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A Proposal of a Diagnostic Protocol for Isolation of *Corynebacterium ulcerans* from Cow's Milk*

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ABSTRACT

***Background*:** Literature about presence *Corynebacterium ulcerans* in milk samples from cows with mastitis is rare and in the literature there are only a few reports. In this study the isolation and identification of *Corynebacterium ulcerans* from mastitis in dairy cows were done. Also, optimization of diagnostic protocols to identify *Corynebacterium ulcerans* was performed.

***Materials, Methods & Results*:** The investigation was performed at the cattle farm that is characterized by closed housing system diary Holstein-Friesian cows during an outbreak of acute mastitis. Milk samples from 298 lactating cows were collected in sterile sampling tubes. Before the collection of quarter milk samples, the udder was thoroughly cleaned with soap and water and rubbed to dry. All collected milk samples were examined for mastitis using California mastitis test, which was carried out by the method first described by Schalm and Noorlander. Equal volumes (5 mL) of commercial CMT reagent and quarter milk were mixed and the changes in milk fluidity and viscosity were observed. Sample portions (0.1 mL each) were inoculated on 10% sheep blood agar, Endo agar and Sabouraud agar as well as on thioglycolate medium and nutrient broth. Primary plates were incubated for 3 days at 37ºC in aerobic conditions. Cultural, morphological and conventional biochemical testing was done. The survey was complemented by double CAMP and plasma coagulation tube test. All 14 isolates developed a synergistic haemolysis with Rhodococcus equi (ATCC 6939) and inverse CAMP phenomenon with *Staphylococcus* aureus and coagulated rabbit plasma. Final diagnosis was confirmed using API Coryne V 2.0 and software program by BioMerieux[1], revealing an identity rate of 99.9%, accuracy rate T = 1, test count = 0.

***Discussion*:** The first fourteen isolates of *Corynebacterium ulcerans* have been identified in our country, on the basis of a diagnostic protocol that is proposed in this paper. In our experience double CAMP test, rabbit plasma coagulation, catalase, oxidase tests and selected biochemical parameters, are sufficient as a diagnostic minimum. In the diagnostics of bacterial agents in cow mastitis, the attention of a bacteriologist is mostly limited to most widespread agents of mastitis, the isolation of which is mandatory pursuant to national legislation (*Staphylococcus aureus* and *Streptococcus agalactiae*). A more important reason for "missing" *Corynebacterium ulcerans* in the diagnosis is its colonial morphology that could resemble organisms of the genus *Staphylococcus*. Complex and expensive diagnostic procedure that is not available to most laboratories is also responsible for the small number of reports of isolation *C. ulcerans*. Furthermore, in routine work *C. ulcerans* could be misidentified with *Staphylococcus intermedius*, because of cultural similarity, positive plasma coagulation tube test and absence of manitol fermentation of both species. This paper is a report on isolation and identification of *Corynebacterium ulcerans* from milk of cows with mastitis, as well as a suggestion of a diagnostic protocol available for routine work in most veterinary microbiology laboratory. Therefore we suggest as the diagnostic protocol double CAMP test to be used as a complementary method to rabbit plasma coagulation tube test.

Keywords: *Corynebacterium ulcerans*, mastitis, diagnostic protocol, double CAMP.

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INTRODUCTION

Corynebacterium ulcerans is a well-known causative agent of diphteryform diseases in humans [16] with potential appearance of pseudomembrane [12] and malignant diphtheria [8]. Some of them were reported in individuals previously vaccinated against diphtheria [18]. *C. ulcerans* is a commensal in animals, which may represent the reservoirs for human infections [23]. Handling of infected dairy animals and consumption of contaminated milk have been associated with respiratory diphtheria-like disease caused by *C. ulcerans* [4]. Furthermore *C. ulcerans* causes alimentary intoxications [2] and cutaneous lesions in humans [21,25]. Reports on presence of *C. ulcerans* in milk samples from cows with mastitis are rare, and there are only few reports in the literature [13-15,17]. Thus, we are of the opinion that reporting the isolation of *C. ulcerans* from milk specimens originating from cows with clinical mastitis and positive in California-mastitis test might be of interest to other researchers in this field. As the isolation and identification of *Corynebacterium* spp. are not performed routinely in most microbiology laboratories involved in cow-mastitis investigation, these pathogens are only infrequently identified. Indeed, other *Corynebacterium* spp., as well as *C. ulcerans*, have been reported in bovine mastitis only in studies involving more extensive characterization [10,15].

