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**Original article**

# Evaluation of the API 50CHB system for the identification and biochemical characterization of *Bacillus larvae*

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**Summary** — We have evaluated the suitability of the API 50CHB system for the identification and biochemical characterization of *Bacillus larvae* White, the etiological agent of American foulbrood, an important disease of the honeybee. Twenty-nine strains of *B larvae*, isolated from outbreaks of American foulbrood in several geographic areas, were assayed with this system. All strains showed positive reaction (acid production) with ribose, D-glucose, D-mannose, *N*-acetylglucosamine, trehalose and D-tagatose. All but one showed positive reaction with glycerol; variable results were observed with salicine and 5-ketogluconate. Only one strain acidified D-fructose and galactose.

***Apis mellifera* / American foulbrood / *Bacillus larvae* / diagnosis / API 50CHB system**

## INTRODUCTION

*Bacillus larvae* White is the etiological agent of American foulbrood, the most serious and widespread bacterial disease in the honey bee (*Apis mellifera* L). The infection affects the larval stage and is easily transmitted through the resistant spores of the bacterium (Bailey and Ball, 1991).

Common beekeeping practice increases the frequency with which spores are transferred from colony to colony. The persistence of the pathogenic agent as spores on beekeeping equipment and in living colonies makes any technical or chemical cure ineffective for complete recovery and the infection can sooner or later reappear. Strict veterinary measures together with equipment sterilizing techniques are applied to control American foulbrood (Ratnieks, 1992).

The clinical diagnosis of American foulbrood is not normally difficult owing to a fairly characteristic symptomatology. Furthermore, the search for the pathogen by means of bacteriological tests is facilitated by the fact that *B larvae* almost always develops in larvae in pure culture (Bailey and Ball, 1991).

Aside from clinical cases, the search for *B larvae* spores can be carried out, on a prevention basis, on material that can be contaminated by spores and therefore could be a potential source of infection. Special test procedures have been created for honey in particular (Sturtevant, 1932, 1936; Hansen, 1984a,b; Hornitzky and Clark, 1991; Shimanuki and Knox, 1991; Steinkraus and Morse, 1992). Literature on the subject provides sufficient information for the isolation and correct identification of *B larvae*, by means of cultural and biochemical tests (Gordon *et al,* 1973; Sneath, 1984; Lloyd, 1986; Hornitzky and Karlovskis, 1989; Alippi, 1991; Bailey and Ball, 1991; Shimanuki and Knox, 1991). Among the biochemical traits used to identify the species of the *Bacillus* genus, acid production from carbohydrates plays an important diagnostic role (Gordon *et al,* 1973; Logan and Berkeley, 1984; Sneath, 1984). For *B larvae*, bibliographic information is available for around 20 carbohydrates (Azuma and Kitaoka, 1965; Furowicz and Zahaczewska, 1972; Gordon *et al,* 1973; Sneath, 1984; Jelinski, 1985a,b).

As an alternative to traditional microbiological methods for identifying *Bacillus* species, the use of the API system has become widespread. In addition to fast execution, this system offers the advantage of high reproducibility. The system involves the determination of the metabolism of 49 carbohydrates and derivatives (API 50CHB strips), plus 12 enzymatic tests (API 20E strips). Using this system, Logan and Berkeley (1984) characterized the *Bacillus* genus organically and described all the representative species, except a few including *Bacillus larvae*.

Our study is based on the API 50CHB system as a quick method for determining the ability of *B larvae* to use various carbohydrates and their derivatives. Our purpose here is to contribute towards an expansion of information at both a diagnostic and biochemical characterization level.

## MATERIALS AND METHODS

### *Cultures*

We examined 29 *B larvae* strains using strain ATCC 9545 as the reference. Strains marked 1066, 1090, 1104 were provided by Dr Jelinsky (Department for Research of Useful Insect Diseases, Veterinary Institute, Swarzedz, Poland); strains 42A, 620, 757 were supplied by Dr V Drobníková (Institute of Apiculture, Dol, Czechoslovakia). The rest were isolated over the past 12 years from 17 infected brood combs from various areas in Italy.

We used Michael's medium (Azuma and Kitaoka, 1965) for strain maintenance and growth. Its composition was the following: yeast extract, 10 g; neopeptone, 10 g; thiamine, 0.1 mg; agar, 15 g; distilled water, 1 000 ml; pH 6.8. The cultures were incubated aerobically at 36°C for the various tests.

