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Detection of *Paenibacillus larvae* Subsp. *larvae* Spores in Honey Samples from Beekeepers of the Taquari Valley, Rio Grande Do Sul State, Brazil

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Abstract: This research aimed at evaluating the presence of *Paenibacillus larvae* subsp. *larvae* spores, which cause the disease known as American foulbrood. 35 samples of honey from the Taquari Valley major beekeepers were analyzed during the months of May and June 2010. The analyses were performed according to the Normative Instruction No. 62. The results revealed no presence of *Paneibacillus larvae* subsp. *larvae* spores in the 20 ml of the evaluated samples.

Key words: *Paenibacillus larvae* subsp · *Larvae* · Honey · Spores · American Foulbrood

INTRODUCTION

Microorganisms can be divided into several groups, but in general, they are classed as benign, indicator, pathogen and spoilage. Benign ones turn one kind of food into another one that still fits for consumption [1]; indicator microorganisms provide information on the hygienic and sanitary conditions of food [2]; pathogen ones, when present in food, cause no organoleptic changes, but can be harmful to human health [3], while spoilage microorganisms cause changes in color, taste and smell of food, making it unfit for human consumption [4].

American foulbrood (AFB) is a disease caused by *Paenibacillus larvae* subsp. *larvae* (*P. larvae larvae*) spores, which contaminate the larvae. It is the most serious worldwide disease that affects *Apis mellifera* bee larvae. Larvae become infected by ingesting spores that germinate in their gut, disseminating the larval tissue in 72 hours [5]. This disease causes considerable economic losses to beekeepers [6].

It is in the spore form that *P. larvae larvae* can infect the hives. This infection occurs when queens from infected hives are introduced in another hive and/or beekeepers use contaminated or poorly cleaned beekeeping equipment during harvest or maintenance. These spores contaminate the honey and the royal jelly, which are the bee larvae's food, causing the disease. This is a facultative anaerobe able to form very different endospores, what can provide viability for at least 35-50 years [7, 8].

According to the Brazilian Institute of Geography and Statistics (IBGE) [9], Brazil's honey production was higher than 33,000 tons in 2005. The 2007 Municipal Livestock IBGE survey shows that the states of Rio Grande do Sul and Paraná are the largest honey producers, 21.2% and 13.3% of total national production, respectively. In 2005, there was a honey production of 340,495 kilograms in the Taquari Valley, while in 2006 this amount was 375,516 kilograms. The main municipalities were Paverama, Arroio do Meio, Progresso, Encantado and Anta Gorda, according to the Regional Taquari Valley Data Bank [10].

According to the Technical Regulation of Honey Identity and Quality - Ordinance No. 367 (September 4, 1997), repealed by the Normative Instruction No. 11 [11], microbiological tests in bee products include: *Salmonella* spp.- *Shigella* spp., total coliforms and yeasts and molds. While *Salmonella* - *Shigella* and total coliforms must be absent in the sample, yeasts and molds have a tolerance of 100 CFU/g. Microbiological quality control on bee products also concerns about *Clostridium botulinum*, another microorganism. Recent researches conducted by Ragazani [12], emphasize the presence of this microorganism in honey samples. The Brazilian Sanitary Surveillance Agency (Anvisa) [13] cautions against the consumption of honey by children under one year

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because they lack the fully formed intestinal flora, what allows the development of *Clostridium botulinum* spores in their vegetative form.

Because *P. larvae larvae* are microorganisms rarely studied in routine laboratories and are specific to bee products, currently, there are few data about them in Brazil.

The aim of this study was to verify the presence of *P. larvae larvae* spores in honey samples collected in the Taquari Valley.

MATERIALS AND METHODS

This research was conducted May through June 2010 in the microbiology laboratory of the Centro Universitário UNIVATES, where 35 honey samples were analyzed. These samples were collected randomly among the largest beekeepers of the Taquari Valley.

The samples were collected August through September 2009 and were sent to the laboratory at room temperature and kept in this way until the processing.

The analytical methodology followed the recommendations of the Normative Instruction No. 62 [14], as described and outlined below.

