The aims of this study are the isolation and identification of possible bacteriological agents in respiratory infections of calves and the optimization of a diagnostic protocol to identify Arcanobacterium haemolyticum. Lesions of lungs from calves with pneumonia were examined. Cultural, morphological and conventional biochemical testing were done. The investigation was complemented by the double CAMP test. Five strains of Arcanobacterium haemolyticum in pure culture were found.

The presence of Arcanobacterium haemolyticum in the lungs of calves with pneumonia was established and, consequently, more attention should be paid to this species in everyday laboratory work.

The cultural similarity of Arcanobacterium haemolyticum to common bacteria like beta-hemolytic Streptococcus spp. and Arcanobacterium pyogenes is probably responsible for rare reports on the isolation of Arcanobacterium haemolyticum in veterinary microbiology. Our results indicate that Arcanobacterium haemolyticum could be or is the etiological agent of pneumonia. Therefore, we suggest the diagnostic
protocol available for routine work in most microbiological laboratories.

Key words: Arcanobacterium haemolyticum, pneumonia, calves, double CAMP

Introduction / Uvod

In human medicine Arcanobacterium haemolyticum is commonly described in cases of infective pharyngitis, sometimes with a characteristic scarlatiniform rash (Carlson et al., 1994; Karpathios et al., 1992; Suvajdžić et al., 2006). Other less commonly reported infections include osteomyelitis, meningitis, brain abscess, cavitary pneumonia, endocarditis and sepsis (Puerto Alonso et al., 2002; Therriault et al., 2008). Bacteremia caused by A. haemolyticum is rare, but a few cases of bacteremia associated with soft-tissue infections in immunocompetent patients were reported (Tan et al., 2006).

However, the isolation of A. haemolyticum from animals appears to be rare. In animals, A. haemolyticum was isolated from bull’s sperm (Richardson and Smith, 1968), sheep’s lungs (Roberts, 1969) and goat’s brain (Younan and Drescher, 1996). A single A. haemolyticum strain was isolated from a periodontal infection of a rabbit (Tyrrell et al., 2002). A. haemolyticum was isolated from piglet’s lungs (Suvajdzic et al., 2002). Arcanobacterium haemolyticum strains obtained from infections of horses were characterized phenotypically and genotypically (Hassan et al., 2009).

According to the available literature, A. haemolyticum originating from calf’s lungs has not been reported yet. Due to our findings, A. haemolyticum may have a possible etiological role as a cause of clinically manifest respiratory tract infections in calves.

Material and methods / Materijal i metode rada

Holstein-Friesian calves with clinically manifest pneumonia were euthanized during an outbreak in a herd and their lungs were examined. Affected calves were 2-3 months old. Homogenization of parts of the lungs with lesions was performed in a thioglycolate medium with silver sand (Oxoid) within two hours after sampling. The homogenate was inoculated on: agar with 10% sheep blood, 3 mm thick (SBA), endo agar (EA), nutrient agar (NA) and thioglycolate broth (TB). On SBA and NA inoculations were performed with and without streaks of Staphylococcus aureus. The streaked plates were incubated at 37 °C in aerobic and microaerophilic conditions and examined after 12, 18, 24, 36 and 48 h. The following aspects of suspect colonies were assessed: embedding, type of colony, colony characteristic including hemolysis diameter ratio, tinctorial status (Gram,
Neisser, Ziehl-Nielsen stains). The CAMP test (with *Rhodococcus equi* and *Staphylococcus aureus*) was performed on the same agar plate as described by Clarridge (Clarridge, 1989). The biochemical activity of the isolates was examined by conventional biochemical tests and commercial kits (API CORYNE, bioMérieux, France). Conventional biochemical tests included: oxidase, catalase, esculin, urea, lactose, xylose, maltose and gelatin. Bacitracin tests and growth in the presence of 0.33% cholic and 12-monoketocholeic acid were also performed.

### Results

Five strains of *A. haemolyticum* were isolated from 5 calves suffering from pneumonia. All five isolates formed visible S-form colonies on SBA and NA, but not on EA. Colony growth did not show dependence on staphylococcal or blood growth factors. The isolates grew better under microaerophilic than under aerobic conditions. After 12 hours of cultivation, a zone of complete hemolysis occurred without visible colony growth. Hemolysis showed non-distinctive edges and spread through the agar with a colony growth at the same time. After 24 hours of cultivation the hemolysis diameter was 2-5 times bigger than the colony diameter (Fig. 1), with colony size of 0.2 mm in microaerophilic conditions and 0.1 mm in aerobic conditions (using digital caliper).