### *Isolation and identification*

Decomposing larvae from broodcombs with the visual characteristics of American foulbrood were streaked directly on slides for microscopic examination of the spores (400 x in phase contrast or 1 000 x in immersion after staining with methylene blue).

A second streak, after dilution in physiological solution, was made on the basal medium. After 2–3 d of aerobic incubation, we examined the isolated colonies. They were whitish, opaque, flattened, with irregular edges and usually with a diameter of 1–3 mm. Microscopic examination highlighted Gram-positive rods with a width of less than 1 μm.

These cultures were subjected to further identification tests illustrated below.

### Catalase

One millilitre of $H_2O_2$ at 3% was added to a 24 h culture grown on a slant of basal medium. This reaction was checked within 1 min (a positive reaction was indicated by the formation of gas bubbles).

### Growth at 20 and 40°C

A 24 h culture was inoculated on a slant of basal medium and incubated at 20°C and 40°C. Data

collection began after 24 h and continued until day 14.

### Growth in medium with 5% NaCl

A small loop of a 24 h culture was inoculated into liquid basal medium (Michael's medium without added agar) plus 5% NaCl. Data collection began after 24 h and continued to day 14.

### Casein hydrolysis

The medium used for this test was composed of: *Solution A*, 10 g skim-milk powder and 90 ml distilled water; *Solution B*, 3 g agar and 97 ml distilled water. The 2 solutions were sterilized separately (121°C for 20 min), brought to 45°C in a double boiler and then mixed. The medium thus prepared (25 ml) was poured into Petri dishes which were then inoculated with the 24 h cultures. Incubation was continued for 7 d. A positive reaction was indicated by clarification of the medium under and around the colony growth area.

### Starch hydrolysis

We used Michael's medium with 1% of soluble starch and poured into Petri dishes. After inoculation and incubation for 3–5 d, when growth had occurred, the surface of the medium was flooded with iodated Lugol solution at 1:5 dilution. A positive reaction was indicated by the formation of a colourless halo around the area where the colony was growing.

All the isolated cultures showed the characters associated with *B larvae* (Sneath, 1984):

Catalase: negative
Growth at 20°C: negative
Growth at 40°C: positive
Growth in 5% NaCl: negative
Casein hydrolysis: positive
Starch hydrolysis: negative

### *Carbohydrate acidification*

We used an API 50CHB system purchased from Biomerieux Italia Spa. The system includes the strip and the inoculation medium. Each strip is made up of 50 microtubes. One tube acts as the control while the others contain substrates with the carbohydrates and their derivatives (heterosides, polyalcohols, uronic acids).

Manufacturer's instructions enclosed with the system were followed and the Michael medium was used for microorganism growth.

For each strain, 2 strips were inoculated with a bacterial suspension in API 50CHB medium with a density equivalent to tube $n = 2$ of the McFarland scale. As required by the system, we took a first reading at 24 h and the definitive reading at 48 h. Visual examination of the phenol red in the inoculation medium suggested a value from 1 to 3 in the intensity of the reaction observed. Very weak reactions were taken as negative. We used *B alvei* strain ATCC 6344 as a quality control for the system. To check the congruence of the initial results, each test was repeated. The API 20E system used to complete the API 50CHB system was not used, as the results from the preliminary tests were not significant because so few positive results were obtained.

## RESULTS AND DISCUSSION

The results from the control tests on *B alvei* strain ATCC 6344 reflect the average profile indicated for this species in the identification chart enclosed with the API system. The following carbohydrates were acidified: glycerol, ribose, adonitol, galactose , D-glucose, inositol, α-methyl-D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, melibiose, saccharose, trehalose, melezitose, D-raffinose, starch, β-gentiobiose and D-turanose. The *B larvae* strains examined acidified a limited number of substrates and produced different colouring intensities (table I). The response was negative for all substrates not included in table I.

When the profile obtained was compared with those described for other species, it was sufficiently discriminatory and thus useful in the identification of *B larvae*.

