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SHORT COMMUNICATION

Biochemical Profile of *Paenibacillus larvae* Repetitive Element Polymerase Chain Reaction (rep-PCR) Genotypes in Bulgaria [1]

Nikolina RUSENOVA [1] Parvan PARVANOV [1]

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[1] Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora 6000, BULGARIA

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Summary

The aim of the present study was to determine the biochemical profile of *Paenibacillus larvae* repetitive element polymerase chain reaction (rep-PCR) genotypes in Bulgaria and to assess the link between genotype and phenotype. A total of 103 isolates (genotype AB, n=21; genotype ab, n=82) and a reference strain NBIMCC 8478 were analyzed using identification system BioLog Gen III and nitrate reducing ability. Genotypes AB and ab showed a particular metabolic fingerprint based on 71 carbon sources provided by BioLog system. Considering the nitrate reducing ability, mannitol and salicin utilization, the strains were distributed into biotypes I, III, IV and VIII. The majority of *Paenibacillus larvae* AB clustered into biotype III while ab were grouped mainly into biotype I. Biotypes I and IV were not found among the tested AB strains. This study showed the obvious link between rep-PCR genotypes AB/ab and biochemical phenotype that can be useful in epidemiologic situations to trace the source of infection and to control the disease.

Keywords: *Paenibacillus larvae, rep-PCR, genotype AB and ab, biochemical phenotype*

Bulgaristanda *Paenibacillus larvae* Repetitive Element Polymerase Chain Reaction (rep-PCR) Genotiplerinin Biyokimyasal Profili

Özet

Bu çalışmanın amacı Bulgaristan'da *Paenibacillus larvae* repetitive element polymerase chain reaction (rep-PCR) genotiplerini belirlemek ve genotip ile fenotip arasındaki ilişkiyi ortaya koymaktır. Toplam 103 izolat (genotip AB, n=21; genotip ab, n=82) ve referans suş olarak NBIMCC 8478 BioLog Gen III identifikasyon sistemi kullanılarak ve nitrat indirgeme kabiliyeti yönünden analiz edildi. Genotip AB ve ab, BioLog system ile 71 karbon temelli özel bir metabolik parmak izi gösterdi. Nitrat indirgeme kabiliyeti, mannitol ve salisin kullanımı göz önüne alınarak suşlar biyotip I, III, IV ve VIII olarak ayrıldı. *Paenibacillus larvae* AB'nin büyük bölümü biyotip III'de toplanırken ab çoğunlukla biyotip I'de gruplandı. Biyotip I ve IV test edilen AB suşları arasında tespit edilmedi. Bu çalışma rep-PCR genotipleri AB/ab ile biyokimyasal fenotipler arasındaki ilişki koyarak epidemiyolojik çalışmalarda enfeksiyon kaynağını tespit etme ve hastalığın kontrolünde faydalı olacaktır.

Anahtar sözcükler: *Paenibacillus larvae, rep-PCR, Genotip AB ve ab, Biyokimyasal fenotip*

INTRODUCTION

Paenibacillus larvae (P. larvae) is the etiologic agent of the most virulent bacterial disease of honey bees, American foulbrood (AFB) [1]. The bacterium is Gram-positive, rod-shaped, catalase negative and spore forming [2]. The infectious form of the agent are only spores which are difficult to be induced *in vitro* but are readily formed in infected larvae [3]. Therefore, spores are transmitted easily in the nest, between colonies and apiaries during the beekeeping practice leading to considerable beekeepers' losses worldwide [3]. American foulbrood is a notifiable disease in many countries including Bulgaria, where regulatory measures are observed to control the disease [4]. Incorporation of the research achievements on conventional and molecular characteristics of the agent into practice

İletişim (Correspondence)
+359 886846327
ninavelrus@yahoo.com

would be a valuable additional epidemiologic tool in AFB surveillance programs.