![Figure 1. Arcanobacterium haemolyticum. Colonies on 10% sheep blood agar, after 24 hours, incubation at 37°C. Note that the hemolysis diameter is 2-5 times bigger than the colony diameter](image)

The Gram stain from young colonies (before 18 h of cultivation) showed gram-positive, thin, gracile rods which occasionally developed a branch effect. After 18 h of cultivation, bacterial cells had a different morphology and were pleomorphic and polychromatic. After 24 h of cultivation rods were Gram-variable,
Figure 2. *Arcanobacterium haemolyticum*. Gram-stain smears made from solid media taken by scraping from depth of agar. Note that bacteria are gram-negative and coccoidal in form.


Figure 3. *Arcanobacterium haemolyticum*. Gram-stain smears from liquid media. Showing gram-variable, thin, gracile, curved rods with blunted ends.


granulated with an impression of the existence of metachromatic granules and a domination of coccoid forms. No presence of metachromatic granules was detected by Neisser staining. Smears made from bacteria taken by scraping from the depth of the agar showed gram-negative coccoid cells (Fig. 2). Contrary to the solid media, smears made of bacteria from liquid media showed Gram-variable, thin, gracile, curved rods with blunted ends (Fig. 3).

The isolates restricted beta hemolysis of *Staphylococcus aureus* and caused synergistic hemolysis with the equi factor (phospholipase C) produced by *Rhodococcus equi*, presenting an “open umbrella” pattern. The presence of the hot-cold effect was not observed around colonies, but it was well observed in synergistic hemolysis in the double CAMP test (Fig. 4).

Table 1. Biochemical characteristics of isolates determined using commercial kit
(API CORYNE, bioMérieux, Marcy-l’Etoile, France) / Tabela 1. Biohemijske karakteristike izolata određene upotrebom komercijalnog kita (API CORYNE, bioMérieux, Marcy-l’Etoile, France)

<table>
<thead>
<tr>
<th>Biochemical Reaction</th>
<th>Biohemijska reakcija</th>
<th>Results of investigated strains</th>
<th>% positive strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reduction</td>
<td>all 5 strains positive</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pyrazinamidase</td>
<td>all 5 strains positive</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Pyrrolidonyl Arylamidase</td>
<td>all 5 strains positive</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>all 5 strains positive</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Beta Glucuronidase</td>
<td>all 5 strains negative</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Beta Galactosidase</td>
<td>all 5 strains positive</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>Alpha Glucosidase</td>
<td>all 5 strains positive</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl- Glucosaminidase</td>
<td>all 5 strains positive</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>ESCulin (- glucosidase)</td>
<td>all 5 strains negative</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>UREase</td>
<td>all 5 strains negative</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>GELatin (hydrolysis)</td>
<td>all 5 strains negative</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Oxidase</td>
<td>all 5 strains negative</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>GLUCosine</td>
<td>all 5 strains positive</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>all 5 strains positive</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>XYLose</td>
<td>all 5 strains negative</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MANitol</td>
<td>all 5 strains negative</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MALtose</td>
<td>all 5 strains positive</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>LACtose</td>
<td>all 5 strains positive</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>all 5 strains positive</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>GLYcoGen</td>
<td>all 5 strains negative</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CATalase</td>
<td>all 5 strains negative</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
All five isolates, investigated by conventional biochemical tests, were oxidase, catalase, esculin, xylose and urea negative, but lactose and maltose positive. No isolates liquefied gelatin. All five isolates were resistant to bacitracin and grew in the presence of 0.33% cholic and 12-monoketocholic acid.

The results of identification obtained using the commercial kit (API CORYNE, bioMérieux) are summarized in Table 1. The results were read using the bioMérieux software program. All strains were identified as *A. haemolyticum* with the probability rate of 99.9% and $T = 0.75$. All five isolates had the activity of nitrate reductase.

