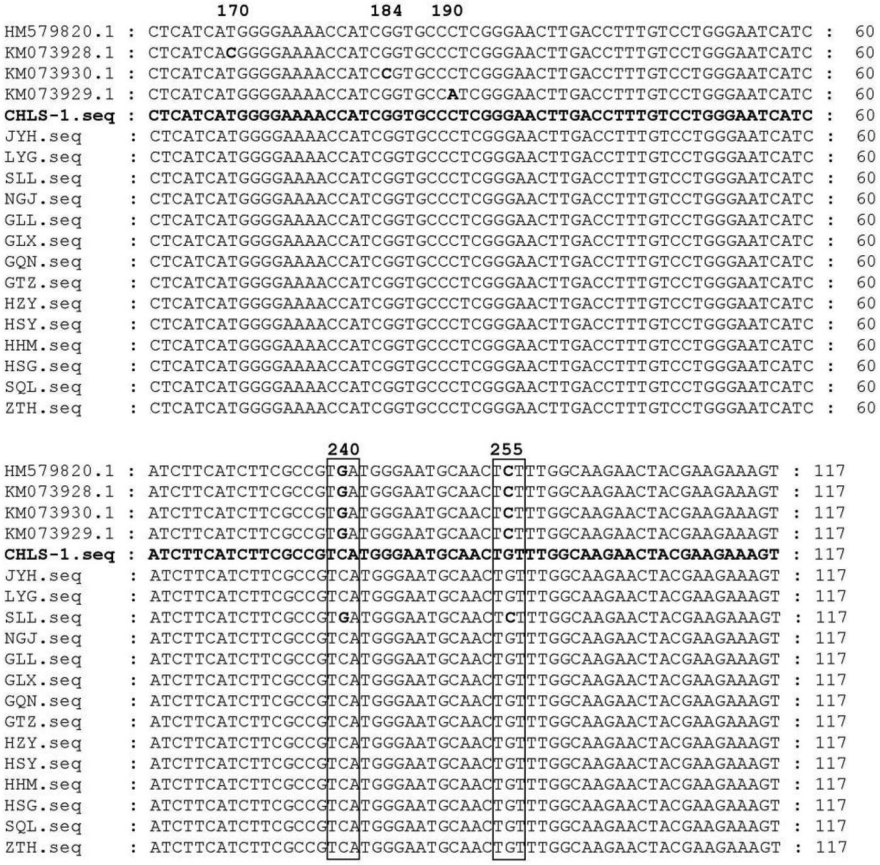

Fig. 2 Nucleotide sequence alignment of the H. longicornis VGSC domain II (IIS4-5) fragment with reference sequences of the acaricide-sensitive and acaricide-resistant strains of R. microplus. HM579820.1 is the R. microplus-sensitive strain, HM073928 corresponds to T170C, KM073930.1 corresponds to G184C, and KM073929 corresponds to C190A. The bold text indicates the sensitive H. longicornis strain

GABA-gated chloride channel gene mutations

The GABA-gated chloride channel gene sequences of *H. longicornis* populations from several regions of China were amplified. A total of 8 single-nucleotide polymorphisms (SNPs), including one nonsynonymous SNP and seven synonymous SNPs, were detected in *H. longicornis*. Five synonymous mutations (G884A, A890T, G965A, T992C, and A998G) were detected in the GTZ population. Two synonymous mutations (G890A and A829C) were observed in the GLL population. The nonsynonymous mutation M295L, corresponding to A885T, was exclusively found in the SQL population, as shown in Fig. 4.