MATERIALS AND METHODS

Cattle farm

The study was carried out in the summer of 2009, during an outbreak of acute mastitis on a large cattle farm situated in the northern part of the Autonomous Province of Vojvodina, Republic of Serbia. The farm is characterized by closed housing system of diary Holstein-Friesian cows. Most of the year, the cows are held in corals, but during the winter animals are tied in a stall barn. The animals are fed silage, dry beet pulp, brewer's grain containing 16% protein and green crop. Milking is performed according to standard regimen, twice a day, with an average milk yield of 5,700 liters. Udder papillae are disinfected before and after milking using chlorine based solutions.

Milk samples

Milk samples from 298 lactating cows were collected in sterile sampling tubes. Before the collection of quarter milk samples, the udder was thoroughly cleaned with soap and water and rubbed to dry. The teats were disinfected with cotton wool moistened with 70% ethyl alcohol and allowed to be air-dried. The first few squirts of milk were discarded. The quarter milk samples were stored in ice container and transported as soon as possible to the microbiological laboratory.

California Mastitis Test (CMT)

All collected milk samples were examined for mastitis using California mastitis test, which was carried out by the method first described by Schalm and Noorlander [20]. Briefly, equal volumes (5 mL) of commercial CMT reagent[2] and quarter milk were mixed and the changes in milk fluidity and viscosity were observed [17,20].

Microbiological examination

Sample portions (0.1 mL each) were inoculated on 10% sheep blood agar, Endo agar and Sabouraud agar as well as on thioglycolate medium and nutrient broth[3]. Primary plates were incubated for 3 days at 37°C in aerobic conditions. Following incubation at 37°C for 18 h, the thioglycolate medium was inoculated onto 3 plates with 10% sheep blood agar, which were consequently incubated at 37°C under aerobic, anaerobic and microaerophylic conditions. Nutrient broth was inoculated at 4°C for 7 days, and subcultures on blood agar were performed at two- day intervals with the aim to exclude presence of *Listeria* spp. All isolates were presumptively identified based on colonial morphology, tinctorial status (using Gram, Neisser and Ziehl-Nielsen methods), rabbit plasma coagulation tube test[4], the production of CAMP phenomenon in double CAMP test with *Rhodococcus equi* (ATCC 6939) and *Staphylococcus aureus*. Double CAMP test was performed on a separate Petri-dish with blood agar using *Staphylococcus aureus* and *Rhodococcus equi* (ATCC 6939) as diagnostic strains inoculated as vertical and paralell lines with an aim of confirmation or exclusion of both CAMP phenomenons by the investigated isolate (horizontal streak): synergistic haemolysis with *R. equi* and inverse CAMP phenomenon with *S. aureus* [5]. For controls at double CAMP test *Streptococcus agalactiae*, *Listeria monocytogenes*, *Streptococcus* non A non B group, *Corynebacterium* sp., *Corynebacterium pseudotuberculosis* and *Arcanobacterium pyogenes* were used. Catalase and oxidase tests on nutritive agar were performed, as

well as biochemical tests: fermentation of lactose and xylose, liquefaction of gelatin and hydrolysis of urea. The definitive biochemical identity of the bacteria was confirmed using API Coryne V 2.0 and software program-BioMerieux[1]. For control in plasma coagulation and catalase test *Staphylococcus aureus* was used, whilst *Pseudomonas aeruginosa* was used as control in oxidase test. Human isolates were used as the controls in staining procedures, i.e.: *Streptococcus pyogenes*, *Mycobacterium tuberculosis* and *Corynebacterium diphteriae* type gravis for Gram-, Ziehl Nielsen- and Neisser-staining, respectively.