In 19 of the 49 carbohydrates tested, our results can be compared with those reported

**Table I.** Results of carbohydrate acidification by *B larvae*. The increasing strength of positive reactions, as assessed from the intensity of phenol red colouring, is given in 3 steps, marked by 1, 2 or 3 asterisks, respectively. The following carbohydrates were not acidified: erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β-methylxyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, amygdalin, arbutin, aesculin, cellobiose, maltose, lactose, melibiose, saccharose, inulin, melezitose, D-raffinose, starch, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate.

| *Origin* | *Strain* | *Glycerol* | *Ribose* | *Galactose* | *D-Glucose* | *D-Fructose* | *D-Mannose* | *N-Acetyl-glucosamine* | *Salicin* | *Trehalose* | *D-Tagatose* | *5-Ketogluconate* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ATCC | 9545 | ** | *** | | ** | | ** | *** | ** | *** | *** | * |
| Czechoslovakia | | | | | | | | | | | | |
| | 42A | *** | *** | | *** | | * | *** | | *** | ** | |
| | 757 | *** | *** | | *** | | *** | *** | * | *** | ** | * |
| | 620 | ** | *** | | *** | | ** | *** | | *** | ** | * |
| Poland | | | | | | | | | | | | |
| | 1104 | *** | *** | | *** | | *** | *** | * | *** | ** | * |
| | 1066 | *** | *** | | *** | | ** | *** | * | *** | ** | * |
| | 1090 | ** | ** | | *** | | ** | *** | * | ** | ** | * |
| Italy | | | | | | | | | | | | |
| | 33p | *** | *** | | *** | | ** | ** | | ** | ** | * |
| | Vx/1 | *** | ** | | *** | | ** | *** | | *** | *** | |
| | Vx/6 | *** | *** | ** | *** | | ** | *** | | *** | * | |
| | 001/1 | *** | *** | | ** | | * | *** | | *** | ** | * |
| | 002/6 | *** | *** | | *** | | ** | *** | | *** | ** | |
| | 003/1 | *** | *** | | *** | | * | *** | | *** | ** | * |
| | 004/6 | *** | *** | | *** | | * | *** | | ** | ** | |
| | 005/4 | ** | *** | | *** | | ** | *** | | *** | ** | |
| | 005/6 | *** | *** | | *** | | ** | *** | | *** | ** | |
| | 36/1 | ** | *** | | *** | | ** | *** | * | *** | ** | |
| | 38/3 | * | *** | | *** | | ** | *** | | *** | * | |
| | 45/1 | *** | *** | | *** | | ** | *** | | *** | *** | |
| | 45/6 | | *** | | ** | *** | *** | *** | * | *** | ** | |
| | 62/5 | *** | *** | | *** | | *** | *** | ** | *** | *** | |
| | 81/2 | *** | *** | | *** | | *** | *** | | *** | *** | * |
| | 112/3 | ** | *** | | *** | | ** | *** | * | *** | ** | |
| | 114/5 | *** | *** | | *** | | * | *** | | *** | * | * |
| | 114/7 | ** | *** | | ** | | * | *** | | ** | ** | * |
| | 117/2 | ** | *** | | *** | | ** | *** | | *** | *** | * |
| | 117/4 | ** | *** | | *** | | ** | *** | | *** | ** | * |
| | 118/9 | * | *** | | *** | | ** | *** | | ** | * | * |
| | Ve11 | *** | *** | | *** | | *** | *** | | ** | ** | |

in the literature (Azuma and Kitaoka, 1965; Furowicz and Zahaczewska, 1972; Gordon *et al,* 1973; Sneath, 1984; Jelinski, 1985a,b). However, it should be kept in mind that the latter were obtained using traditional macro methods. Partial discrepancies appeared for some substrates: xylose, galactose, fructose, mannitol and sucrose. As regards xylose, our data agree with those of Jelinski (1985b) whose research on 110 strains found that no strain could acidify this substrate. Azuma and Kitaoka (1965), on the other hand, report 11 positive results out of 12 but only after a long incubation time. Galactose and fructose, in our tests, were acidified by only one strain each. Jelinski (1985b) did not note any positive results for these 2 carbohydrates, but Azuma and Kitaoka (1965) observed a positive reaction which was quite slow in appearing. Production of acid from galactose was also reported by Furowicz and Zahaczewska (1972). Acidification of mannitol is variable; we found always it to be negative. In Jelinski's tests (1985a,b) it was positive in 17% of the cases while Azuma and Kitaoka (1965) reported only negative data. It was always positive for Furowicz and Zahaczewska (1972) and variable for Gordon *et al* (1973). Lastly, for saccharose, our data agree fully with Jelinkski's (1985b) and partly with those of Azuma and Kitaoka (1965) who observed acidification in 5 strains out of 12 but, here too, it was very slow.

