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PCR-RFLP analysis of insecticide resistance to pyrethroids, organophosphates and carbamates in *Musca domestica* L.

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ABSTRACT

Introduction. Zoophilic flies play a significant role in animal disease transmission, and insecticide resistance being a relevant veterinary issue globally is an obstacle to effective fly population control. Molecular methods are more commonly used to monitor and diagnose insecticide resistance in insect populations.

Objective. The study aims to assess distribution of the main mutations associated with resistance to pyrethroids, organophosphorus compounds and carbamates in three field populations of *Musca domestica* L. collected in 2021–2023 in livestock facilities of the Tyumen Oblast.

Materials and methods. Genotyping of *CYP*, *vssc* and *ace-2* genes was performed using polymerase chain reaction and restriction fragment length polymorphism analysis.

Results. One mutation in the *vssc* gene (L1014F) associated with resistance to pyrethroids and two mutations in the *ace-2* gene (G342A, G342V) conferring resistance to organophosphorus compounds and carbamates were found. The resistant allele L1014F was present in 40–70% of the tested insects of all three populations with 30–55% frequency. The G342A allele was found in 10 and 60% of insects from two populations with frequencies of 5 and 30%, respectively. The G342V allele was detected in 40% insects of only one population with a frequency of 25%.

Conclusion. The results obtained indicate the potential for conferring resistance to pyrethroids, organophosphorus compounds and carbamates in the studied populations of *Musca domestica*, which should be taken into account when selecting disinsectants for livestock-keeping facilities and protecting animals from insects. Further molecular tests of *Musca domestica* flies from the regions bordering the Tyumen Oblast will be useful for developing a strategy to contain spread of resistant alleles in local populations.

Keywords: house flies, insecticides, insecticide resistance, resistance markers, molecular diagnosis

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Анализ инсектицидной устойчивости к пиретроидам, фосфорорганическим соединениям и карбаматам у *Musca domestica* L. методом ПЦР-ПДРФ

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РЕЗЮМЕ

Введение. Значимым фактором в распространении заболеваний животных являются зоофильные мухи, контроль численности которых осложняется проблемой инсектицидной резистентности, актуальной для ветеринарии и медицины во всем мире. Для мониторинга и диагностики устойчивости к инсектицидам в популяциях насекомых все большее применение находят молекулярные методы.

Цель исследования. Оценка распространения основных мутаций, ассоциированных с резистентностью к пиретроидам, фосфорорганическим соединениям и карбаматам, в трех природных популяциях *Musca domestica* L., собранных в 2021–2023 гг. в животноводческих помещениях Тюменской области.

Материалы и методы. Методом полимеразной цепной реакции с анализом полиморфизма длин рестрикционных фрагментов выполнено генотипирование генов *CYP*, *vssc* и *ace-2*.

Результаты. Выявлена одна мутация в гене *vssc* (L1014F), связанная с устойчивостью к пиретроидам, и две мутации в гене *ace-2* (G342A, G342V), обеспечивающие резистентность к фосфорорганическим соединениям и карбаматам. Резистентный аллель L1014F присутствовал у 40–70% исследованных особей всех трех популяций с частотой 30–55%. Аллель G342A обнаружен у 10 и 60% особей двух популяций с частотой 5 и 30% соответственно. Аллель G342V выявлен у 40% особей только одной популяции с частотой 25%.

Заключение. Полученные результаты свидетельствуют о потенциале формирования устойчивости к пиретроидам, фосфорорганическим соединениям и карбаматам в исследованных популяциях *Musca domestica*, что необходимо учитывать при выборе средств для дезинсекции животноводческих помещений и защиты животных от насекомых. Дальнейшие молекулярные исследования *Musca domestica* из граничащих с Тюменской областью регионов будут полезны для выработки стратегии по сдерживанию распространения резистентных аллелей в локальных популяциях.