Biochemical properties of *P. larvae* have long been a subject of scientific interest. Carbolytic, proteolytic and lipolytic activities of the bacterium have been studied [5]. However, the researchers' reports have been somewhat in disagreement with each other. Jelinski [5] proposed a scheme to biotype *Bacillus larvae (B. larvae*, now *P. larvae)* based on nitrate reduction, acid production from mannitol and salicin. Hence, biotypes I-VIII were possible. These variable features might be of importance in distinguishing the biochemical types of the bacterium in disease outbreaks. With the development of molecular typing methods different genotypes of *P. larvae* have been recognized. The most exploited molecular techniques for typing *P. larvae* were the repetitive element polymerase chain reaction (rep-PCR) [6-9] and pulse-field gel electrophoresis (PFGE) [10,11]. There is still little information about the link between different genotypes established with rep-PCR and their phenotype [1,7].

Nothing is known about the biochemical profile of *P. larvae* genotypes in Bulgaria- that was the rationale to perform the present study. Hence, the aim of this work was to characterize genotypes of the agent determined in Bulgarian apiaries with the commercial identification system BioLog Gen III and by their nitrate reducing ability. The link between genotype and biochemical phenotype was also assessed.

MATERIAL and METHODS

Isolation, PCR Identification and rep-PCR Genotyping of P. larvae Isolates

A total of 103 *P. larvae* isolates and a reference strain NBIMCC 8478 were included in the study. Strains were isolated from brood combs with clinical symptoms of AFB originating from apiaries located in different regions of Bulgaria. Isolation and PCR identification of the isolates were done as previously described [12].

Genotyping of the isolates was based on rep-PCR protocol using BOX A1R and MBO REP1 primers developed by us [9].

Biochemical Profile of P. larvae rep-PCR Genotypes in Bulgaria

Biochemical profile of the isolates with known genotype was determined by the commercial identification (ID) system BioLog Gen III (Hayward, USA). Microplates were processed following the company's protocol. Briefly, protocol A was used to identify and characterize *P. larvae*. The isolates were cultured on trypticase soy agar (Fluka, India) supplemented with 5% defibrinated sheep blood and the inocula were prepared in a broth medium, procured by the manufacturer until achieving the cell density in the range of 90-98% T. The plates were filled with 100 µL of the inocula and incubated at 33ºC for 48-72 h aerobically. Then, the plates were analyzed using the computer system software OmniLog. After identification with the BioLog system, only the substrates providing a carbon source (n=71) were taken into consideration to characterize the strains. In addition strains were analyzed by their nitrate reducing ability and grouped into biotypes as described by Jelinski [5].

RESULTS

Isolated strains were identified by multiplex PCR protocol for detection of the fragments of 16S rRNA and metalloproteinase (Mlp) genes of *P. larvae*. Specific amplicon length of 973 bp (16S rRNA gene) and 273 bp (Mlp) was generated which confirmed the species affiliation.

Molecular typing with rep-PCR using BOX A1R and MBO REP1 primers resulted in two genotypes of the studied isolates. Most of them referred to genotype ab (n=82) followed by AB isolates (n=21). The reference strain NBIMCC 8478 belonged to ab genotype.

All tested isolates were confirmed as *P. larvae* by the BioLog Gen III system. The biochemical profile of *P. larvae* genotypes AB and ab determined with BioLog microplates is given in *Table 1*. Genotype AB and ab isolates were able to metabolize D-trehalose, N-acetyl-D-glucosamine and N-acetyl-β-D-mannosamine. All AB isolates used D-fructose and D-mannitol as carbon sources, while for ab isolates the results were 5% and 18%, respectively. Glucose was utilized by 100% of ab and 90% of *P. larvae* AB. All ab isolates used glycerol as a carbon source, positive reactions for the AB genotype were 10%. Zero percent of genotype AB metabolized D-turanose, L-alanine and L-lactic acid. None of the tested ab isolates used D-melibiose as for AB values were also low - 5%.

Considering the nitrate reducing ability, mannitol and salicin utilization, the strains were distributed into biotypes I, III, IV and VIII. Only the reference strain referred to biotype V. The majority of *P. larvae* AB clustered into biotype III (90%) while ab were grouped mainly into biotype I (62%). Biotypes I and IV were not found among the tested AB strains. Biotype VIII represented 10% of AB and 13% of ab isolates *(Fig. 1)*.

