

CHARACTERIZATION OF *ARCANOBACTERIUM HAEMOLYTICUM* ISOLATES ORIGINATING
FROM PNEUMONIC PIGLETS
(THE FIRST ISOLATES FROM PIGLET LUNGS IN YUGOSLAVIA)

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(Received 11. May 2002)

Arcanobacterium haemolyticum is rarely isolated from clinical material either in human or veterinary medicine. As for our country, there is only one official report with regard to the isolation of *A. haemolyticum* from material of animal origin. Pathologically altered parts of the lungs from piglets with pneumonic symptoms, were streaked aerobically and microaerophilically and the suspect colonies were tested with a double CAMP test. The positive inverse CAMP with *Staphylococcus aureus* and synergistic haemolysis with *Rhodococcus equi* prompted us to direct our investigations to *A. haemolyticum*. The API Coryne Bio Merieux set was used to make the diagnosis and later evaluated using a software programme from the same manufacturer. The diagnosis was: *Arcanobacterium haemolyticum* with an identity probability of 99,9 % and $T = 0,75$. The only departure from the identification table was recorded for α - glucosidase, which was negative in all the tested isolates.

Key words: *Arcanobacterium haemolyticum*, piglets, pneumonia

INTRODUCTION

Arcanobacterium haemolyticum is increasingly referred to as a clinically important isolate in human medicine. Most of the references point to its role in causing respiratory disorders (Karpathios *et al.*, 1992; Carlson *et al.*, 1994; Mackenzie *et al.*, 1994; Pichichero, 1995; Gaston and Zurewsky, 1996) or skin ulceration (Clarridge, 1989). Although some earlier reports minimized its aetiological role, lately there have been many that draw attention to the possible pathogenic importance of *A. haemolyticum* (Banck and Numan, 1986; Miller *et al.*, 1986; Cambier *et al.*, 1992; Alos *et al.*, 1995). Some of the diseases caused by this bacterium may have serious clinical conditions and consequences (Waagner, 1991; Chang *et al.*, 1991; Givner, 1992; Karpathios *et al.*, 1992; Esteban *et al.*, 1994; Ford, 1995). Although the causative agent has been isolated in all age classes (Karpathios *et al.*, 1992; Coman *et al.*, 1996) it is most commonly isolated in young adult individuals (Carlson *et al.*, 1994 and 1995; MacKenzie *et al.*, 1994; Coman *et al.*, 1996). In everyday routine work it is very often overlooked for numerous reasons (Clarridge, 1989).

For the strains isolated from animals there are data pertaining to bull sperm (Richardson and Smith, 1968) sheep lungs (Roberts, 1969) and goat brain (von Younan and Drescher, 1996). According to the available literature, *A. haemolyticum* originating from piglet lungs has not been reported yet. Its pathogenic role is not altogether clear and requires further classification. It is definitely pathogenic to laboratory animals (MacLean *et al.* 1946; Herman, 1961) and there are some indications for pathogenicity to farm animals (Holt *et al.* 1994).

Since our isolates were pure cultures and piglets exhibited signs of respiratory tract infection while alive, it may have a possible aetiological role in causing clinically manifest infections of the porcine respiratory tract.

MATERIALS AND METHODS

The origin of the material

As clinically manifest infections of the respiratory tract were present in the herd, we examined the lungs of pneumonic Swedish Landrace piglets, aged 2-3 months, after they had been sacrificed.

Laboratory identification

Not later than 2 hours post sampling, pathoanatomically prepared parts of the lungs, were treated in the laboratory by homogenization with silver sand in a thioglycolate medium. The homogenate was stained according to Gram and inoculated on the following culture media: blood agar with 10% sheep blood and endo nutrient agar. Blood and nutrient agars were simultaneously inoculated with and without growth lines of *Staphylococcus aureus*. The streaked plates were incubated at 37°C, aerobically and microaerophilically, and read after 24, 48 and 96 h. Cultivation under anaerobic conditions was done on a thioglycolate medium, incubated at 37 °C and 42 °C. Subculturing from the thioglycolate medium was performed on blood agar after 48 h under aerobic, anaerobic and microaerophilic conditions.