Ключевые слова: комнатная муха, инсектициды, инсектицидная резистентность, маркеры устойчивости, молекулярная диагностика

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INTRODUCTION

Insects are a significant factor in the spread of various human and animal diseases [1, 2], including synanthropic and zoophilic flies, in particular *Musca domestica* L. house fly (*Diptera*: *Muscidae*) [3, 4]. The ability of adult *M. domestica* to be a mechanical vector of such pathogens as helminth eggs, protozoa, viruses and bacteria, including antibiotic-resistant strains, has been demonstrated in a number of studies [4, 5, 6, 7]. Thus, *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni* causing bovine respiratory diseases were recovered from *M. domestica* collected at feedlots from animals suffering from bovine respiratory disease symptoms [5]. When homogenates prepared from house flies from US dairy and livestock farms were tested, tetracycline and florphenicol resistance genes with prevalence ranging from 5 to 95.8% were identified in recovered bacteria [6]. The ability of Newcastle disease virus to persist in an infectious dose in the gut of flies for four days after feeding with infected milk and for one day in chicken droppings has been shown under laboratory conditions [7], which increases the risk of disease spread via flies present in poultry farms. Given the veterinary importance of zoophilic flies, it is necessary to control their numbers.

Despite the great interest in pest control biological methods, the chemical method based on the use of synthetic insecticidal agents remains widely used. Synthetic pyrethroids, neonicotinoids, organophosphorus compounds (OPCs), and carbamates are most often used for protecting animals from insects and disinsecting livestock premises both in Russia and abroad [4, 8]. *M. domestica* quite rapidly develop resistance against insecticides when used intensively: for example, more than 20-fold increase of resistance to permethrin [9] and alpha-cypermethrin [10] was revealed under laboratory conditions over 10–20 generations. According to a number of studies, resistance to pyrethroids (deltamethrin, permethrin, beta-cyfluthrin, cypermethrin) was observed in house fly field populations in China [11, 12], Pakistan [9], Iran [13], USA [14], Saudi Arabia [10, 15], the Moscow and Kaluga Oblasts of the Russian Federation [8]. In the Tyumen Oblast, tolerant and exceptionally highly pyrethroid-resistant field populations were also recorded [16, 17]. OPC-resistant house fly populations were found, for instance, in China [12], Iran [18], and Saudi Arabia [15, 19]. Insecticide resistance of *M. domestica* field populations makes it difficult to control their numbers.

The molecular target of pyrethroids is voltage-sensitive sodium channels (*vssc*), and the presence of mutations in the genes encoding this protein, i.e. knock-down resistance (*kdr*), is recognised as a marker of resistance to pyrethroids [14, 20]. Of the five known alleles associated with target insensitivity and, consequently, pyrethroid resistance of insects, the *kdr* (L1014F) and *kdr-his* (L1014H) are the most frequently investigated [13, 14, 20]. Target insensitivity is often combined with another major mechanism of pyrethroid resistance, namely enhanced detoxification of insecticides via cytochrome P450-dependent monooxygenases (*CYP*). A confirmed molecular marker of this type of resistance is the presence of a 15-base pair (bp) insertion in the *CYP6D1* gene [21, 22]. Acetylcholinesterase (AChE), encoded by the *ace* gene, is a key enzyme of the cholinergic system and a major target of OPC and carbamate insecticides, which block the transmission of nerve impulses at cholinergic synapses. Resistance to OPC and carbamates may result from insensitivity of AChE due to mutations in

the *ace* gene or due to mutations in the carboxylesterase gene, leading to an increase in the hydrolytic activity of the enzyme with respect to OPC [20, 23]. *M. domestica* is known to have only one AChE-encoding gene, *ace-2* [24], and six major mutations associated with resistance to OPC and carbamates have been described in detail: V260L, A316S, G342A, G342V, F407Y, and G445A [25, 26, 27].

Analysis of insecticide resistance in *M. domestica* field populations in Russia is traditionally carried out using toxicological methods [8, 17, 28], which allow establishing the presence of a stable phenotype and the level of resistance and do not explain the mechanisms underlying insecticide resistance [29]. The resistance mechanisms are defined and the potential for its formation is assessed using biochemical and molecular methods [30], and these steps are critical for rationalized selection of insecticidal agents and development of insecticide application schemes. As compared to traditional toxicological methods, molecular tests provide more complete information on the population structure, and the combination of toxicological and molecular methods allows objective assessment of the level of adaptation of the population to insecticide load [31]. Among molecular methods for detecting mutations associated with insecticide resistance, PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) is used [32]. The PCR-RFLP method is cost-effective, easy to implement and requires only basic molecular genetic equipment; it is widely available and is a good alternative to sequencing.

The aim of the study was to test *Musca domestica* flies collected from three field populations in the Tyumen Oblast for the presence of mutations in *CYP*, *vssc* and *ace-2* genes associated with resistance to pyrethroids, OPC and carbamates by PCR-RFLP.