### Discussion / Diskusija

The isolates formed visible colonies slightly slower (24-36 h) and they were smaller (0.1-0.2 mm) than in other investigations. Maclean's discovery of *A. haemolyticum* was made from throat cultures on human blood agar, on which, at 24 h, colonies were 0.75 mm in diameter. After 48 h of incubation, colonies were about 1.5 mm in diameter (Maclean et al., 1946). Rabbit and human blood agar yielded the same colonial morphology of *A. haemolyticum*, but sheep blood yielded much smaller colonies that became hemolytic after 48 h of incubation (Hermann, 1961). In our opinion these differences were the consequence of primoisolation in aerobic and microaerophilic conditions with 3% CO$_2$ on 10% blood agar in this investigation. All the isolates formed visible colonies faster on SBA than NA, and in microaerophilic than in aerobic conditions. The effect of the atmosphere on growth rate and colony size has already been noticed in subcultures grown under aerobic, anaerobic and microaerophilic conditions (Clarridge, 1989; Cummings et al., 1993). The colonies were embedded, of buttery consistency, easy to emulsify and to pick up (except from the depth of the agar). All the isolates formed complete hemolysis with non-distinctive edges on SBA. This is in full agreement with the results of other investigators (Clarridge, 1989; Coman et al., 1996; Cummings et al., 1993). The only difference between our isolates and those first described was that ours did not form a hot-cold hemolysis (Maclean et al., 1946). However, the presence of the hot-cold effect was well observed in synergistic hemolysis in the double CAMP test (Fig. 4). All five investigated isolates formed visible S-form colonies on SBA and NA and, to our knowledge, only two authors described the R form of this bacterial species (Carlson et al., 1994; Fell et al., 1977).

It is almost impossible to find investigators with different results regarding tinctorial characteristics of *A. haemolyticum*. Maclean described pin point gracile rods up to the 18th hour with a further tendency towards granular forms and "swelling", thus visually imitating species of the genus *Streptococcus*. Gram instability occurred after 24 h, as well (Maclean et al., 1946). The same author pointed out the tinctorial similarity with *Streptococcus spp.*, *A. pyogenes*, *C. ulcerans* and *C. pseudotuberculosis*. They retained the rod form in broth cultures,
whilst being markedly coccoid if scraped from the depth of an agar plate (Clarridge, 1989). All investigators that studied this microorganism, pointed out 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, Listeria, A. pyogenes, E. rhusiopathiae*. To our knowledge acid resistant isolates have not been described so far.

In our research we found the inhibition of hemolysis of *S. aureus* with *A. haemolyticum* (inversa CAMP). This phenomenon is diagnostically significant for this species (Coman et al., 1996; Lammler and Blobel, 1988). We also found synergistic hemolysis with *Rhodococcus equi* resembling an "open umbrella". This corresponded to findings on human isolates and our previous experience with animal isolates (Clarridge, 1989; Suvajdzic et al., 2002).

The results obtained by conventional biochemical tests were in agreement with literature data. Among the results obtained by a commercial kit and software program (API CORYNE, bioMérieux, Marcy-l’Etoile, France), the only parameter that differed from the identification table was nitrate reductase activity (according to the bioMérieux identification table nitrate reduction should have been positive in only 4%). Our results about nitrate reductase activity are consistent with the reports of Collins and Cummins, 1986; and Clarridge, 1989. In the ninth edition of Bergey’s Manual of Determinative Bacteriology it can be found that most strains reduce nitrates (Holt et al., 1994). Some authors report opposite data regarding nitrate reduction of the species. Maclean’s strains (Maclean et al., 1946) did not reduce nitrates supplemented with 20% serum. Krech and Hollis expected a negative reaction (Krech and Hollis, 1991).