RESULTS

Out of 298 examined milk samples from cows with clinical mastitis and positive California-mastitis test result, 14 isolates were suspected as *Corynebacterium ulcerans / pseudotuberculosis*.

All 14 suspect strains formed visible colonies on 10%-sheep blood agar after 18 h of incubation. The colonies were whitish, shiny, smooth and clearly margined, with a narrow β-haemolysis zone. After 24 h, the colonies resembled smaller colonies of haemolytic staphylococci, approximately 1 mm in diameter (Figures 1 & 2).

Subcultures on thyoglycolate medium revealed bacterial growth in all incubation conditions; however, the best growth was observed in microaerophylic conditions. The isolates survived at 4°C, but the phenomenon of "cold enrichment" was not observed. The growth of colonies on a nutritive agar was observed after 24 h, but their size was significantly smaller then of those grown on blood agar, reaching a diameter up to 0.5 mm. Gram-staining revealed Gram-positive rods and coccoid forms. Existence of metachromatic granules and acid-resistance was excluded by Neisser--staining and Ziehl-Nielsen staining, respectively. All the investigated strains coagulated rabbit plasma. In a double CAMP test all examined strains developed both CAMP phenomenons: a synergistic haemolysis with *Rhodococcus equi* (ATCC 6939), and inverse CAMP phenomenon with *Staphylococcus aureus* (Figure 3).

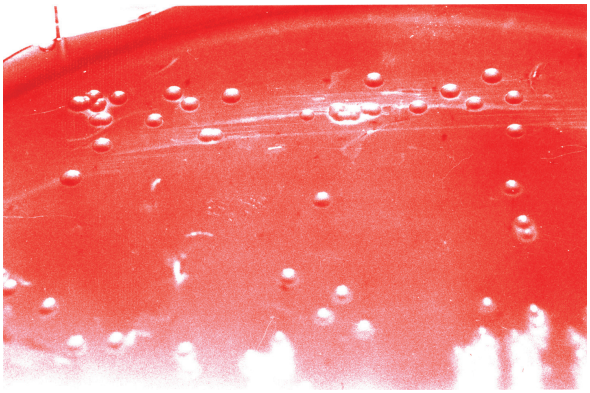

Figure 1. Colonies of *Corynebacterium ulcerans* on 10% sheep blood agar after 24 h, incubation at 37°C.

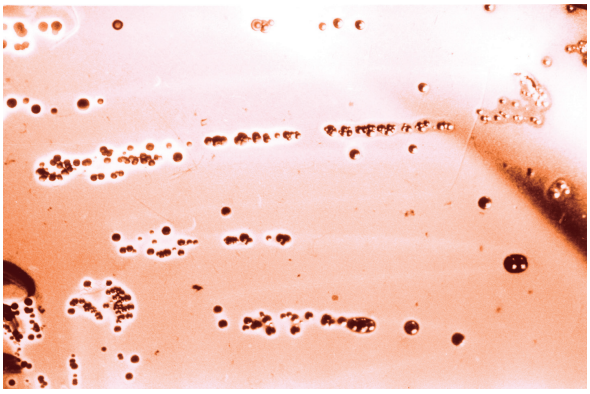

Figure 2. Colonies of *Corynebacterium ulcerans* with a narrow β-haemolysis zone on 10% sheep blood agar after 24 h, incubation at 37°C.