MATERIALS AND METHODS

The study was aimed at *M. domestica* flies of three field populations: Nov (56.53700°, 65.24238°), Cha (56.781583°, 65.96014°), Nik (55.55352°, 70.62864°) collected in livestock facilities of the Tyumen Oblast in 2021–2023. The first generation (F1) was obtained from the collected insects of each population under insectarium conditions, 3–5 day old adult flies were frozen and stored at –80 °C before they were used for testing.

DNA was isolated from adult flies (5 females and males of each population) using alkaline lysis [33]. The amplification process was performed with GeneExplorer GE-96G (Bioer, China) using an individual primer pair for each gene. P1, P2, P3, P4 primers were used for genotyping mutations in the *vssc* gene, and AceF and AceR primers taken from the study of X. Qiu et al. [32] were used for the *ace-2* gene. For genotyping of mutation in the *CYP6D1* gene, the S35 and AS2 primers and restrictase were used according to F. D. Rinkevich et al. [34]. The amplification conditions were identical except for the temperature of primer annealing (Table 1): at 95 °C for 5 min, further at 95 °C for 20 s, at 62–53 °C for 30 s, at 72 °C for 30 s (5 cycles), at 95 °C for 20 s, at 60–51 °C for 30 s, at 72°C for 30 s (35 cycles), at 72 °C for 10 min. The PCR reaction mixture included: 1 µL of total DNA; 4 µL of 5X ScreenMix-HS PCR prepared mix (Eurogen, Russia); 0.3 µL of each primer (25 µM); 14.4 µL of purified sterile water (18.2 µS/cm). The restriction enzymes and test conditions are indicated in Table 1. Visualization of restriction results was performed through electrophoresis with 2% agarose gel containing ethidium bromide.

RESULTS AND DISCUSSION

The prevalence and frequency of mutations associated with resistance to pyrethroids and OPCs have been investigated in *M. domestica* field populations in Denmark [35], Turkey [36], Iran [26, 37], USA [14, 34], Kazakhstan [22], United Arab Emirates (UAE) [38] and other countries. Regarding *M. domestica* populations in the Russian Federation, resistance to pyrethroids and other insecticides was previously assessed using mainly toxicological methods [8, 17, 28]. Data on molecular test results of the house fly field populations and the genetic potential for insecticide resistance in local populations of the Russian Federation have not been published in the open access.

Sse9I and Fat I restrictases are used *for* ***vssc genotyping*** with PCR-RFLP. The Sse9I restrictase cuts the amplicon into 2 fragments of 96 and 60 bp, respectively, in the presence of the L1014F mutation. The L1014H mutation is detected using the Fat I enzyme, which, in the presence of the mutation, cuts the 220 bp amplicon into fragments of 170 and 50 bp long, respectively [22]. Combining the both test results, we identified the following genotypes (Fig. 1): 1014 (L/L), 1014 (L/F), 1014 (F/F). The L1014F mutation was detected in 70% of the tested flies of the Nov and Cha populations and in 40% of the flies of the Nik population (Table 2).

Table 1
PCR-RFLP assay conditions

Gene	Primers (5′–3′)	Annealing temperature, °C	Amplicon length, bp	Restrictase	Mutation	Restriction conditions
vssc	P1. GTGCTGTGCGGAGAGTGG P2. GAAGCCTCCATCCTGGGAG	60	156	Sse9I	L1014F	3 h – 55 °C; 20 min – 65 °C
	P3. AGCTGTATACCCTTCTTCT P4. CGAAGTTGGACAAAAGCAAA	51	220	Fat I	L1014H	
CYP6D1	S35. AGCTGACGAAATTGATCAATCAGT AS2. CATTGGATCATTTTTCTCATC	59	732–711	Hpy 188III	CYP6D1v	1 h – 37 °C; 20 min – 65 °C
ace-2	AceF. CGGTGCATTTGGGTTTCTAC AceR. CGTAACCGCTAAGATCTGCTG	57	609	Mh1 I	G342	3 h – 37 °C; 20 min – 80 °C
				Aco I	G342A	3 h – 37 °C; 20 min – 65 °C

Table 2
Distribution of detected mutations associated with insecticide resistance in three populations of *M. domestica* in the Tyumen Oblast