The following aspects of suspect colonies were assessed: embedding, type of colony, colony characteristics including haemolysis diameter ratio, tinctorial status (staining according to Gram, Neisser, Ziehl-Nielsen) and the production of a CAMP phenomenon (with *Rhodococcus equi* and *Staphylococcus aureus*). In addition, oxidase and catalase tests, as well as the reaction to molecular oxygen were investigated. The biochemical activity of the isolates with regard to esculin, urea, lactose, gelatine, xylose and maltose was tested also. The identification was confirmed by using a commercial kit (API CORYNE - BioMerieux). A human isolate was used as the control strain. *Streptococcus pyogenes* was used as the control for Gram staining, *Corynebacterium diphtheriae* for Neisser staining and *Mycobacterium tuberculosis* for Ziehl - Nielsen staining. The last two were human isolates obtained thanks to the kindness of Dr Miloš Malenković from the Institute of Microbiology, Novi Sad and Dr Borka Lovodić from the Institute of Lung Diseases and Tuberculosis, Sremska Kamenica. *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used as positive controls for the oxidase and catalase tests. *Actinomyces pyogenes* and *Listeria ivanovii* strain CAMP-BRNO 5884 were obtained through the kindness of Dr Branka Vidić and employed as positive controls for the double CAMP test.

RESULTS

Culture characteristics. All five investigated isolates formed visible colonies on agar with 7-10 % sheep blood after 18 - 36 h incubation aerobically at 37 °C. The colonies were distinctly small, barely visible, with zones of complete haemolysis 2 to 5 times greater in diameter than the colony that eventually was formed (Fig. 1). The morphology of the colony (all its parameters) was S-form, whereas the appearance and consistency of the colonies imitated *Listeria species*, *L. ivanovii* more than *L. monocytogenes*, *Streptococcus* (sometimes more like *S. agalactiae*, other times more like *S. pyogenes*) and all "Miller" forms of other haemolytic representatives of the genus *Streptococcus*. On days 4 and 5, a central elevation was noticed using a slight enlargement of the microscope (lens x 10). This was not milky white, as in *A. pyogenes*, but more translucent - almost greyish and similar to the mentioned competitive genera (Fig.2). The colonies grew poorly on the nutrient agar with a characteristic "powdery" appearance very similar to *Actinomyces* colonies, but much faster.

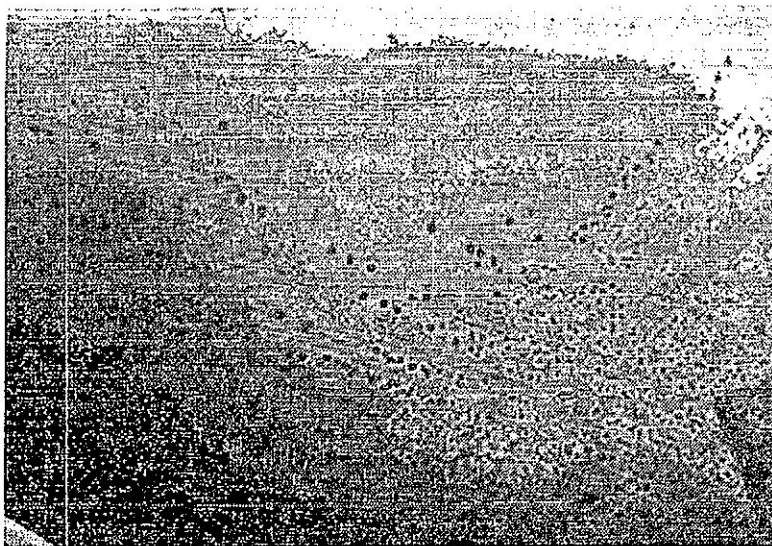


Figure 1. *Arcanobacterium haemolyticum* colonies on blood agar (24 hrs post incubation)

Tinctorial characteristics

Pleomorphism and polychromasia of gracile rods were present in all "age" categories of colonies, being more pronounced when the preparation was made from a solid, than from a liquid medium. The tendency towards a coccobacillary form culminated in younger cultures, obtained from the deep in the agar, after picking off the bacterial growth (Fig.3). The similarity to *Streptococcus* genus was such (coccoid cells) that it was necessary to make a preparation from liquid medium together with a control isolate of haemolytic *Streptococcus*. In liquid medium *A. haemolyticum* did not have the characteristic arrangement but was diffuse or in "clusters" in comparison with *Streptococcus* which formed chains. As