Discussion

H. longicornis is widely distributed in 441 counties in China (Zhao et al. 2020). It can transmit nearly 30 pathogens that harm humans and animals, posing a serious challenge to medicine and veterinary medicine (Zhao et al. 2020; Nwanade et al. 2022). Monitoring the

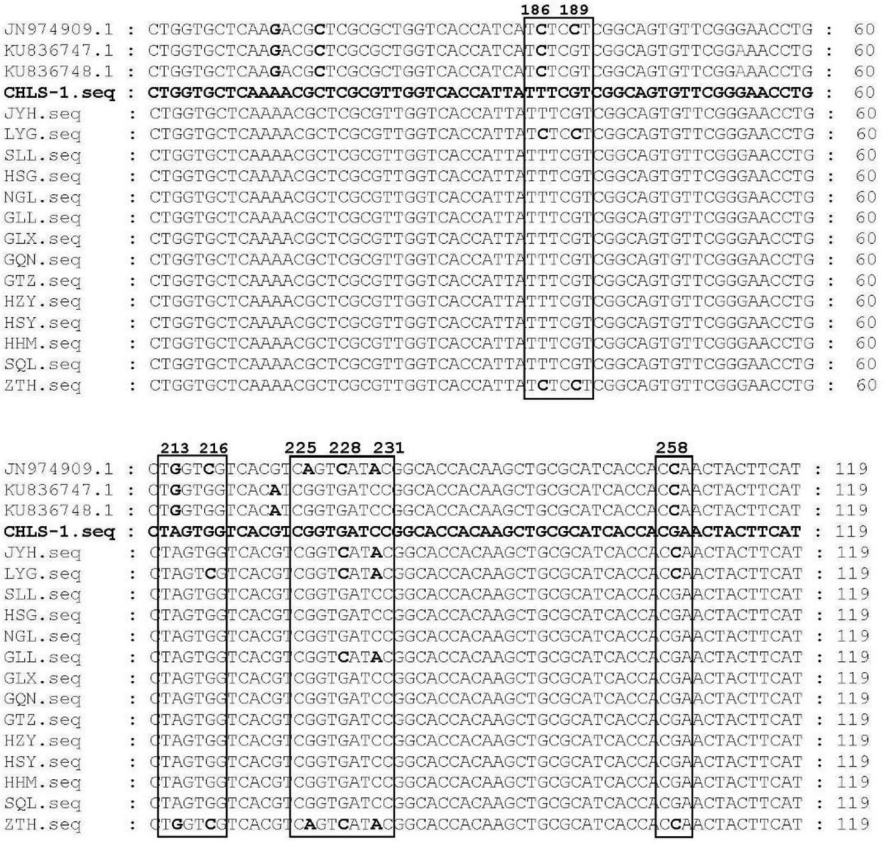

Fig. 3 Nucleotide sequence alignment of the β AOR fragment of H. longicornis with reference sequences of R. microplus and R. sanguineus. JN974909 is an R. microplus strain, whereas KU836747 and KU836748 are R. sanguineus strains. The bold text indicates the sensitive H. longicornis strain