Population	Number of flies	Proportion of flies with L1014F mutation, %	Number of flies with the genotype			Allele frequency, %	Proportion of flies with mutation, %		Number of flies with the genotype			Allele frequency, %	
			L/L	L/F	F/F	F	G342A	G342V	G/G	G/A	G/V	A	V
Nov	10	70	3	3	4	55	0	0	10	0	0	0	0
Cha	10	70	3	4	3	50	60	0	4	6	0	30	0
Nik	10	40	6	2	2	30	10	40	5	1	4	5	25

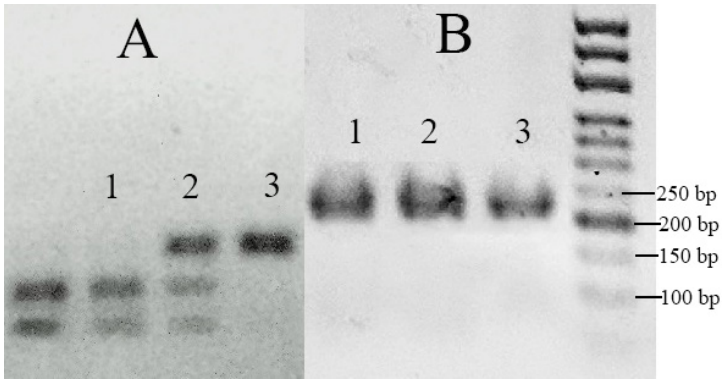

Fig. 1. Electrophoregram for PCR-RFLP amplification products of the vssc gene region: A – using Sse9I restrictase; B – using Fat I restrictase; 1 –1014 (F/F), 2 – 1014 (L/F), 3 – 1014 (L/L)

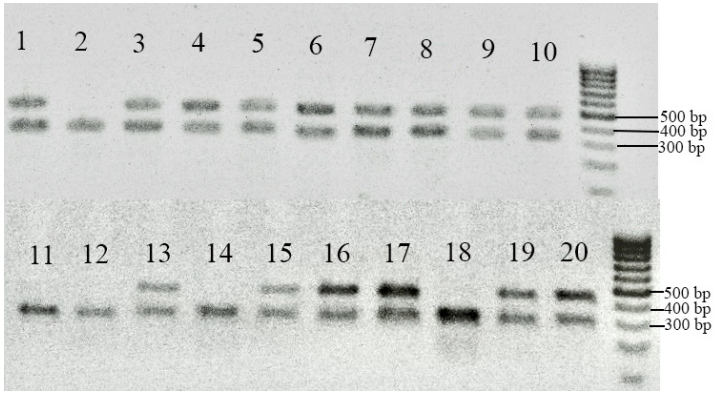

Fig. 2. Electrophoregram for PCR-RFLP amplification products of CYP6D1 gene region using Hpy 188III restrictase: 1–20 – different M. domestica species

Hpy 188III restrictase is used ***for CYP genotyping*** with PCR-RFLP. The resistant allele *CYP6D1v1* is characterised by a 15 bp insertion that disrupts the recognition sequence of the Hpy 188III enzyme. As a result, after restriction, fragments of 432 and 279 bp will be characteristic of the wild-type genotype, and 732 bp will be characteristic of the genotype carrying the mutation [34]. No resistant allele of *CYP6D1v1* was detected during the study, but Figure 2 shows that in some flies the 432 bp band is additionally cut by the Hpy 188III enzyme.

PCR-RFLP assay of the ace-2 gene was performed using Mh1 I and Aco I enzymes. The Mh1 I restrictase has a restriction site (GGC) that is characteristic of the wild-type genotype, 342G. After restriction, the two fragments of 361 and 248 bp detected in the electrophoregram are indicative of a wild type genotype, and a 609 bp fragment is indicative of the G342A or G342V mutation. The Aco I restrictase identifies the G342A mutation and cuts the amplicon into 2 fragments of 361 and 248 bp long. Thus, combining the two assays allows the detection of 6 different genotypes [32]. In our study we managed to detect 3 different genotypes (Fig. 3). G342A or G342V mutations were found in the Nov population. In the Nik population, the proportion of flies with G342A and G342V mutations was 10 and 40%, respectively. In the Cha population, only G342A mutation was detected in 60% of flies (Table 2).