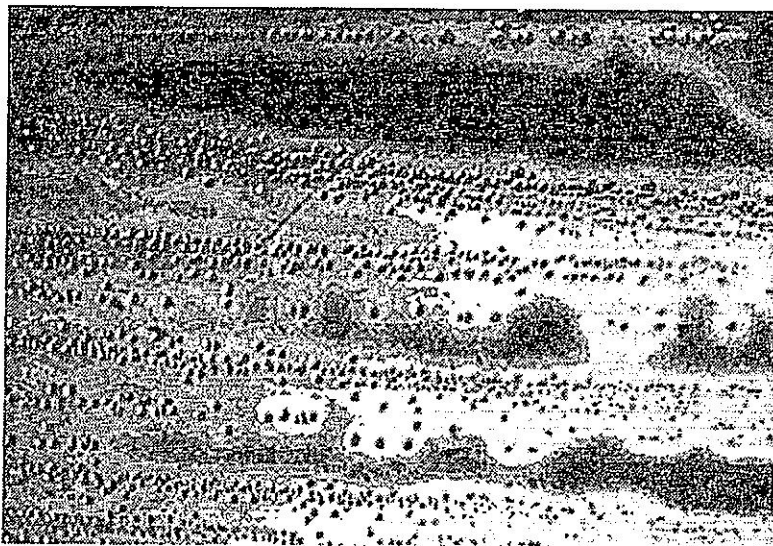


Figure 2. *Arcanobacterium haemolyticum* colonies on blood agar (96 hrs post incubation)

in the case of *A. pyogenes*, pleomorphism and polychromasia gave an impression that metachromatic granules existed, which were eliminated by Neisser staining. The isolates were not acidoresistant.

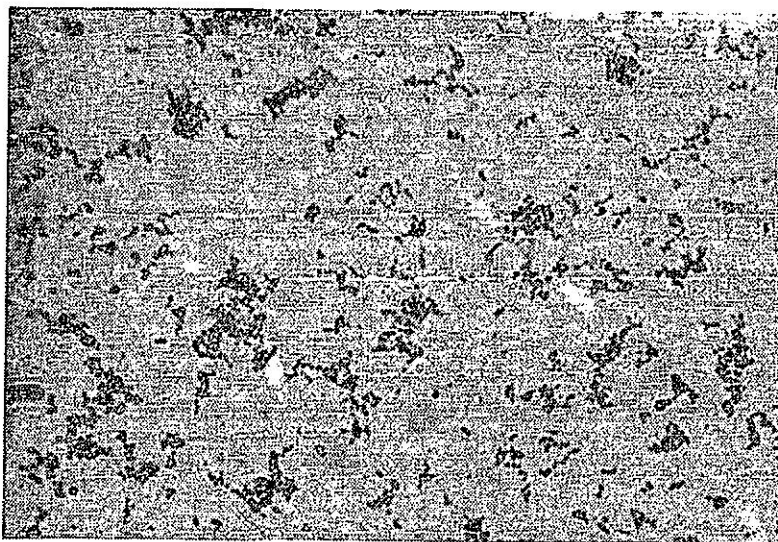


Figure 3. *Arcanobacterium haemolyticum*, a preparation from a 24-hour culture, Gram stained. Material taken from the deep in the agar - predominance of coccoid forms.

Oxidase, catalase, plasma, bacitracin and double CAMP tests. One out of five investigated isolates had an abortive catalase reaction, qualified as negative in comparison with the positive control (*Staphylococcus aureus*). All isolates produced a marked CAMP phenomenon with *R. equi*, with an "open umbrella" shaped pattern and a wide inhibition zone of haemolysis of *S. aureus*.

The initial crescent form of the CAMP phenomenon with *R. equi*, (up to 18 h) also imitated the initial crescent appearance of *L. ivanovii*. However *L. ivanovii* did not possess phospholipase D, responsible for the restriction of staphylococcal haemolysis. Prolongation of the incubation, after 24h, affected the appearance of the CAMP phenomenon with *A. haemolyticum* ("open umbrella" growth) and *L. ivanovii* ("closed umbrella" growth) (Fig.4). The result of the double CAMP test was fully in agreement with the biochemical identification of all the investigated isolates. No isolate coagulated plasma, nor exhibited susceptibility to bacitracin.