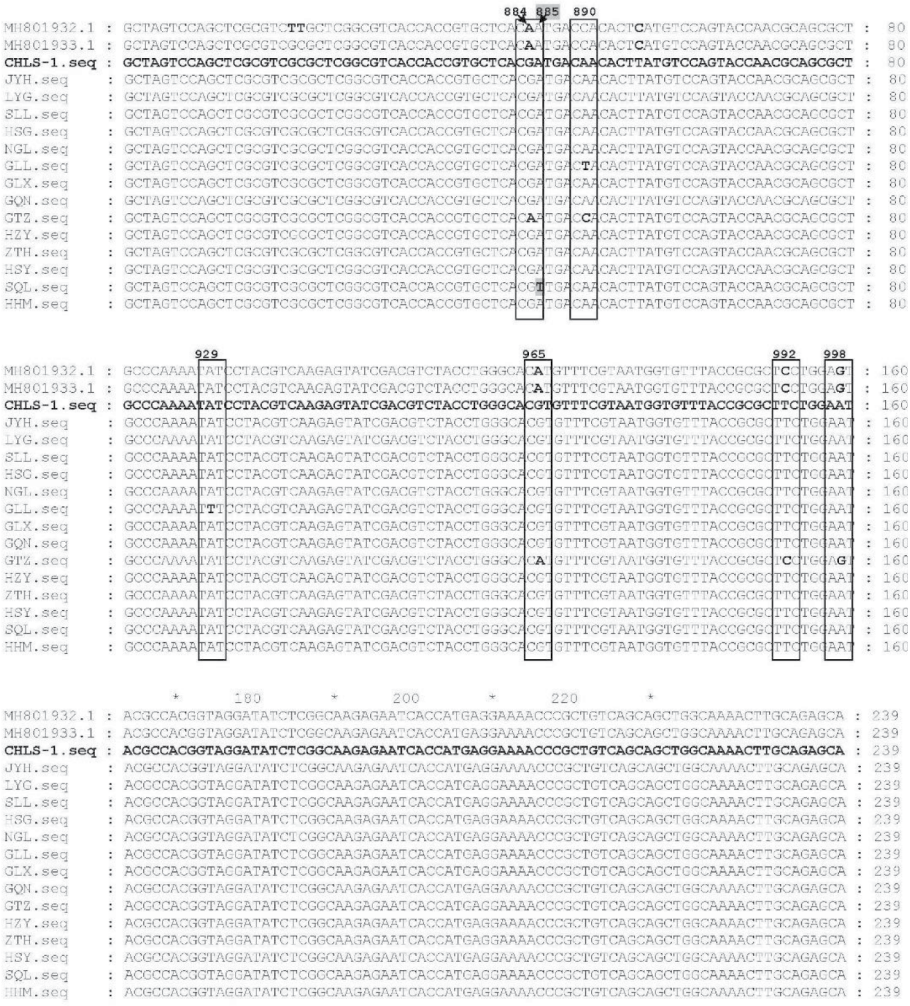

Fig. 4 Nucleotide sequence alignment of the GABA-gated chloride channel gene fragment of H. longicornis with reference sequences of sensitive and resistant R.microplus strains. MH801932.1 is a resistant strain of R. microplus, whereas MH801933.1 is a sensitive strain of R. microplus. The bold text indicates the sensitive H. longicornis strain

resistance status of *H. longicornis* and understanding its potential resistance mechanisms are crucial for controlling and managing resistance. Therefore, in this study, a complete workflow for monitoring the resistance of *H. longicornis* to five common neurotoxic acaricides was developed. For the first time, the molecular marker of *R. microplus* was used on *H. longicornis*. The molecular markers used for prediction included VGSC, β AOR, and GABA-gated chloride channel genes, which are associated with resistance to synthetic pyrethroids, amitraz, and fipronil, respectively (Casida and Durkin 2015; Taylor Wells et al. 2015; De Rouck et al. 2023). Through molecular markers, resistance-related genes can be quickly analysed to predict and track drug resistance in the early stages of field populations

of *H. longicornis* to prevent the increasingly serious problem of tick resistance, which are important parts of resistance management strategies.

Most resistance studies have focused on one-host tick, *R. microplus* (Obaid et al. 2022), whereas few studies have investigated three-host ticks, including *H. longicornis* (Obaid et al. 2025b). According to extensive reports, the resistance of one-host ticks to acaricides is often greater than that of three-host ticks (Singh and Rath 2014; Ouedraogo et al. 2021). One-host ticks are exposed to acaricides on the same host at all stages of their life, posing significant chemical challenges. The life stages of three-host ticks may be exposed to acaricides inconsistently among different hosts, and most of their life cycle is spent outside the host (Singh and Rath 2014).