In total, 3 (L1014F, G342A, G342V) out of 5 tested mutations were identified using the PCR-RFLP. The distribution frequencies of the resistant alleles in the three populations are presented in Table 2. The *kdr* mutation (L1014F) was found in the hetero- and homozygous state in 7 out of 10 flies of the Nov and Cha populations and in 4 out of 10 flies of the Nik population. The *kdr-his* mutation (L1014H) was not detected in any of the three populations. Test results for field populations of *M. domestica* in Turkey showed that the frequency of *kdr* and *kdr-his* alleles was 8 and 20%, respectively [36]. A survey of six field populations of the house fly in Kazakhstan showed the presence of the *kdr* allele in one of the populations with a frequency of 5% and the *kdr-his* allele in another population with a frequency of 14.3% in the heterozygous state [22]. Interestingly, the L1014F mutation was not reported in the Iranian population of *M. domestica*, and the percentage of *kdr-his* polymorphism (L1014H) was low at 4.7% [37]. On the contrary, in the USA, the *kdr* (L1014F) mutation was present in all six studied populations of house flies found in poultry and livestock farms, and *kdr-his* (L1014H) mutation was present in five populations. The frequency of *kdr-his* and *kdr* alleles varied widely in the populations, ranging from 12.5–28.1% and 7.1–76.6%, respectively [14]. A recent paper reported the detection of the *kdr* allele in *M. domestica* flies from the United Arab Emirates with the frequencies ranging from 9.4 to 46.9% [38]. The frequency of the resistant *kdr* allele (30–55%) in house fly populations in the Tyumen Oblast is comparable to that of populations from the USA and the UAE.

According to literature data, the knockdown resistance was first reported in house flies in the 1950s as insensitivity of sodium channels to the action of dichlorodiphenyltrichloroethane (DDT). It was later found that such resistance was associated with a nucleotide substitution (cytosine for thymine) in the *vssc* gene, resulting in the replacement of leucine with phenylalanine at position 1014 (L1014F) of the sodium channel alpha subunit [39]. As a result, structural changes in the protein molecule occur, affecting the interaction of the insecticide with the target. This

mutation also leads to the formation of resistance to pyrethroids, as they have a similar mechanism of action to DDT. The L1014F mutation, in addition to *M. domestica*, has been found in other two-winged insects (e.g., *Culex* and *Anopheles* mosquitoes, *Haematobia* fatheads), red cockroach (*Blattella germanica*), cat flea (*Ctenocephalides felis*), rat flea (*Xenopsylla cheopis*), triatomine bugs (e.g., *Triatoma infestans*), and other arthropods [39, 40].

One of the sufficiently described mechanisms of resistance to pyrethroids in insects is the enhancement of detoxification mediated by cytochrome P450-dependent monooxygenases (*CYP*) [41]. This type of insecticide resistance in *M. domestica* is associated with increased expression of the *CYP6D1* gene in the presence of 15 bp insertion (*CYP6D1v1* allele) [34]. In the USA, the resistant *CYP6D1v1* allele was detected with a frequency of > 75% in 5 studied populations of *M. domestica* [14]. According to V. Taşkın et al., the frequency of *CYP6D1v1* in house fly population from Turkey was 39% [36]. In Kazakhstan, this allele was present in 3 out of 6 populations of *M. domestica* with a much lower frequency: 4.4–6.3% [22]. In our study, PCR-RFLP assay did not reveal an insertion characteristic of the resistant allele of *CYP6D1v*; however, a mutation described earlier for *M. domestica* laboratory culture was detected in flies from the Nov and Cha populations [42]. Freeman J. C. et al. rightly pointed out in their study that *CYP6D1v1* is only partially responsible for the increased expression level of *CYP6D1* [14]. Due to the high evolutionary plasticity of *CYPs*, their other representatives or other mutations not yet described may be involved in the formation of resistance to insecticides – in general, and pyrethroids – in particular, in local *M. domestica* populations.

Detection of a rather large percentage of flies with the *kdr* mutation among *M. domestica* of the three field populations under study is not surprising, since, according to the surveys, pyrethroids (mainly deltamethrin and cyfluthrin) had been used for premise disinsection and animal protection from annoying insects for several seasons in livestock farms where the flies were collected. The use of these insecticides in this case served as a selection factor that apparently allowed the *kdr* (L1014F) mutation to gain a foothold in the populations under study. It is believed that in the presence of the *kdr* (L1014F) mutation, a higher level of pyrethroid resistance is formed than in the presence of the *kdr-his* (L1014H) mutation [36, 37]. In order to slow down the emergence of populations highly resistant to pyrethroids, it is advisable to replace pyrethroids with insecticides with a different mechanism of action (e.g., pyrroles, oxadiazines, insect growth regulators, etc.) in the studied livestock farms.

