The use of antigens derived from Bacillus thuringiensis bacteria for further differentiation

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Abstract

This study is devoted to studying Bacillus thuringiensis antigens and their insecticide activity as critical feature in bacterial differentiation. 190 samples were examined for flagellar antigenicity as well as the insecticidal activity exhibited. From a serological perspective, 122 isolates (64.2%) were attributed to 8 H-serogroups, including 3 non-typeable and 65 unverified. The dominant serotype was H3abc (82% frequency); H6 was less frequent (8.5%). The other 6 serotypes accounted for a low frequency of occurrence (up to 1.5%). Of the 190 isolates tested, 125 (65.8%) formed bipyramidal and 63 (33.2%) represented spherical inclusions. All H3abc isolates contained bipyramidal inclusions. The same applied to H8ab and H7 isolates. Insecticide activity was noted in 70.1% of populations. 128 samples were toxic to both species (Bombyx mori, Aedes sp.). Another 3 samples were toxic only to B. mori, and 2 for Aedes sp. Of the samples that showed toxicity for both species, 97.6% belonged to bipyramidal paraspore inclusions (H3abc). All H7 samples were toxic to two insect species. Monotoxic B. thuringiensis against Aedes sp. were found only among organisms producing spherical parasporal inclusions in the cell. Examples of such microorganisms include an isolate of H4ab/43 serotype.

Introduction

Recently, strains of *Bacillus thuringiensis* were discovered that had toxic activity against two-winged and rigid-winged insect species(Ben-Dov, 2014; Zghal et al., 2018; Domínguez-Arrizabalaga et al., 2020; Cao et al., 2022). In this regard, the larvicidal activities of these strains may be broader than previously thought, as it was assumed that they were effective only against lepidopterans. During the last three decades, an intensive search for natural isolates that could be of economic importance for the control of insect pests has been carried out (Xiao & Wu, 2019). Screening procedures have yielded tens of thousands of isolates that are in both publicly and privately owned collections (Pinto et al., 2012). These isolates in collections, therefore, could be reservoirs that would act as toxins in pest control.

B. thuringiensis produces insecticidal proteins, the primary type of crystalline (Cry) proteins (Bravo et al., 2007). Actively growing vegetative cells are non-toxic since they synthesize proteins that do not produce crystals. At the same time, some strains synthesize Cry proteins that are endotoxic to some species of Coleoptera. Thus, Δ-endotoxins mainly contain cytolytic (Cyt) and Cry proteins (Pardo-López et al., 2019). However, Cyt and Cry proteins have different sequence homology, although they assume similar action modes relative to cell lysis, resulting in irreversible damages in the mid-gut of insects (Bravo et al., 2007).

There are several reasons for the increased interest in *B. thuringiensis* . In particular, many insects are becoming resistant to insecticides. At the same time, the presence of resistance to strains of*B. thuringiensis* may also be increasing. Classification by pathotype of *B. thuringiensis* strains is difficult because not only the great diversity of δ endotoxic genes is known (Aronson, 2002), but also their multiplicity even within one strain (Palma et al., 2014). Therefore, it is necessary to search for reliable methods to classify*B. thuringiensis*

strains. Serotyping and classification according to biochemical features proved to be the most effective methods. Serotyping of *B. thuringiensis* was developed by examining flagellar antigens (De Barjac & Frachon, 1990). Two issues remain relevant: a) whether serotyping reflects the full diversity of *B. thuringiensis* ; b) to what extent serotyping may be relevant to genetic type exchanges between species and subspecies related to *Bacillus cereus* .

The two above bacteria are very similar in biochemical traits and genetic properties (Wei et al., 2019). Comparative analysis of conserved genes in the core genomes and pangenomes of related *Bacillus*species revealed numerous overlapping loci in these strains. Poornima et al. (2012) also reported that these bacteria's genetic and phenotypic properties are almost indistinguishable. They synthesize parasporins, a new functional category of inclusion proteins capable of destroying cancer cells first identified in the *B. thuringiensis* isolate. Although this strain possesses parasporal inclusions typical of *B. thuringiensis* species, an in-depth study is required on its morphological and molecular differences relative to other related species.

Furthermore, it has been shown that *B. cereus* species cannot be reliably identified via standard biotyping (Yusuf et al., 2018). Hence, biochemical experiments may not be sufficient for differentiation. Instead, an insecticidal crystalline protein has been used as a distinguishing feature to differentiate these bacteria (Pardo-López et al., 2013).