DISCUSSION

The present study has shown that the ID system BioLog GEN III can be successfully used for the correct identification of *P. larvae*. In addition to the probability values, BioLog provides information about the biochemical profile of analyzed isolates. Findings also revealed that the

Table 1. *Biochemical profile of P. larvae (genotype AB and ab) strains determined with BioLog Gen III microplates. Results are presented as percent positive reactions of using the respective carbon source*

Tablo 1. *P. larvae (genotip AB ve ab) suşlarının BioLog Gen III mikroplate ile belirlenen biyokimyasal profili. Sonuçlar ilgili karbon kaynağı kullanılarak yüzde pozitif reaksiyon olarak ifade edilmiştir*

Well	A4	A8	B3	B5	B6	B7	B8	C1	C2	C3	C9	D2	D5	E3	E9	G2	G4
Substrate	D-trehalose	D-Turanose	D-Melibiose	D-Salicin	N-Acetyl-D-Glucosamine	N-Acetyl-β-D-Mannosamine	N-Acetyl-D-Galactosamine	α-D-Glucose	D-Mannose	D-Fructose	Inosine	D-Mannitol	Glycerol	L-Alanine	L-Serine	Methyl Pyruvate	L-Lactic Acid
P. larvae genotype AB n = 21	100	0	5	10	100	100	43	90	52	100	33	100	10	0	10	29	0
P. larvae genotype ab n = 82	100	9	0	33	100	100	24	100	5	5	100	18	100	20	34	91	7

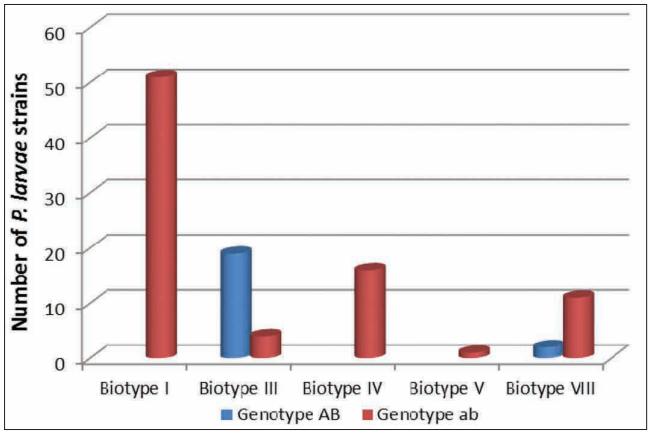

Fig 1. Distribution of Bulgarian *P. larvae* genotypes AB/ab into biotypes according to Jelinski [5]; Biotype I (reduction of nitrate to nitrite - ; acid production from mannitol - ; acid production from salicin -); Biotype III (reduction of nitrate to nitrite - ; acid production from mannitol + ; acid production from salicin -); Biotype IV (reduction of nitrate to nitrite - ; acid production from mannitol - ; acid production from salicin +); Biotype V (*P. larvae* NBIMCC 8478, reduction of nitrate to nitrite + ; acid production from mannitol - ; acid production from salicin +); Biotype VIII (reduction of nitrate to nitrite - ; acid production from mannitol + ; acid production from salicin +); + = positive reaction; - = negative reaction

Şekil 1. Bulgaristan *P. larvae* genotipleri AB/ab'nin Jelinski'nin bildirdiğine yönteme göre [5] biyotiplere dağılımı; Biyotip I (nitratı nitrite indirgeme - ; mannitolden asit üretimi - ; salisinden asit üretimi -); Biyotip III (nitratı nitrite indirgeme - ; mannitolden asit üretimi + ; salisinden asit üretimi -); Biyotip IV (nitratı nitrite indirgeme - ; mannitolden asit üretimi - ; salisinden asit üretimi +); Biyotip V (*P. larvae* NBIMCC 8478, nitratı nitrite indirgeme + ; mannitolden asit üretimi - ; salisinden asit üretimi +); Biyotip VIII (nitratı nitrite indirgeme - ; mannitolden asit üretimi + ; salisinden asit üretimi +); + = pozitif reaksiyon; - = negatif reaksiyon

system was a good alternative to PCR for detection of the agent, especially where the molecular techniques are not yet applicable. However, the ID results are obtained after 48-72 h incubation due to the slow growth and metabolic activity of *P. larvae*.