In this study, the resistance level of *H. longicornis* to three pyrethroids ranged from S to III, with the highest resistance factor being 29.57, which is consistent with the range of three-host tick resistance levels in other countries. In the study by Obaid et al. (2025a), the survival rate after 24 h (12.49%) of exposure to the highest concentration of deltamethrin (6 mg/mL) was only used to indicate the possible existence of *H. longicornis* species in China that are resistant to deltamethrin. Sensitive strains were not selected, and the resistance level was not indicated. Ouedraogo et al. (2021) reported that *Amblyomma variegatum* of Burkina Faso is sensitive to cypermethrin, but the reference sensitive strain used was *Rhipicephalus geigyi*. *R. geigyi* is a one-host tick, whereas *Amblyomma variegatum* is a three-host tick, whose resistance is typically greater than that of a three-host tick. The RF_{50} derived from this sensitive strain may be lower. However, most studies on three-host ticks have focused on *H. anatolicum* in India. Prerna et al. (2019) reported that the resistance of *H. anatolicum* to deltamethrin in the western region of Punjab Province, India, ranged from level I to level II. Similarly, in India, Nandi et al. (2015) reported that *H. anatolicum* has a resistance level of I to III to deltamethrin. In the report by Shyma et al. (2012), 10 regions in India had level I resistance to deltamethrin and cypermethrin, whereas one region (Moga) had level II resistance to deltamethrin. In India, they have their own laboratory-screened sensitive strains (IVRI-II) (Shyma et al. 2012). This study also referred to the sensitive strain of *H. longicornis* in China (Diao et al. 2025); therefore, the RF_{50} obtained is more accurate. This study referred to the grading method of India (Shyma et al. 2012), with clear and precise resistance levels.

The intensity of amitraz resistance in this study is consistent with observations from other countries. According to Evans, the *Amblyomma variegatum* population in East and West Africa has an RF of up to 234.9 for amitraz (Evans and Maqueira 2005). The highest RF_{50} of the *Amblyomma scutum*-resistant population in Brazilian national parks is 56.2 (Cardoso et al. 2023). The *Hyalomma anatolicum anatolicum* population in India developed level II resistance to amitraz (Singh et al. 2015). In this study, the RFs of amitraz (4.50–56.97) were greater than those of the other chemical acaricides. Approximately 90% of the ticks in the field population have a resistance level of level II or above to amitraz. This scenario is related to the widespread use of amitriptyline as a substitute for organophosphates and synthetic pyrethroids in the twentieth and twenty-first centuries (FAO 2004). Notably, using the traditional LPT method to detect resistance to amitraz resulted in a high slope in the dose–mortality curve, making it impossible to calculate the LC_{50} and LC_{99}. Therefore, in this study, the improved LPT method was chosen; nylon cloth was used instead of filter paper according to the FAO regulations, and the experimental time was extended from 24 to 48 h (FAO 2004; Miller et al. 2002, 2007; Li et al. 2004).

Three-host ticks from different countries exhibit widespread resistance to fipronil. Monitoring the resistance of *Amblyomma mixtum* in Mexico revealed a mortality rate of 65.3% at the distinguishing concentration of fipronil, indicating possible resistance (Bravo-Ramos et al. 2025). Resistance to fipronil has also been reported in four populations in the United States (Eiden et al. 2015). In Brazil, the resistance rate of *R. sanguineus* to fipronil is abnormally high (Becker et al. 2019). In Turkey, the resistance ratio of *R. sanguineus* to fipronil ranged from 1.23–15.87 (Koc et al. 2022). In this study, the resistance rate of *H. longicornis* to fipronil was also high (100%), and except for a few field populations (HHM and HSG), the resistance of the other field populations was between level I and level II. The resistance situation is generally consistent with the reference literature. HHM and HSG may be related to frequent dosing by individual sheep owners, as it is understood that sheep in these areas take acaricides every two months. In this study, regardless of the type of acaricide used, the resistance factors were very broad. This result occurred because the collection sites were all unorganized farms, and farmers chose the type, concentration, and frequency of administration themselves, resulting in different resistance factors. In this study, only the farmers from the two farms provided feedback on their use of acaricides. This information should be included in a survey questionnaire on the frequency of acaricide use on each farm in future experiments.