Some of the factors that distinguish these two bacteria are the pathogenicity of samples *B. cereus* causing gastrointestinal disorders, while *B. thuringiensis* is also involved in diarrhoeal epidemics. Insecticidal crystalline proteins (δ-endotoxin) encoded by*cry* genes have been reported as one of the distinctive features*of B. thuringiensis* (Bravo et al., 2007). Since using a biomarker to differentiate the *B. cereus* is quite complicated and time-consuming, a highly effective identifier is urgently required to replace previous ones with less efficient performance, sometimes providing false results. Based on transcription regulator genes and Cry protein genes for *B. thuringiensis* (Pardo-López et al., 2013), a study was conducted to test and compare the effectiveness of the developed biomarker to the existing Cry protein marker (*cry* 2) in distinguishing *B. thuringiensis* from *B. cereus* strains. Cry2 was found to be the most abundant crystalline protein in *B. thuringiensis* (Bravo et al., 2007).

It has been established that *B. thuringiensis* is quite commonly associated with the feces of many animals, particularly herbivorous mammals (Rahman et al., 2022). Therefore, this work aimed to investigate fecal animal isolates to determine flagellar (H) antigenic serotypes, as well as the insect pathogenic activity of *B. thuringiensis*strains necessary for their differentiation. The data indicated daily plant material consumed with food, in which high concentrations of these bacteria were present. In practical applications, obtained results may assist in studying the toxicity of *B. thuringiensis* serotypes against several types of human cancer cells that would help treat tumors.

Methods and materials

Bacterial isolates and cultivation conditions

A total of 190 samples of *B. thuringiensis* were studied. All samples were found in the zoo. To perform serological studies, the bacteria were grown at a temperature of 37°C for a period of 5 hours. Nutrient broth with a pH value of 7.5 was used for this purpose. The broth consisted of 15 g of meat extract, the same amount of polypeptone, as well as 1000 ml of distilled water and 3 g of sodium chloride. Bacterial growth was carried out on nutrient medium (nutrient agar consisting of 1 liter of broth, 30 g of agar). The temperature regime was 28°C and the cultivation time was 5-6 days. This was necessary for tests on pathogenic activity for insects, as well as for observations of sporulated cultures using a microscope.

H-serotyping

The study of flagellate H serotypes obtained from fecal samples was used. For this purpose, an agglutination method was used; the study was performed on a slide according to the generally accepted recommendations (De Barjac & Frachon, 1990). The test was performed by referring the H antiserum against *B. thuringiensis* H-serotypes 1-58. Hence, motile isolates were termed non-typeable as non-reactive against the reference H

antiserum; isolates without flagellation were found; the presence of a strong degree of autoagglutination was considered as well. Bacterial serotypes were identified according to the nature of their flagellate H-antigens. However, antisera prepared to the H antigens may share several common antibodies. Monospecific antisera containing one particular antibody were prepared by saturating aliquots of basal antisera with selected antigens one at a time and removing undesirable antibodies by centrifugation after completing the precipitation reaction. Such monospecific antisera allow the detailed typing bacteria with common antigenic subfactors as serotypes.

DNA Isolation and Conventional PCR Assay

Cultures of isolates were incubated in LB overnight at 30 °C and DNA was extracted according to Carozzi et al. (1991).

The oligonucleotide primers used in the study were as follows (5'-3'):

GTTATTCTTAATGCAGATGAATGGG, CGGATAAAATAATCTGGGAAATAGT.

The test tube for PCR was filled with 1 ml of a solution containing 10 pmol of a primer for cry2 and 2 ml of DNA. The PCR conditions:

- denaturation (95 degC, 5 min, 1 cycle);
- denaturation (95 degC, 30 sec, 35 cycles);
- annealing (55 degC, 30 sec, 1 cycle);
- elongation (72 degC, 6 min, 1 cycle).

After amplification, an agar gel solution (1.5%) was used for gel electrophoresis and identification.

Morphology of parasporal inclusions

Cultures sporulated from 5 to 6 days were observed using a phase-contrast microscope to morphologically identify paraspore inclusions (Chai et al., 2014). Biochemical and phenotypic characterization and identification of 190 *B. thuringiensis*isolates were performed based on esculin hydrolysis, lecithinase, hemolytic activity, and motility activity (El-Kersh et al., 2016).