The higher *Diptera* have only one AChE-encoding gene and, accordingly, mutations providing resistance to OPCs and carbamates in this group of insects were found only in the *ace-2* gene. Such mutations individually or in combination lead to amino acid substitutions close to the catalytic triad of the active centre of the enzyme, affecting the orientation of the amino acids of the triad and limiting the access and/or binding of bulk insecticides (enzyme inhibitors) in the substrate centre of the protein [25]. Six such mutations have been described in detail for *M. domestica*: V260L, A316S, G342A, G342V, F407Y and G445A [25, 28]. In addition to *M. domestica*, resistance to OPCs and carbamates is known to be formed by a similar mechanism in other insect species, such as the green meat fly *Lucilia*

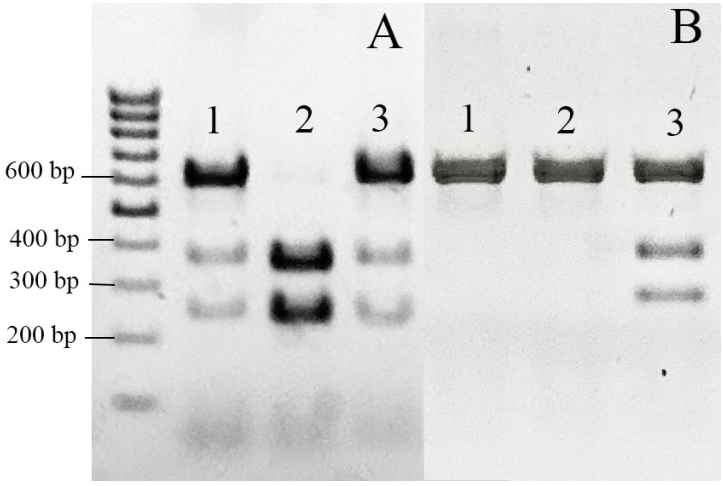

Fig. 3. Electrophoregram for PCR-RFLP amplification products of ace-2 gene region: A – using Mh1 I restrictase; B – using Aco I restrictase; 1 – 342 (G/V), 2 – 342 (G/G), 3 – 342 (G/A)

cuprina [43], *Drosophila melanogaster* [44, 45], and tephritid fruit flies *Bactrocera oleae* [46] and *Bactrocera dorsalis* [47]. In their study S. Başkurt et al. [48] indicated equivalent substitutions of amino acid residues in the AChE molecule for *M. domestica* and *D. melanogaster*. Literature data indicate that mutations underlying resistance of the house fly to OPCs and carbamates are widespread worldwide. Thus, resistant alleles G342A and G342V were found in flies of field populations of *M. domestica* of the USA, China, Iran, Kazakhstan [14, 22, 26, 49]. In house fly populations from Kazakhstan, G342A and G342V resistant alleles were found with a frequency of 27–48 and 0–20%, respectively [22]. G342A and G342V mutations were detected in 30 and 40% of *M. domestica* flies from Iran, respectively [26]. In our study, the G342V resistant allele was only present in the Nik population (the mutation was present in 40% of flies) with a frequency of 25%, the G342A allele in the Nik (in 10% of flies) and Cha (in 60% of flies) populations with a frequency of 5 and 30%, respectively, and these mutations were not detected in the Nov population. It is assumed that the allele with the G342V mutation plays a more significant role in AChE insensitivity and the formation of a high level of resistance to certain insecticides compared to that with G342A mutation [14, 25, 49].

CONCLUSION

In this study, PCR-RFLP assay showed presence of the *kdr* allele (L1014F), responsible for resistance to pyrethroids, with a frequency of 30–55% and the G342A/V alleles associated with resistance to OPCs and carbamates, with a frequency of 5–30% in flies from three and two field populations of *M. domestica* in the Tyumen Oblast, respectively. The presented data indicate the potential for formation of resistance to pyrethroids, OPCs and carbamates in the studied populations. On the basis of the obtained results it is possible to recommend replacement of these insecticides during disinsection of livestock facilities with preparations from other groups in order to mitigate the spread of resistant alleles in local populations of *M. domestica*. Further molecular studies of insects from different regions of the country are required to assess more fully the situation regarding resistance to pyrethroids, OPCs and carbamates and the potential for its formation in *M. domestica* in Russia.

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