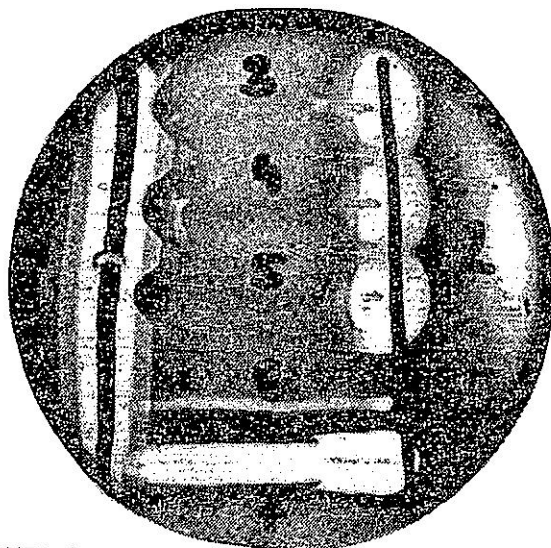


Figure 4. Double CAMP test.

Key Note:

Left vertical line = *Staphylococcus aureus*

Right vertical line = *Rhodococcus equi*

3, 4 and 5 = *Arcanobacterium haemolyticum*

6 = *Actinomyces pyogenes*

7 = *Listeria ivanovii* CAMP Brno 5884 (a "closed umbrella" -shaped pattern)

Note: Inverse CAMP phenomenon with *S. aureus* (left) and synergistic haemolysis with *equi* factors in the shape of an open umbrella (right)

Biochemical characteristics

All five investigated isolates departed from the same parameter, i.e. alpha Glucosidase, although they were expected to be 92% positive according to the identification table, with 99.9% probability. Such a uniform departure from the identification strain, ($T=0,75$), was considered to be excellent. All the other investigated characteristics, in all five isolates, were in agreement with the identification table. Thus, pyrazinamidase, alkaline phosphatase, beta

glucuronidase, N-acetyl- glucosaminidase, glucose, ribose, maltose, lactose and sucrose were positive, while pyrrolidonyl arylamidase, esculin, urease, gelatine were negative, as well as the catalase and oxidase tests (Table 1).

Table 1: Biochemical characteristics of the investigated isolates, with the human isolate as a positive control and the Identification table showing % positive reactions after 24h at 35 - 37 °C

	Reaction	Isolate reaction			
		Isolates	Human isolate	Identification table	
		1	2	3	4
NIT	NITrate reduction	0/5	-	0	1
PYZ	PyraZinamidase	5/5	-	10	98
PyrA	Pyrrolidonyl Arylamidase	0/5	-	100	70
PAL	ALkaline Phosphatase	5/5	+	65	85
β-GUR	beta GlucURonidase	0/5	-	100	36
β-GAL	beta GALactosidase	5/5	-	91	89
α-GLU	alpha GLUcosidase	0/5	+	94	92
β-NAG	N-Acetyl-β Glucosaminidase	5/5	-	41	89
ESC	ESCulin (β Glucosidase)	0/5	-	0	0
URE	UREase	0/5	-	0	0
GEL	GELatine (hydrolysis)	0/5	-	88	0
O	Oxidase	0/5	-	0	0
GLU	GLUcose	5/5	+	100	100
RIB	RIBose	5/5	+	100	91
XYL	XYLose	0/5	-	100	0
MAN	MANitol	0/5	-	0	0
MAL	MALtose	5/5	+	97	94
LAC	LACtose	5/5	+	91	100
SAC	Sucrose	5/5	-	50	44
GLYG	GLYcoGen	0/5	-	11	0
CAT	CATalase (ESC or GEL test)	0/5	-	0	0

Key: 1. The investigated isolates

2. The human isolate identified as *Arcanobacterium haemolyticum* (positive control)

3. *A. pyogenes*

4. *A. haemolyticum*

3 and 4= an approximative strain (Code Book Bio Merieux)

For all the five investigated isolates the identity of 99.9% had T = 0,75, thus qualifying the identification as excellent. The negative tests = 1 (alpha glucosidase = 92%).

The human strain that served as a control had 99.9%, with $T = 0.39$ and a good identification mark. The negative tests = 3 (Pyrazinamidase = 98%, N-acetyl- glucosaminidase = 89% and beta galactosidase = 89%).