Tests for insecticidal activity

Eggs of *Bombyx mori* were kindly provided by employees of the National Scientific Center "Institute of Experimental and Clinical Veterinary Medicine" (Kharkiv, Ukraine). Spore-forming samples obtained from fecal samples were tested for insecticidal activity against*Bombyx mori* and*Aedes* mosquitoes. The *Aedes* larvae were withdrawn from the population maintained in the laboratory. The testing was conducted using the previously described methodology (Ahmed et al., 2017).

Statistical data processing

The mean and standard deviation (SD) were estimated for statistical analysis of the obtained data. Analysis of Variance (ANOVA) was employed to assess the difference in the mean values of the analyzed criteria using Microsoft Excel and Statistica 10 software (De Smith, 2018). Differences in the obtained results were considered significant at P [?] 0.05 based on the Student's test.

Results

The results of experiments with pre-extracted populations of *B. thuringiensis* have demonstrated the importance of applying H-serotyping to elucidate the properties of serotypes. After more than 30 years, the H-serotype classification of *B. thuringiensis* remains the most effective. This classification makes it possible to better distinguish the thousands of samples of the strain that are available worldwide.

H serotyping

The work presents the results of analyzing the H-serotypes of *B. thuringiensis* isolates secluded from the feces of 20 animal species. Of the 190 isolates, 166 were from 14 mammalian species, 20 were obtained from 5

reptile species and 4 bird species. The obtained bacterial populations were assigned to 8 serotypes: H31, H6, H3abc, H7, H4ab/43, H5ab/21, H8ab, and H9. There were 3 atypical samples in the analyzed populations, as well as 65 that were not verified. Serotype analysis showed that H3abc dominated, accounting for 82.0% of the 122 serotyped isolates, H6 serotype was much less common (8.5%). The remaining 6 serotypes accounted for only up to 1.5% (Fig. 1). The resulting isolates could be multiples of the same organism.

[Fig. 1 here]

The PCR images of *B. thuringiensis* serotypes are given in Fig. 2.

[Fig. 2 here]

In the feces of several herbivores, isolates belonging to H3abc were ubiquitous, accounting for 89% of *B. thuringiensis* populations. Examples included the feces of anthropoids and bears.

Arginine hydrolase (ADH) was used as a factor for serotype identification. Urease reducing enzymes, nitrate reducing enzymes, and enzymes involved in the degradation of sucrose, mannose, cellobiose, and salicin were also used. This also includes enzymes involved in the production of acetyl methyl carbinol (AMC). The latter reaction may be negative only in some cases. All of the above features were used to analyze different antigenic subgroups belonging to different serotypes as well as to analyze a number of isolates that belonged to certain serotypes. Isolates belonging to the same serotype may have certain differences. In particular, six isolates of serotype H8ab were similar in the main characteristics. At the same time, to confirm the hypothesis of similar main characteristics in one serotype, it is necessary to test a larger number of samples.

Morphology of parasporal inclusions

The morphology of parasporal inclusions produced by true fecal isolates can be divided into four different groups (Fig. 3).

[Fig. 3 here]

These include bipyramidal, spherical, irregularly shaped or irregularly pointed. Of the 190 isolates tested, 125 (65.8%) formed bipyramidal inclusions, and 63 (33.2%) were spherical. All H3abc isolates demonstrated bipyramidal inclusions. The same was true for H8ab and H7 isolates (Table 1).

[Table 1 here]

Insecticidal activity

Fecal isolates *of B. thuringiensis* were analyzed in terms of the oral toxicity of the resulting sporulated bacterial cultures against insect larvae to identify the properties of the obtained serotypes. Of the 190 isolates tested, 133 (70.1%) showed insecticidal activity, 128 killed *B. mori* and *Aedes* sp. larvae, 3 isolates were monotoxic to *B. mori,* and 2 more were capable to destroy only*Aedes* s*p.,* proving their biological selectivity against laboratory test objects. The results are detailed in Table 2.

[Table 2 here]

All H3abc samples obtained with bipyramid inclusions were toxic to both insect species, i.e., to both *B. mori* and *Aedes*mosquitoes. The obtained H5ab/21 isolates exhibited double toxicity. Monotoxic *B. thuringiensis* against *Aedes* sp. were found only among organisms forming spherical parasporal inclusions. Examples included an H4ab/43 serotype isolate.