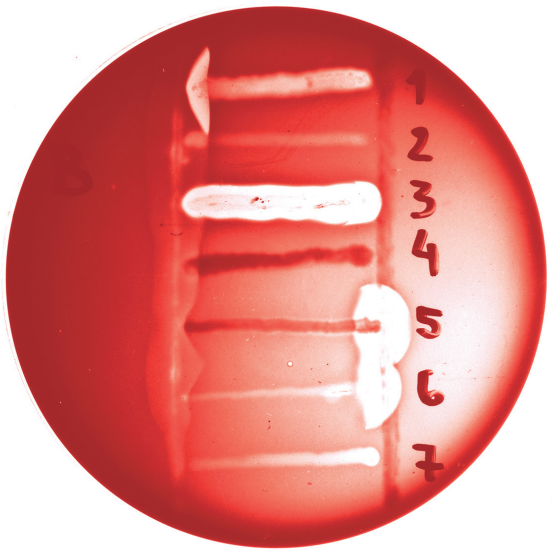

Figure 3. Double CAMP test, Legend: 1. *Streptococcus agalactiae*; 2. *Listeria monocytogenes*; 3. *Streptococcus* non A non B group; 4. *Corynebacterium* sp.; 5. *Corynebacterium pseudotuberculosis*; 6. *Corynebacterium ulcerans*; 7. *Arcanobacterium pyogenes*. Left vertical line is *Staphylococcus aureus* and right vertical line is *Rhodococcus equi* (ATCC 6939). Note: synergistic haemolysis with *Rhodococcus equi* and inverse CAMP phenomenon on *Staphylococcus aureus*.

Oxidase-test and catalase test with 3% H_2O_2 revealed a negative and a strongly positive result, respectively. The isolates resulted in neither lactose and xylose fermentation, nor gelatin liquefaction. On the other hand, all investigated isolates hydrolysed urea

and starch. Bacteriological diagnosis was confirmed using API Coryne V 2.0 and software program1, revealing an identity rate of 99.9%, accuracy rate T = 1, test count = 0. The identification rate was evaluated as excellent (Table 1).

Table 1. Biochemical characteristics* of strains of Corynebacterium ulcerans isolated from milk samples of cows with clinical mastitis.

Tests	Reactions	Results biochemical reactions	
		Tested strains	Percentage of positive reactions
NIT	NITrate reduction	0/14	1
PYZ	PYraZinamidase	0/14	1
PyrA	Pyrrolidonyl Arylamidase	0/14	1
PAL	ALkaline Phosphatase	14/14	99
β-GUR	beta-GlucURonidase	0/14	0
β-GAL	beta-GALactosidase	0/14	1
α-GLU	alpha-GLUcosidase	14/14	99
β-NAG	N-Acetyl-β-Glucosaminidase	0/14	1
ESC	ESCulin (β-Glucosidase)	0/14	1
URE	UREase	14/14	99
GEL	GELatine (hydrolysis)	0/14	1
O	Oxidase	0/14	0
GLU	GLUcose (Fermentation)	14/14	100
RIB	RIBose (Fermentation)	14/14	99
XYL	XYLose (Fermentation)	0/14	1
MAN	MANnitol (Fermentation)	0/14	1
MAL	MALtose (Fermentation)	14/14	99
LAC	LACtose (Fermentation)	0/14	1
SAC	Sucrose (Fermentation)	0/14	13
GLYG	GLYcoGen (Fermentation)	14/14	99
CAT	CATalase	14/14	100

*API Coryne V 2.0 and software program-BioMérieux.

DISCUSSION

Using this protocol, 14 *C. ulcerans* strains isolated from milk samples of cows with mastitis were identified. All identifications of *C. ulcerans* strains were confirmed applying the API Coryne V 2.0 - diagnostic kit and software[1].

This study demonstrated that the morphological, cultural and tinctorial traits of the isolates corresponded with the literature data [19]. In this study, plasma coagulation caused by *C. ulcerans* was observed, which corresponds with our previous experience. However, there are no references on such experiences in the available literature. Gomes *et al*. [11] reported that the coagulase tube test resulted in the formation of a thin layer of fibrin embedded in rabbit plasma by the non-toxigenic BR-CAT5003748 strain *C. diphtheriae*. All of our isolates protected erythrocytes from lysis (inverse CAMP phenomenon) but caused synergistic haemolysis with *Rhodococcus equi*. Soucek and Souckova [22] reported that only phospholipase D produced by *Arcanobacterium haemolyticum*, *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* can protect erythrocytes from lysis by the *staphylococcus* β-toxin. Bernheimer *et al*. [3] described gradual decomposition of erythrocyte membrane sphyngomyelyns influenced by phospholipase D excreted by Corynebacteria. Barksdale *et al*. [1] defined the production of phospholipase D as a crucial