In conclusion, the biochemical profile we found for *B larvae* using the API 50CHB system is sufficiently discriminatory compared with other species in the same genus. The substrates of most value in differentiating *B larvae* should be noted: fructose (almost always negative), aesculin (negative), D-tagatose (positive). In fact, the overall response from these 3 carbohydrates appears sufficient to be able to identify *B larvae* correctly. The API 50CHB system could also be used for the biochemical characterization of strains of *B larvae* on the basis of variations in carbohydrate acidification.

**Résumé — Évaluation du système API 50CHB pour l'identification et la caractérisation biochimique de *Bacillus larvae*.** *Bacillus larvae* White est l'agent de la loque américaine, maladie très répandue dans les colonies d'abeilles et pour laquelle sont prévues des mesures d'éradication par les services vétérinaires. Le système API 50CHB a été testé dans ce but. Le test, appliqué à 29 souches de provenances géographiques différentes (Italie, Pologne, Républiques tchèque et slovaque), a permis de définir pour *B larvae* un profil biochimique concernant l'acidification des 49 substrats (hydrates de carbone et leurs dérivés) prévus par la méthode. Le substrat de développement utilisé avait la composition suivante : extrait de levure, 10 g ; néo-peptone, 10 g ; thiamine, 0,1 mg ; agar, 15 g ; eau distillée, 1000 ml ; pH 6,8. Toutes les souches examinées ont présenté une réaction positive (acidification) avec le ribose, D-glucose, D-mannose, *N*-acétylglucosamine, tréhalose, D-tagatose ; mis à part une souche, toutes ont acidifié le glycérol ; des résultats variables ont été observés avec la salicine et le 5-chétogluconate ; une seule souche a acidifié le galactose et le D-fructose. Le profil biochimique obtenu pour *B larvae* au moyen du système API 50CHB est suffisamment caractéristique pour différencier *B larvae* des autres espèces du même genre. Les substrats les plus discriminatifs sont le fructose (presque toujours négatif), l'esculine (négatif) et le D-tagatose (positif).

***Apis mellifera* / *Bacillus larvae* / loque américaine / diagnostic / système API 50CHB**

**Zusammenfassung — Bewertung vom API 50CHB System als eine diagnostisch anerkannte Methode zur Erkennung und biochemischen Kennzeichnung von *Bacillus larvae*.** Es erfolgt eine Beurteilung für die Anwendung des API 50CHB Systems zur Identifizierung und biochemischen Kennzeichnung von *Bacillus larvae*, dem Erreger

der amerikanischen Faulbrut. Diese Krankheit ist bei Bienen verbreitet und das API 50CHB System ist als Test für ein Abtöten der Völker durch die tierärztlichen Behörden vorgesehen. Der Test, der auf 29 Stämme unterschiedlicher geographischer Herkunft (Italien, Polen, Tschechoslowakei) angewendet wurde, hat für *Bacillus larvae* die Aufstellung eines biochemischen Profils ermöglicht, das sich auf die Säurebildung aus 49 vom System vorgesehenen Substraten (Kohlenhydrate und ihre Derivate) bezieht. Das verwendete Aufzuchtmedium hatte die folgende Zusammensetzung: 10 g Hefeextrakt, 10 g Neopepton, 0,1 g Thiamin, 15 g Agar-Agar, 1000 ml destilliertes Wasser, pH 6,8. Bei allen geprüften Stämmen fand eine Säurebildung mit Ribose, Glukose, Mannose, *N*-Acetyl-Glucosamin, Trehalose und D-Tagatose statt. Mit Ausnahme von einem Stamm reagierten alle mit Glycerol. Unterschiedliche Reaktionen wurden bei Salicin und 5-Keto-Gluconat beobachtet. Mit Galactose und Fructose fand nur bei einem Stamm eine Säurebildung statt. Die Unterschiede im biochemische Profil, die mit dem API 50CHB System erhalten wurden, sind ausreichend, um Keime von *Bacillus larvae* von anderen Arten der gleichen Gattung abzugrenzen. Die Besonderheiten bei der Metabolisierung der folgenden Substrate sollen besonders hervorgehoben werden: Fructose (fast immer negativ), Esculin (negativ), D-Tagatose (positiv).

***Apis mellifera* / amerikanische Faulbrut / *Bacillus larvae* / Diagnose / API 50CHB System**

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