The insecticidal activity of the bacterial serotypes is an integral biochemical component when characterizing different serotypes. The studies carried out mainly with recently characterized serotypes allow the hypothesis that biochemical traits, although important, cannot be used for analysis within or between serotypes. This method, combined with H serotyping, may be effective when certain traits remain unclear. In addition, there are suggestions as to how reliable the microscopy methods are. These methods have proven effective for many bacteria, in addition, their results are comparable with those obtained by traditional methods. In this regard, in some cases, it is possible to use not only traditional methods but also microscopic methods.

Discussion

According to several studies, strains of *B. thuringiensis* tend to be associated with the feces of animals living in national parks or kept in zoos (Swiecicka, et al., 2002; Noda et al., 2009; Djenane et al., 2017). In particular, this organism was quite frequently revealed in the feces of animals that are herbivores. Besides, daily plant-based food intake implies consumption of *B. thuringiensis* in high concentrations. The authors analyzed the pathogenic activity and distribution of antigenic H serotypes in *B. thuringiensis*bacteria, samples of which were obtained from feces.

The data showed that fecal populations of *B. thuringiensis* in terms of serology were diverse and represented by at least 9 H-serotypes with an undefined serogroup/H serogroup. A typical feature of results obtained in this study is that serotype H3abc was identified predominantly in fecal samples belonging to 12 herbivore animal species. In particular, the presence of this serotype was extremely high in the feces of chimpanzees, gorillas, tapirs, two kinds of bears (white and black), as well as rabbits. Hence, all 39 isolates of chimpanzees were serologically assigned to this serotype. Similarly, 25 isolates from polar bears were identified as H3abc. Noteworthy is that 2-3 serotypes could sometimes be detected even in a single fecal sample. Thus, a fecal sample isolated from a rabbit contained 3 serotypes (one H3abc serotype, one H6, and one non-typeable).

Researchers have previously reported that *B. thuringiensis*bacteria can be found on the phyllosphere of different plants (Swiecicka, 2008; Monnerat et al., 2009; Dubey et al., 2017). Serotype H3abc has also been identified as a typical natural flora member of H serotype on phylloplane (Jeong et al., 2017). Hence, the obtained H3abc isolates may originate from natural populations of phylloplane. More likely, however, that the source of some isolates under study is insecticides extracted from microbes. These insecticides can be applied to various plant crops (vegetables, agricultural crops). Thus, insecticides based on H3abc serotype *of B. thuringiensis* are popular in controlling agricultural insect pests (Mendoza-Almanza et al., 2020). Most *B. thuringiensis* toxins identify their specific target through bounding of specific cell membrane receptors. Cry proteins are the best-known toxins representing *B. thuringiensis,*with numerous related studies having been published. Cry is cytotoxic to insect larvae, affecting important crops by recognizing certain types of plant membranes using specific receptors such as cadherin, aminopeptidase-N, and alkaline phosphatase. These toxins mainly affect mosquitoes that are vectors of human diseases such as *Anopheles*spp (malaria), *Aedes* spp (dengue, Zika, and chikungunya) and*Culex* spp (Nile fever and Rift Valley fever), respectively (Mendoza-Almanza et al., 2020).

Previous research reported that *B. thuringiensis* strains without a pronounced insecticidal activity outperformed insecticidal isolates in natural environments in several countries (Lone et al., 2017). Other laboratory study reports have concluded that *B. thuringiensis*isolates with non-insecticidal Cry proteins overperform insecticides in natural ecological niches, comprising over 90% of natural populations in soils and phylloplane (Aboul-Soud et al., 2019). These data contrast with the present findings that insect pathogenic activity was detected in 70.1% of fecal samples. Another important result is that the majority of pathogenic isolates belonged to serotype H3abc.

Global efforts are currently focused on discovering local *B. thuringiensis* isolates with unique anticancer properties. Thus, parasporins are a group of non-insecticidal crystalline proteins with potential and specific antitumor activity *in vitro* (Aboul-Soud et al., 2019). However, despite the significant therapeutic potential of PS-producing *B. thuringiensis* strains, knowledge on the effects of these proteins remains limited. *Thuringiensis* has been found to have unique biological activities. Among them are cytotoxicity specific to certain human cancer cells (Mizuki et al., 2000; Aboul-Soud et al., 2019; Mendoza-Almanza et al., 2020), lectin activity against mammalian red blood cells (Torres-Quintero et al., 2018; Onofre et al., 2020), and activity against trichomonads (Lee et al., 2017). Future research will include tests of parasporal proteins, e.g., animal feces, which may affect indicators of biological activity unrelated to pathogenicity.