This is the first study concerning the biochemical profile of *P. larvae* rep-PCR genotypes in Bulgaria. A relatively small number of the BioLog Gen III substrates were utilized by both AB and ab genotypes included in the present work. Out of the 71 carbon sources, 17 were used. It was found that only three substrates were metabolized by 100% of isolates from genotypes AB and ab, namely D-trehalose, N-acetyl-D-glucosamine and N-acetyl-β-D-mannosamine. In a study of Neuendorf et al.[7] regarding the biochemical characterization of German *P. larvae* genotypes (AB, Ab, ab) using BioLog Gram positive identification test panel, the authors obtained similar results for D-trehalose (92% AB) and N-acetyl-D-glucosamine. With respect to N-acetyl-β-D-mannosamine the findings were not comparable. None of the German ab isolates and 19% of AB have used this substrate as carbon source. The same study exhibited the AB genotype as one with a most striking metabolic pattern, since it was the only able to utilize the carbohydrates D-fructose and D-psicose, and the only strains not capable to use glycerol. Also, the authors did not find any isolate which could use turanose or L-alanine. However, among tested isolates in our study we detected 5% of ab to metabolize D-fructose, 10% of AB able to use glycerol, 9% and 20% of ab to use turanose and L-alanine, respectively. Regarding some of the substrates incorporated in the test panel, similar to our results were reported by Carpana et al.[13] and Dobbelaere et al.[14] who evaluated the API 50CHB and BBL Crystal systems for identification and biochemical characterization of *B. larvae*. Jelinski [5] also observed consistent and variable biochemical properties of the studied 110 reference and field strains *B. larvae*. It has to be considered that in this three studies and in the past years' reports the genotypes of the bacterium were not known.

Alippi and Aguilar [15] observed no obvious linkage between the biochemical type and the genotype of the analyzed isolates. The results in this study are not comparable with those of Alippi and Aguilar [15] because of the different primers used for the rep-PCR molecular typing of the strains. An association between genotypes

and their biochemical phenotype was found by Neuendorf et al.[7] using the BioLog Gram positive panel with 95 carbon sources as mentioned above. Particular metabolic features of Bulgarian AB and ab isolates were also found based on the 71 carbon sources. Additionally, the strains were biotyped according to Jelinski [5] testing their nitrate reducing ability that was not included in the Biolog microplates layout. Thus the link between genotype and biotype became even more apparent. Biotypes I and IV correlated with the ab genotype. The majority of AB genotype belonged to biotype III versus 5% for ab strains. The reference strain belonged to biotype V. Interestingly, we found strains possessing characteristics for biotype VIII whereas according to Jelinski [5] no strains were known by that time. Pentikäinen et al.[11] also used the proposal of Jelinski [5] to biotype Finnish *P. larvae* strains. The authors found biotype V as the commonest followed by biotypes IV and I. Pentikäinen et al.[11] established a relationship between the genotype determined by pulse-field gel electrophoresis and the biotype. Obviously biotyping based on the work of Jelinski [5] combined with the genotype can be very useful to address the epidemiologic problems in AFB outbreaks.

The precise comparison between genotype and biochemical phenotype of the strains originating from different countries is impossible due to the different biochemical tests and ID systems or used genotyping methods and probably the geographically related metabolic features of the studied strains. However, this study has shown a clear connection between the rep-PCR AB/ab genotypes and the biochemical type. We suggest that biotyping using ID systems or the accepted biochemical scheme based on three substrates could add to the genotype specific characteristics and might improve the epidemiologic investigations with regard to tracing the source of infection and controlling the disease.

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