In this study, all the tick populations were resistant to three or more chemical acaricides (10/10), especially cyhalothrin and amitraz (10/10). These findings indicate that the resistance of *H. longicornis* to multiple acaricides is common in China; this phenomenon also occurs in arthropods in other countries (FAO 2004; Abbas et al. 2014; Ferreira et al. 2025). *R. microplus* in Brazil was confirmed to exhibit multidrug resistance to cypermethrin, amitraz, ivermectin, and chlorpyrifos (Villar et al. 2020). Ferreira reported resistance to multiple acaricides, including amitraz, cypermethrin, ivermectin, chlorpyrifos, and fipronil, with 98% of the population resistant to three or more acaricides (Castro et al. 2019; Ferreira et al. 2025). Cross-resistance between type I and type II pyrethroid acaricides has been documented. Moyes et al. (2021) advised against switching between different pyrethroid acaricides to reduce resistance in *Anopheles gambiae* until the mechanisms of action of these acaricides were determined. The three acaricides tested in this study, i.e., cyhalothrin, cypermethrin, and deltamethrin, are type II pyrethroids, and 40% of the population exhibited mild cross-resistance to all three acaricides. Therefore, prompt monitoring of the resistance of *H. longicornis* field populations is necessary to control and prevent the spread of acaricide resistance among ticks nationwide.

The C190A mutation in VGSC was not detected, possibly because of a low RF. In this study, the highest RF for deltamethrin was observed for the field population of *H. longicornis* from the HSY, with a value of 24.2. The nonsynonymous mutation C190A in the VGSC domain II (IIS4-5) was found only in the field population of *R. microplus*, with an RF of up to 34.9 for deltamethrin (Ziapour et al. 2017). In this study, two nucleotide mutations (G240C and G255C) were detected. These two mutations have also been reported in *H. longicornis* (Obaid et al. 2025a). With respect to β AOR mutations, the A225G mutation was discovered in the genus *Rhipicephalus* (*R. appendiculatus*, *R. evertsi*, *R. sanguineus*, and *R. decoloratus*) (Shyma et al. 2015), whereas the G225A mutation was detected in *H. longicornis* in this study. Similar mutations include C189G, C216G, and A231C in the genus *Rhipicephalus* (Koh-Tan et al. 2016) and G189C, G216C, and C231A in *H. longicornis*. With respect to the GABA-gated chloride channel gene, three synonymous mutations (G884A

and A890C/T) in the TM2 region and the corresponding synonymous mutations M295L, A885T, and A929T in the TM2 and TM3 junction regions and G965A, T992C and A998G in the TM3 region were detected. The novel nonsynonymous mutation M295L in the TM2 region may alter GABA-gated chloride channel function, resulting in acaricide resistance. Further validation and functional characterization studies are needed to determine whether the M295 mutation in the TM2 domain of the GABA-gated chloride channel gene confers resistance to fipronil. The A268S/L mutation was also not detected in the population of *R. microplus* resistant to fipronil in northern Uruguay, and a molecular marker for fipronil resistance in the *R. microplus* population in northern Argentina has not been reported (Castro et al. 2019). In this study, no nonsynonymous mutations were found, and most of the results revealed synonymous mutations that did not alter amino acid sequences. It is plausible that the mechanisms underlying pyrethroid resistance differ between *R. microplus* and *H. longicornis*, possibly because of variations in the structure or expression of target-site genes or in the detoxification pathways involved in pyrethroid metabolism.

This is the first study in China to determine acaricide cross-resistance and resistance to multiple acaricides in *H. longicornis*. The cross-resistance of *H. longicornis* to pyrethroids is common in China, but resistance levels are often low. However, the pronounced resistance to amitraz and fipronil warrants attention. Based on these findings, the five acaricides investigated in this study should be used judiciously in future tick management programs, preferably in rotation with other control options. This study highlights the importance of monitoring continuous acaricide resistance and provides valuable insights for developing effective tick management strategies, preventing the spread of resistance and guiding the development of new acaricides.

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Author contribution PWD, QYR, and GYL contributed to the conception and design of the study; BZ, WGL, YYZ, GQG, JXL, and HY organized the collection of samples; PWD organized the bioassays; PD and MKO conducted the experiments; PWD, QYR, JXL, MKO, and GYL revised the manuscript and contributed to the interpretation and analysis of the data. All authors have read and approved the manuscript.

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Data availability All the data generated or analysed during the current study are included in this published article.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval This study was approved by the Research Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (LVRI, CAAS), China (permission no. LVRI-AEC-2023–043). During tick collection, all the rabbits were handled in accordance with the Animal Ethics procedures and guidelines of the People's Republic of China.

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