Conclusions

After 30 years, the classification of *B. thuringiensis* strains based on H serotyping remains an effective method for distinguishing strains. This classification is based on stable and distinctive features. Therefore,

H serotyping of *B. thuringiensis* isolates secluded from feces of 20 animal species was performed in laboratory conditions. Of the 190 isolates, 166 represented 14 mammalian species, 20 were obtained from 5 species of reptiles, as well as from 4 species of birds. The obtained bacterial populations were assigned to 8 H serotypes: H31, H6, H3abc, H7, H4ab/43, H5ab/21, H8ab, and H9. The studied populations consisted of 3 atypical, as well as 65 non-verified isolates. Among the 8 serotypes studied, the most common was H3abc (82.0% of cases) out of 122 serotyped samples, less common was H6 (8.5%), the remaining 6 serotypes accounted for no more than 1.5%.

Parasporal inclusions in true fecal isolates can be divided into 4 different groups in terms of morphology. These included bipyramidal, spherical, or irregularly shaped. Of the 190 isolates tested, 125 (65.8%) formed bipyramidal inclusions, and 63 (33.2%) were spherical. All H3abc isolates demonstrated bipyramidal inclusions. The same was true for H8ab and H7 isolates. Fecal isolates *of B. thuringiensis*containing sporulated cultures have been analyzed for oral toxicity against insect *larvae* to elucidate the properties of the obtained serotypes. Of the 190 isolates tested, 133 (70.1%) showed insecticidal activity, 128 killed *B. mori* and *Aedes* sp. Other 3 isolates showed effects only on *B. mori* , and 2 showed effects only on *Aedes* sp., proving their biological selectivity against laboratory test objects. All H3abc isolates with bipyramid inclusions were double-toxic to silkworms and mosquitoes. In addition, all H5ab/21 isolates exhibited dual toxicity. Monotoxic *B. thuringiensis*against *Aedes* sp. were recorded only in cultures of the spherical group. These samples belonged to the H4ab/43 serotype.

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Ethics approval . The authors declare that the work is written with due consideration of ethical standards. The study wasconducted in accordance with the ethical principles approved by the Ethics Committee of Federal State Budgetary Educational Establishment of Higher Education "Sechenov First Moscow State Medical University" (Protocol 13 of 13.09.2022).

Consent to participate . Informed consent was signed by participants.

Consent for publication . Not applicable

Authors' contributions . Both authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by ES and AA. The first draft of the manuscript was written by ES and AA. All authors read and approved the final manuscript.

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Table 1

Distribution of *B. thuringiensis* serotypes by morphological characteristics of the studied parasporal inclusions.

Serotypes B. thuringiensis	**Quantity of isolates tested**	**Morphology of parasporal inclusions**
H3abc	122	Bipyramidal
H6	48	Spherical
H7	1	Bipyramidal
H4ab/43	4	Spherical
H5ab/21	1	Bipyramidal
H8ab	1	Bipyramidal
H9	6	Spherical
H31	5	Spherical
Atypical isolates	2	Misshapen

Table 2

Data on insecticidal activity of *B. thuringiensis* obtained via tests.

Activity	LC50±SD (μg/ml)	LC90±SD (μg/ml)	Slope±SD
Toxic to both *B. mori* and *Aedes* sp. larvae	4.7±0.55	9.6±1.58	5.5±0.006
Toxic to *B. mori*	4.1±0.67	32.4±12.51	1.7±0.02
Toxic to *Aedes* sp.	2.3±0.33	15.2±5.72	2.0±0.05

LC – lethal concentration

Figure captions

Fig. 1. Shares of *B. thuringiensis* serotypes detected.

Fig. 2. PCR screening of *B. thuringiensis* strains from different serovars. The arrow indicates the zmaR PCR amplicon. Positive strains are marked with (+).

Fig. 3. Morphology of *B. thuringiensis* isolates in nutrient agar. 2A – general view of the colonies; 2B – the general view of one of the colonies; 2C – a photograph obtained by the phase-contrast method (magnification x1000). Arrows without lines indicate parasporal crystals. Arrows with lines indicate bacterial spores.