**Zaključak / Conclusion**

In our opinion, we established the presence of this species in the lungs of calves with pneumonia and its role in the etiology of the disease. We consider that in everyday work more attention should be paid to *Arcanobacterium haemolyticum* when issuing findings. Microbiologists in human medicine can misidentify this microorganism as *Streptococcus* non-A non-B group. When the samples are of animal origin this species can be confused with *Arcanobacterium pyogenes* which is frequent and an expected organism in animal specimens. In our experience, the double CAMP test, oxidase, catalase, esculin, xylose, urea, lactose, maltose, bacitracin test and gelatin liquefaction are sufficient as a diagnostic minimum, therefore we suggest this protocol as the diagnostic routine. The recommended tests are inexpensive and available to every routine laboratory and completely correspond to the API CORYNE bioMérieux commercial kit.
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References / Literatura

IZOLACIJA ARCANOBACTERIUM HAEMOLYTICUM IZ PLUĆA TELADI SA PNEUMONIJOM I PREPORUKA DJAGNOSTIČKOG PROTOKOLA

Ljiljana Suvajdzic, Jelena Ašanin, Bjanka Lako, A. Potkonjak, V. Sakač, Ivana Čabarkapa, Nataša Stojaković

Cilj ovog rada bila je izolacija Arcanobacterium haemolyticum kao potencijalnog uzročnika respiratorne infekcije teladi sa preporukom dijagnostičke procedure prilikom njegove identifikacije u laboratorijama koje se bave rutinskom dijagnostikom. Bakteriološki su ispitani izmenjeni delovi pluća teladi sa znacima pneumonije, zatim su izolatima ispitane kulturelne, morfološke i tinktorijalne osobine. U daljoj dijagnostici su korišteni klasični biohemiji testovi i dvostruki CAMP test. Dijagnoza je potvrđena komercijalnim kitovima ApiCoryne (bioMérieux, France), a dobijeni rezultati su očitavani softverskim programom istog proizvođača. Kako je Arcanobacterium haemolyticum izolovan u čistoj kulturi kod svih žrtvovanih životinja, mislimo da je on mogući uzročnik pneumonije teladi koja se razvila tokom života. Takođe smo mislili da u rutinskom radu zbog njegove kulturelne sličnosti sa češće prisutnim i zato očekivanim bakterijskim rodovima i vrstama, kao što su Arcanobacterium pyogenes u uzorcima porekla od životinja i Streptococcus vrsta u kliničkim uzorcima porekla od ljudi, dolazi do propusta i u humanoj i u veterinarskoj bakteriološkoj dijagnostici. Rezultati dobijeni primenom klasičnih biohemijskih ispitivanja koja su dopunjena dvostrukim CAMP testom nisu se razlikovali od rezultata dobijenih primenom bioMérieux, kitova, koji nisu dostupni većini rutinskih laboratorija, kako zbog cene potrošnog materijala, tako i zbog zahtevnijeg izvođenja i očitavanja rezultata. Pristupačnost i jednostavnost izvođenja, kao i tačnost dobijenih rezultata, podržavaju primenjeni dijagnostički proces, kao metod izbora u dokazivanju ove bakterijske vrste.

Ključne reči: Arcanobacterium haemolyticum, pneumonija, telad, dvostruki CAMP
Цель этой работы была изоляция Arcanobacterium haemolyticum как потенциального возбудителя респираторной инфекции телят с рекомендацией диагностической процедуры при его идентификации в лабораториях, занимаемые рутинной диагностикой. Бактериологически испытаны измениённые части легких телят с симптомами пневмонии, затем изолятами испытаны диагностические тесты и двукратный CAMP тест. Диагноз подтверждён коммерческими китами ApiCoryne (bioMérieux, France - Франция), а полученные результаты, отчитанные с помощью программы такого же производителя. Как Arcanobacterium haemolyticum изолирован в чистой культуре у всех животных, мы думаем, что он возможный возбудитель пневмонии телят, развившейся в течение жизни. Также мы думаем, что в рутинной работе из-за его культурального сходства с часто присутствующим и потому ожидаем бактериальным родом и видом, как Arcanobacterium pyogenes в образцах происхождением от животных и Streptococcus видов в клинических образцах происхождением от людей, приходит до двукратного теста и в гуманной и в ветеринарной бактериологической диагностике. Результаты, полученные применением классических биохимических испытаний, дополненные двойным CAMP тестом не различались от результатов, полученных применением bioMérieux, китов, которые не доступные большинству рутинных лабораторий, как из-за цены потребляемого материала, так и из-за более требовательного исполнения и отчитание результатов. Доступность и несложность выполнения, словно и точность полученных результатов, поддерживают применённую диагностическую процедуру, как метод выбора в доказывании этого бактериального вида.

Ключевые слова: Arcanobacterium haemolyticum, пневмония, телята, двойной CAMP тест