DISCUSSION

Culture characteristics

Our isolates formed visible colonies, 0.1-0.3 mm in diameter 18-36 h after inoculation, this being slightly slower than in other investigations (MacLean *et al.*, 1946; Krech and Hollis, 1991, Clarridge, 1989), but in agreement with the studies that, like us, used sheep blood and aerobic culturing (Coman *et al.*, 1996). We are of the opinion that this is the consequence of culturing the primoisolate in aerobic conditions and atmospheric conditions with 3% CO. The effect of the atmosphere on growth rate and colony size already had been noticed in subcultures grown under aerobic, anaerobic and microaerophilic conditions. Provided the remaining identification conditions were identical, the fastest and most abundant growth occurred on a microphilic medium. Other researchers (Clarridge, 1989; Cummings *et al.*, 1993) have noticed and described in detail the same phenomenon. The fact that we used sheep blood, not human blood, like McLean *et al.*, 1946, can also account for the faster appearance of larger colonies (0.75 mm in diameter in 24h) in their other investigations involving human erythrocytes (Hermann, 1961; Clarridge, 1989, Gaston and Zurowsky, 1996).

The other properties of colonies (embedding, butter consistency, easy picking up (except from deep in the agar), easy emulsification and haemolysis (complete sometimes marginal due to incomplete haemolysis, growth, dependence on agar thickness) are fully in agreement with the results of other investigators (Bergey, 1986, Clarridge, 1989; Cummings *et al.* 1993, Coman, 1996). MacLean *et al.* (1946) was the first to recognize them as an undescribed species. The only difference between our isolates and those first described is that ours did not form a double zone of haemolysis, independent of the warm-cold effect. The only authors, in literature available to us, who described an R form of this bacterial species are Fell, 1997 and Carlson *et al.*, 1994. The latter group found some 25% of this bacterial species in the R form, not haemolytic, in 138 isolates 91% being from the human respiratory tract.

In 1946 McLean and coworkers described pin point gracile rods up to the 18th hour, which was confirmed by numerous other investigators (Collins *et al.*, 1986; Clarridge, 1989) with a further tendency towards granular forms and "swelling", thus visually imitating species of the genus *Streptococcus*. Gram instability occurred after 24 h, too.

It is almost impossible to find investigators with different results with regard to the tinctorial characteristics of *A. haemolyticum*. MacLean *et al.* (1946) pointed out the tinctorial similarity with *Streptococcus* spp.; *A. pyogenes*, *C. ulcerans* and *C. pseudotuberculosis*. Clarridge (1989) claimed that they retained the rod form in broth cultures, whilst being markedly coccoid if scraped from the depth of an agar plate. All the investigators that dealt with this problem, from MacLean and coworkers in 1946 until Clarridge and coworkers in 1995, pointed out the Gram instability and the impression of the existence of metachromatic granules (eliminated by adequate staining), and showed that pleomorphism and

polychromasia disappeared after 24 h. They also warned about possible confusion with both Gram positive and Gram negative gracile rods (Clarridge, 1989), particularly with species and genera that are or can be culturally similar: *Streptococcus*, *Listeriae*, *A. pyogenes*, *E. rhusiopathiae*. To our knowledge acidoresistance of isolates has not been described so far.

Catalase, oxidase, plasma, bacitracin and double CAMP tests. One of the investigated isolates had an abortive catalase reaction, sporadically occurred in other investigations (Collins, 1986). Oxidase, bacitracin and plasma tests were negative in all the five isolates, this not being denied in the literature.

The inhibition of haemolysis of *S. aureus* with *A. haemolyticum* as diagnostically significant for the species, was described by Zaharova and Kubelka, (1960), and later confirmed by numerous other investigators (Součková and Souček, 1972; Lamler, 1988; Clarridge, 1989 and Clarridge and Spiegel 1995; Krech, 1991; Comon, 1996). The authors use the term "inverse" CAMP (Clarridge JE, 1996) and "reverse" CAMP (Clarridge, 1989 and Clarridge and Spiegel, 1995; Funke et al., 1997). We prefer the term "inverse" CAMP, as the term "reverse" CAMP has been accepted in the literature for synergistic haemolysis of alfa toxins of *Cl. perfringens*, *Cl. paraperfringens*, *Cl. bifermentis* and *Cl. sordelli* with *S. agalactiae*, (Gubash, 1980).