marker in the genus *Corynebacterium*, because only *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* produce it. The gene encoding *Arcanobacterium haemolyticum* phospholipase D, which is responsible for the inverse CAMP-reaction, has been cloned and sequenced and showed some similarities to the corresponding genes of *C. pseudotuberculosis* and *C. ulcerans* [7]. Synergism with *Rhodococcus equi* is corresponding with our previous experience and literature data [5,6,24]. *C. ulcerans* always produced both, inverse CAMP phenomenon and synergistic hemolysis with *R. equi*.

This study demonstrated that results of catalase and oxidase tests, as well as biochemical tests (fermentation of lactose and xylose, liquefying of gelatin and hydrolysis of urea and starch) corresponded with the literature data for all examined isolates [9,19].

Biochemical features of examined strain confirmed by API Coryne V 2.0 and software program1, were in accordance with the identification table. The obtained results confirmed the identity of *C. ulcerans* with an identity rate of 99.9% and an accuracy rate T = 1 [9,26]. Isolation of *C. ulcerans* from nor human nor animal specimens has not been officially reported in Serbia, and international reports are very rare, as well. In this study, the first 14 isolates of *C. ulcerans* were identified in Serbia. Since *C. ulcerans* was isolated in pure culture from milk samples, we believe that it is the causative agent of the mastitis, what is in accordance with the work of some other authors [13-15].

We are of the opinion that colonial resemblance of *C. ulcerans* and *C. pseudotuberculosis* with species of the genus *Staphylococcus* is the main reason for "missing" these agents in the diagnostics. Crucial explanation for such "missing" is the fact that they, same as some staphylococci, produce plasma coagulation in the test tube. As common diagnostic minimum in most bacteriology laboratories includes tube coagulation test and mannitol fermentation test, we are of the opinion that introduction of double CAMP test is necessary. This test proves presence of phospholipase D enzyme that prevents erythrocyte-lysis caused by hemolysin of *Staphylococcus aureus* (inverse CAMP test) and synergistic hemolysis with *R. equi*. This enzyme is produced by *C. ulcerans*, *C. pseudotuberculosis* and *A. haemolyticum*, but it is not produced by any of *Staphylococcus* strains. Thus, positive double CAMP test, along with positive plasma tube test indicates presence of only two bacterial species - *Corynebacterium ulcerans* and *Corynebacterium* pseudotuberculosis, whilst *A. haemolyticum* is plasma-negative and resembles streptococci. The species *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* may differ from one another by their ability of glycogen or starch degradation (always-positive *Corynebacterium ulcerans* and always-negative *Corynebacterium pseudotuberculosis*).

CONCLUSIONS

The obtained results strongly emphasize the necessity of confirming or excluding *C. ulcerans* in milk samples originating from cows with mastitis. Application of double CAMP test along with plasma coagulation test would enable differentiation of *C. ulcerans* x *C. pseudotuberculosis* from staphylococci. Introduction of starch hydrolysis test would enable differentiation of *C. ulcerans* and *C. pseudotuberculosis* in most cases. The main reasons for suggesting this diagnostic protocol are its reliability, inexpensiveness and simple usage which is convenient for bacteriology laboratories with considerable daily routine. Furthermore, the diagnosis of *C. ulcerans* / *C. pseudotuberculosis* itself presents a valuable diagnostic achievement in the routine practice independent of the differentiation of these two species.

SOURCES AND MANUFACTURES

[1]BioMérieux SA, Marcy l'Etoile, France.

[2]Avatar rapid mastitis test Kit, Alvetera Gmbh, Germany.

[3]Oxoid Limited, Wade Road, Basingstoke, Hampshire, United Kingdom.

[4]Torlak, Institute for Virusology, vaccine i serums, Beograd, Republic of Serbia.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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