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Rapid detection of phosphine resistance in the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrychidae) from China using ARMS-PCR

Yujie Lu [1, +, *], Chenguang Zhang [1,+], Zhenyan Wang [1], Xiaoping Yan [2], Robert N. Emery [3]

[1]. College of Food, Science and Technology, Henan University of Technology, Zhengzhou, Henan Province, China. 450001
[2] Sinograin Chengdu Grain Storage Research Institute Chengdu, Sichuan Province, China. 610091
[3]. Biosecurity and Regulation, Entomology Branch Agriculture and Food Department of Primary Industries and Regional Development Australia Perth, WA 6151
*Corresponding author: Yujie Lu. Email address: luyujie1971@163.com;
+these authors contributed equally to the work
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Abstract

The lesser grain borer, *Rhyzopertha dominica* is one of the serious cosmopolitan stored grain pests worldwide. High phosphine resistant *R. dominica* has been reported in several countries. The evolution of strong phosphine resistance is a major challenge for continuous application of the fumigant. Rapid detection of phosphine resistance level is a prime key to implement an appropriate strategy for control the stored-product pests. Dihydrolipoamide dehydrogenase (DLD) is a key metabolic enzyme mediating the phosphine resistance in population of *R. dominica*, *Tribolium castaneum* and *Caenorhabditis elegans*. Analysis of the DLD sequences deposited in GenBank revealed that the P45/49S mutation was the most common one in many PH3-resistant stored-product pest insects. This information now enables direct detection of resistance using molecular diagnosis in field populations. We herein propose a method for rapid detection of phosphine resistance in *R. dominica* according to P49S point mutation of the DLD gene. Our data provides evidence that the ARMS-PCR method can be used for early warning of phosphine resistance in *R. dominica* in field conditions.

Tetra-primer amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method, in which two pairs of specific primers are applied was carried out based on the single nucleotide mutation in dihydrolipoamide dehydrogenase (DLD) gene. An ARMS-PCR assay was designed for diagnosing CCT to TCT (=P49S in amino acid sequence) mutation in the DLD gene sequences of *R. dominica*. The method employs four primers to amplify a common band from DNA containing the SNP and amplification representing each of the two allelic forms. Primers were designed to amplify fragments of differing sizes for each allele band in order to resolve using agarose gel electrophoresis. Primers were designed using primer1 software (http://cedar.genetics.soton.ac.uk/public_html/primer1.html). To increase the specificity of the reaction, a mismatch is introduced at the 3'end of each of the two allele-specific primers. The results of Taqman@ MGB-probe real-time PCR showed that there were no false-positive results in ARMS-PCR. In addition, the Food and Agriculture Organization (FAO) standard discriminating dose phosphine bioassay for eight *R. dominica* populations collected from China validated that the tetra-primer ARMS-PCR method was an accurate and sensitive method to diagnose *R. dominica* phosphine resistance level. The phosphine concentrations of 20 ppm phosphine for identification of weak resistance in *R. dominica* and 300 ppm for strong resistance according to FAO method were used. The sequences of the DLD gene were isolated from eight geographic populations of *R. dominica* collected from China. Further analysis of the DNA sequences revealed that the single amino acid mutated from proline to serine at the position #49 in the DLD enzyme, which are corresponding to CCC and TCC in nucleotide sequences of laboratory susceptible strain and strong phosphine resistant population of *R. dominica,* respectively. Genomic DNA was extracted from sixteen individuals from each of resistant *R. dominica* population and used as templates for PCR to generate 283-bp common band, 208-bp resistance phenotype and/or 130-bp susceptible phenotype band respectively. The discriminating fragments were a 130-bp band for susceptible (codon CCC) allele and a 208-bp band for mutant (codon TCC) alleles respectively. Therefore, genotype scoring was made according to the basis of presence/absence of 130bp and 208bp fragments, when the individual beetle was either heterozygous for the resistance allele or homozygous susceptible or homozygous resistant. A TaqMan real-time reaction was performed in parallel for P49S allele. In each reaction, a substantial increase in HEX fluorescence indicated a homozygous susceptible genotype, a substantial increase in FAM fluorescence indicated a homozygous resistant genotype and an increase in both signals indicated a heterozygote. The observed and expected ARMS results were conducted from eight populations previously discriminated by the phosphine discrimination dose. DLD allele frequencies in eight *R. dominica* populations were examined by the ARMS-PCR method to test all individuals in susceptibility bioassay. Overall, P49S allele frequency ranged from 9% to 94% in populations collected in this study. Chi-square analysis was carried to determine whether the genotype ratios at each population deviated from Hardy–Weinberg equilibrium. Significant changes in the allele frequencies across genotype frequencies within populations were used to identify phosphine resistance associated with the P49S resistance mutation in *R. dominica*. The phosphine resistance frequency of different geographical populations of *R. dominica* were diagnosed by FAO recommended method with discrimination 20ppm for weak resistance and 300ppm for strong resistance. The phosphine resistance frequency of different geographical populations of *R. dominica* were diagnosed by FAO recommended method with discrimination 20ppm for weak resistance and 300ppm for strong resistance. The results indicated that ARMS-PCR assay was easy to use, more sensitive and specific to detect the P49S mutation in phosphine resistant samples of *R. dominica* than previously used bioassay methods. Previous techniques for gene mutation detection are based on the polymerase chain reaction; many of them require post-PCR manipulations, such as isotopes irradiation, restriction enzymes, are required to two PCRs rounds. For example, using the (RFLP) typing method involved restriction and endonuclease digestion of PCR products, while some mutation sites were hard to find by appropriate restriction enzyme. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis (Campbell 2008), mass spectrometry (MALDI-TOF-MS) assays (Park et al. 2008) and direct DNA sequencing methods are a sensitive, accurate and elegant diagnostic method, but they require expensive equipment and