Sample Preparation: The honey samples were heated in a water bath (DeLeo, Brazil) at 45°C to reduce viscosity, what facilitates handling and the homogenization of the spores. After this, 20 ml of each sample were moved aseptically to centrifuge tubes (Cornig-Hexasysten, Brazil), where 30 ml of sterile phosphate buffered saline (Nuclear, Brazil) were added. Subsequently, this mixture was well homogenized.

Centrifugation: The centrifugation (Excelsa, Brazil) of the samples was performed at 3000 x g for 30 minutes to provide the concentration of the spores. After the centrifugation, the supernatant was discarded carefully and the sediment was suspended again in 1.0 ml of buffer PBS, transferred to a sterile screw cap test-tube and heated in a water bath at 80°C for 10 minutes.

Isolation and Screening: 0.1 ml of the suspension previously prepared was moved to a dry surface of plates (J-Prolab, Brazil) containing ABL agar (Agar *Bacillus larvae*) (Acumedia, EUA) and carefully spread with a Drygalski loop (Labware, China). On another plate also containing ABL agar, the suspension was inoculated by streaking, with a loop of 10 ìl (J-Prolab, Brazil). The two plates of each of the 35 samples were incubated inverted in an incubator at 36 ± 1ºC for five days, noting any possible growth after 48 h. Suspected colonies of *P. larvae larvae* (usually flat, dull, with a smooth grainy surface, with or without a high center of a higher density, with slightly irregular edges, with or without halo of lipolysis [14] on the ABL agar were isolated in nutrient agar without yeast extract (Acumedia-Michigan, EUA).

Confirmation tests for the suspected colonies on the ABL agar were subjected to the catalase test by removing an aliquot of the culture and transferring it to a glass slide containing a drop of hydrogen peroxide 3% (Vetec, Brazil). The formation of bubbles indicates a positive catalase. For samples with negative catalase, aliquots were removed from the colony, transferred to tubes containing inclined milk agar with thiamine (ALT) (Nalgon, Brazil) and incubated for 24 h to 48 h at 36 ± 1ºC. These cultures were kept for further testing. The Gram staining (Laborclin, Brazil) was performed on the suspected colonies. The *P. larvae larvae* are usually Gram-positive and long and thin, but can have various sizes in varying length chains. There was growth in nutrient agar without yeast extract in the inclined tube incubated at 36 ± 1ºC for three days, but *P. larvae larvae* grow sparsely in nutrient agar without yeast extract. An aliquot of suspicious colonies was transferred to ALT and the colonies were incubated in reverse at 36 ± 1ºC for three days. After the incubation, the potential presence of a transparent halo around the colonies was observed. The *P. larvae larvae* decompose casein up to three days, while colonies of these microorganisms are usually white when present in ALT and have a subtle and delicate appearance (a little dense). According to the Normative Instruction No. 62, *P. larvae larvae* cultures are Gram-positive, fine, medium length to long, arranged in chains, with negative catalase, a positive reaction when submitted to hydrolysis of casein associated with the typical growth in ALT and a sparse growth in nutrient agar without yeast extract after a three-day incubation.

The results of the honey analysis must be expressed as presence or absence of *P. larvae larvae* in 20 ml of honey.

RESULTS AND DISCUSSION

During May and June 2010, a total of 35 honey samples were tested for *P. larvae larvae* according to the methodology described in the Normative Instruction No. 62 [14]. Among them, only one sample showed a suspicious result of growth in agar ABL. The suspected colony of *P. larvae larvae* was isolated and submitted to catalase and showed a weak positive result. The Gram staining (Figure 1) showed Gram-positive bacteria arranged in chain.

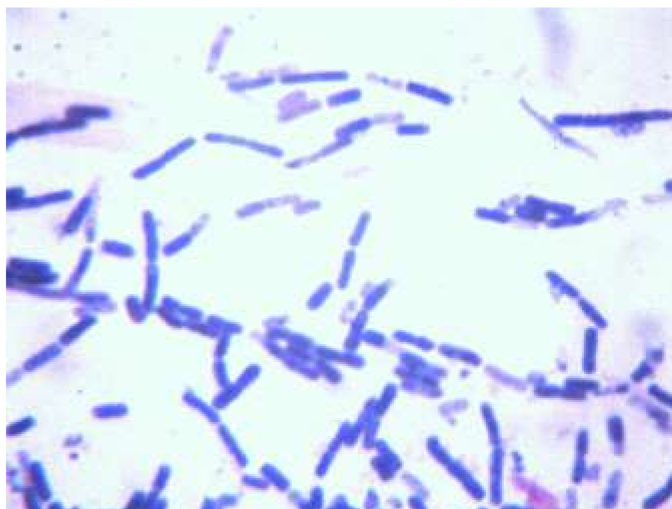

Fig. 1: Isolated suspect colony of *Paenibacillus larvae* subs. *larvae*, Gram-positive arranged in chain. Source: Unianálises Microbiology Laboratory. Amplification: 1000 times.