Under the term "double CAMP" we mean an investigation of the relation of the observed isolate to *Staphylococcus aureus* and *Rhodococcus equi* on the same Petri dish as described by Clarridge, (1989) and Clarridge and Spiegel (1995) as well as Suvajdžić et al. 1996; 1998a and b.

The synergistic haemolysis with *R. equi*, caused by our isolates, did not differ from that described earlier Clarridge (1989) and Clarridge and Spiegel (1995), or from the haemolysis induced by the human isolate.

The literature contains controversial data with regard to biochemical characteristics of the species. MacLeans strains (MacLean et al. 1946) did not reduce nitrates supplemented with 20 % serum. In 1986 Collins and Cummin claimed that most of the strains reduce nitrates, this being confirmed by Clarridge in 1989. In the BioMerieux identification table nitrate is reduced by every hundredth isolate. Krech and Hollis (1991) and Clarridge (1989) expected a negative reaction. In the ninth edition of Bergeys Manual of Determinative Bacteriology it can be found that most strains reduce nitrates. The fact that one of our five isolates reduced nitrates does not seem unusual in this context. The human isolate, that served as the control, did not reduce nitrates.

As the approximative strain in the BioMerieux table is negative in 8% cases alpha glucosidase being negative in four out of five isolates is less of a problem. The human isolate that served as the control was alpha glucosidase positive.

With regard to the parameters that most investigators considered to be essential, our isolates showed no departure nor from the BioMerieux identification table. The isolates did not lyse coagulated serum as *A. pyogenes* did. Neither did they liquefy gelatine in two or three weeks, let alone in four to six days. The human isolate, did not dissolve gelatine either. The majority of studies, manuals and identification tables show that bacteria of this species are inert to gelatine (Cummings et al., 1986, Krech and Hollis, 1991, Collins and Cummins, 1986, Holt, 1994, Clarridge and Spiegel, 1995).

The human strain that served as the control departed from the BioMerieux table in three parameters: pyrrolidonyl arylamidase (98%), N-acetylglucosaminidase (89%), beta galactosidase (89%). This departure resulted in an identity assessment of 99,9%, with a certainty level $T=0,39$ and good identification. It differed from our isolates in the same way, our isolates being positive with regard to all three parameters.

CONCLUSION

As the characteristics of our isolated strains were in agreement with those of *A. haemolyticum* of human origin, which served as the control, and as the Bio Merieux software programme rated the identification of *A. haemolyticum* as excellent, we are of the opinion that we have proved the presence of this causative agent in the lungs of pneumonic piglets. We consider that in everyday routine work more attention should be paid when issuing the findings, particularly in those cases when the diagnosis is *Streptococcus* NON A NON B group for isolates of human origin, or when the strains are of animal origin and the diagnosis is *Arcanobacterium* (*Actinomyces*, *Corynebacterium*) *pyogenes*.

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KARAKTERIZACIJA IZOLATA *ARCANOBACTERIUM HAEMOLYTICUM* IZ PLUĆA PRASADI SA PNEUMONIJOM

SUVAJDŽIĆ LJILJANA, AŠANIN RUŽICA, KNEŽEVIĆ N I KOŠARČIĆ SLAVICA

SADRŽAJ

Arcanobacterium haemolyticum se retko izoluje iz kliničkog materijala i u humano i u veterinarskoj medicini. U našoj zemlji postoji samo jedan zvaničan izveštaj o izolovanju *A. haemolyticum* iz materija poreklom od životinja. U ovom ispitivanju patološki izmenjeni delovi pluća od prasadi sa znacima pneumonije, zasejavani su aerobno i mikroaerofilno, a suspektne kolonije su ispitane dvostrukim CAMP testom. Pozitivan inverzan CAMP sa *Staphylococcus aureus* - om i sinergistička hemoliza sa *Rhododococcus equi* usmerili su naše ispitivanje u pravcu *A. haemolyticum*-a. Dijagnoza je postavljena API Coryne setom Bio Merieux a potvrđena softverskim programom istog proizvođača sa verovatnoćom identiteta od 99,9% i uz stepen sigurnosti $T=0,75$. Jedino odstupanje od identifikacione tabele bilo je odsustvo -glukozidazne aktivnosti u svim ispitivanim uzorcima.