have complex processing. Fast and economical assays that can be performed with standard PCR instruments are highly desirable for diagnostic analyses and for scientific studies of large numbers of pests. Individual *R. dominica* from distinct geographic populations can be discriminated into resistant homozygote, resistant heterozygote and susceptible homozygote from electrophoretogram after ARMS-PCR assay. Our TaqMan@ MGB probe assay could discriminate P49S mutation in DLD gene according to fluorescence labeling intensity variation and confirmed ARMS-PCR result as well. Our results show that the rapid detection of phosphine resistance in *R. dominica* populations in China provides important information to grain industries for decision-making in pest management strategies. In addition, our results suggest that this method could be applied for the detection of phosphine resistance in other grain pests, such as *T. castaneum* and *Sitophilus oryzae*, whose DLD genes have been sequenced. Our methods could be conducted on dead insects or insect fragments. Indeed, we evaluated consumable, running and capital cost for each method. The ability to quickly diagnose the resistance of these strains would be of great benefit. Furthermore, ARMS-PCR method for identifying the resistance locus mutation provides an opportunity to valuate level of phosphine resistance in other key pest species such as *Cryptolestes ferrugineus*, *S. oryzae* and *Sitophilus zeamais*. In addition, this technology could be extended to solve other pesticides resistance. The development of ARMS-PCR does not require generation of phosphine gas in the laboratory; also does not need collection and culture of field populations. Furthermore, the results are easier to assess with naked eye.

Keywords: Phosphine resistance, Lesser grain borer, Taqman@ probe, ARMS-PCR, Dihydrolipoamide dehydrogenase

Determınatıon of toxıcıty of gaseous ozone agaınst adult stages of German Cockroach (*Blatella Germanıca* L.)

Uğur Güz[1] , Hasan Tunaz[1],, Mehmet Kubilay Er,[1] Ali Arda Işikber [1]

[1]Kahramanmaraş Sütçü İmam University, Agriculture Faculty, Plant Protection Department, Avşar Campus, 46100 Kahramanmaraş Turkey
*Corresponding author: htunaz@ksu.edu.tr
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In this study, the effects of two different concentrations of ozone gas (16.7 and 33.3 mg / L) against *Blatella germanica* adults at different exposure times (10, 20, 30, 40 and 50 minutes) were investigated under laboratory conditions. It was determined that the ozone gas had a noticeable effect on mortality of *B. germanica* adults. In general, ozone gas caused higher paralyisis-mortality rates of *B. germanica* adults than mortality rates of *B.germanica* adults at both concentrations and all exposure times. A concentration of 33.3 mg / L of ozone gas with 40 and 50 minute exposure times killed all cockroach adults after 24 hours. On the other hand, 16.7 mg / L concentration of ozone gas with 50 minute exposure time killed 90% of the *B. germanica* adults after 24 hours. When ozone gas is evaluated in terms of exposure time to *B. germanica* adults, the concentration of 33.3 mg / L of ozone gas with 10-20 minute exposure times caused 65 % adult mortality, with 30 minute exposure time caused 90% adult mortality and with 50 minute exposure times caused 100 % adult mortality after 24 hours. At a concentration of 16.7 mg / L of ozone gas, as the exposure times increased, the adult mortalities gradually increased after 24 hours and the adult mortality reached 90% with 50 minute exposure times. All these results show that ozone gas (33.3 mg / L) with 40-50 minute exposure times can successfully control *B.germanica* adults.

Keywords: Ozone gas, *Blatella germanica*, mortality, biological efficacy.

1. Introduction

Cockroaches are insect species that have remained unchanged since ancient times (Appel, 1995). There are approximately 3,500 species of cockroach in the world (Atkinson et al, 1991). Most types of cockroaches are insect species that live in outdoor environments. However, a few cockroach species are found in the living areas of insects. One of the cockraoch species found in people's