Fig. 2: Suspicious growth of *Paenibacillus larvae* subs. *larvae* obtained in ALT agar plate.

The growth of the suspect colony on a nutrient agar surface in an inclined tube without yeast extract was abundant, unlike what might be characteristic for *P. larvae larvae* (low growth).

Regarding the decomposition of casein and typical growth in ALT, there was no decomposition (without halo formation) and the colonies had a creamy color (not characteristic), as shown in Figure 2.

The data show the absence of *P. larvae larvae* in 20 ml of the 35 honey samples tested. In general, there are few studies of *Paenibacillus larvae* in bee products in Brazil, what makes it difficult to compare this study with other pervious researchers' studies. One of the pioneering work about this pathogen was published by Schuch [15]. These researchers detected the presence of *P. larvae larvae* spores in products of a warehouse in the state of Rio Grande do Sul. Imported honey and pollen available in the warehouse, a comb (brood, pollen and honey) collected from a healthy hive, honey stored in an apiary and adult bees were analyzed. The results of this study show that the imported honey and pollen, three groups of the adult bees and the honey of the comb were contaminated. The authors also mention that this study was the first official isolation of *P. larvae larvae* spores in bee products obtained within a hive as well as in adult bees in the Brazilian territory. Undoubtedly, the methodology used in this study corroborated with the introduction of an official methodology [14] for research on this pathogen.

As far as we have information, this study of this pathogen in honey is one of the pioneers carried out in the Taquari Valley. There are no reports in literature demonstrating the existence of *P. larvae larvae* in Brazilian honey. Probably this is due to lack of data since the study of this pathogen is not legally required.

In Uruguay, a national study conducted in 2001 and 2002 to evaluate the presence and quantity of *P. larvae larvae* spores in 101 honey samples from 19 provinces was conducted by Antúnez [16]. The samples were randomly collected and analyzed by combining molecular bacteriology and the molecular method by amplifying a fragment of the 16S rRNA gene of *P. larvae larvae*, using the polymerase chain reaction (PCR). The results revealed that in 52 samples (51.5%) the colonies were typical of the pathogen in J agar supplemented with nalidixic acid. The authors also claim that the PCR technique used in this study was very useful to identify *P. larvae larvae*, since in many cases the accompanying microflora grows abundantly, as in the case of *P. alvei*, that covers the whole plate.

Many references in literature employ the PCR method for rapid detection of *P. larvae larvae* [6, 8, 17, 18]. Govan [6] state that false-positive colonies that eventually grow in the semi-selective medium containing nalidixic acid and pipemidic acid prevent rapid confirmation of *P. larvae larvae*. Therefore, the authors claim that the PCR method can be a great tool once it allows rapid confirmation of the presence of pathogens in food. Bakonyi [17] developed and evaluated a PCR method for direct detection of *P. larvae larvae* in honey samples and compared it with the isolation and the biochemical characterization. They concluded that, although the isolation and the biochemical identification method (BioLog) had higher sensitivity and specificity, the PCR method proved to be a valuable technique for screening honey samples in large scale, especially due to its speed and cost.

CONCLUSION

Based on our results and the discussion presented above it is possible to conclude that the 35 honey samples verified in the Taquari Valley did not show the presence of spores (very important to confirm the AFB disease), what shows the good quality of the honey produced in this region.

For future studies more samples of honey and/or bee products from different apiaries of the major Brazilian producing regions should be subjected to analysis to obtain data regarding the presence of this pathogen as well as its isolation in hives. Probably, the confirmation of suspected colonies of *P. larvae larvae* through molecular methods is an important step to avoid mistakes arising from false interpretations.

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