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Detection of *Mycoplasma bovis* from Hayflick-agar media by polymerase chain reaction

Jūratė Šiugždaitė

Lietuvos veterinarijos akademija,
Tilžės 18,
LT-3022 Kaunas, Lithuania

Forty-five nasal swab samples were collected from randomly selected calves aged under 3 months without clinical symptoms of pneumonia. Mycoplasmas were isolated from the nasal swab samples of 14 from 45 calves (31.1%).

For confirmation of *Mycoplasma bovis* species there was used a polymerase chain reaction (PCR) procedure on purified DNA of mycoplasma culture. Primers MBOURC2-L, MBOURC2-R were used to amplify a 1626 bp fragment of *Mycoplasma bovis* chromosomal DNA. Amplified products were analysed by agarose gel electrophoresis. The expected 1626 bp fragment was obtained in 11 from 14 *Mycoplasma bovis* field strains tested. According to the obtained data, the PCR method correlated well (78.6%) with cultural and biochemical characteristics of *Mycoplasma bovis.*

Key words*: Mycoplasma bovis,* cultural, biochemical, characteristics, PCR method

INTRODUCTION

Many agents are involved in respiratory diseases of calves. *Mycoplasma bovis* is an early agent of respiratory diseases in calves [6, 7].

Mycoplasma bovis was first isolated from cases of severe mastitis in cattle in the USA [5]. *Mycoplasma bovis* is associated with a variety of antibiotic-resistant bovine diseases such as chronic pneumonia, arthritis, mastitis and genital tract infection [9, 10]. The common methods of identifying infected animals still involve direct culture methods of up to 7 days duration with immunological speciation done with conventional antisera [3, 4]. Antisera often cause cross-reactivity with other mycoplasma species [8]. It is very important to develop a sensitive and rapid diagnostic procedure for *Mycoplasma bovis* detection.

Significant efforts have been made for the development of new techniques, including rapid and sensitive DNA hybridization probes [1, 2]. PCR technology is the most promising due to its rapidity, economical convienence and sensitivity, since it can detect a few microorganisms in clinical samples [11].

The aim of this study was detection of *Mycoplasma bovis* field strains from Hayflick agar medium by PCR procedure.

MATERIAL AND METHODS

The study was carried out in 2000. All newborn calves received colostrum from their dams within 2 h after their birth. The animals were fed twice a day. In the study were included randomly selected 45 calves aged under 3 months without clinical symptoms of pneumonia. *Mycoplasma bovis* strains were isolated from nasal swab samples.

The samples were cultivated on Hayflick-agar. Before PCR amplification Hayflick-agar plates were incubated for 3–7 days at 37°C in a CO_2 incubator. The colonies were observed in a Zeiss Axiovert 35

inverted light microscope. Isolates were identified on the grounds of cultural and biochemical characteristics (films and spots production). As the positive control reference strain 5063 was used.

After 7 days of incubation the mycoplasma colonies from Hayflick agar medium were mixed with Master mix reagents. 25 µl Ultra pure distilled water (Gibco BRL), 65 µl 10 × PCR buffer (Gibco BRL), 6.5 µl 10 m M dNTP mix buffer (Gibco BRL), 52 µl Mg Cl_2 (Gibco BRL), Primers 13 µl MBOUVR C2 -R, 13 µl MBOURVC -L (Gibco BRL), 3.25 µl Ampli Taq DNA polymerase were used for 14 samples (Sigma). The positive control was *Mycoplasma bovis* reference strain 5063. The negative control was ultra pure distilled water (Gibco BRL).

The amplification (49 µl master Mix and 1 µl of sample) was perfomed on a Perkin Elmer Geneamp PCR system 2400. The thermocycler conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s, finally 72°C for 5 min.

The amplified products were analysed by gel electophoresis, using 1.2% agarose gel (Agarose MP, Multi purpose agarose, Boehringer Manheim) containing ethidium bromide (0.8 mg/ml) for 30 min at a constant 100 V, in 1 × TBE buffer. A molecular size DNA marker (DNA ladder *Mycoplasma bovis* PCR products, Gibco BRL) was run concurrently. The gels were examined by UV illumination for presence 1626 bp band. PCR product obtained after amplification of *Mycoplasma bovis* DNA was photographed by using the computer programme (Kodak digital Science 120).

RESULTS AND DISCUSSION

The observation of calves was continued till 3 months of age. Fourty-five nasal swab samples were collected from randomly selected calves without clinical symptoms of pneumonia. Mycoplasmas were isolated from nasal swab samples of 14 from 45 (31.1%) calves (Table 1). After cultivation (from 3 to 7 days) on 14 Petri plates with Hayflick-agar media, the mycoplasma colonies were smaller (50–500 µm in diameter) and had the "fried egg" morphology. The film and spots were characteristic of *Mycoplasma bovis*. The wrinkled film that develops on the medium surface consists of cholesterol and phospholipids. The tiny black spots that appear beneath and around the colonies are attributed to the deposition of calcium and magnesium salts of fatty acids liberated by the lipolytic activity [4, 5]. PCR sensitivity was tested on purified DNA on mycoplasma culture. Fourteen isolated mycoplasma cultures for confirmation of *Mycoplasma bovis* species were examined by *Mycoplasma bovis* primers MBOUVRC2-L, MBOUVRC2-R.

The expected 1626 bp fragment was obtained in 11 from 14 *Mycoplasma bovis* field strains tested (78.6%) (Table 2).

By comparing the product bands with the bands from a known molecular-weight marker, it is easy to identify the product fragments which are of

Table 1. **Mycoplasmas isolated from calve nasal swab samples**

No. of samples	No. of calves	Mycoplasma growth on solid Hayflick media	Film and spots formation
1.	4246	+	+
2.	4125	+	+
3.	4245	+	+
4.	4336	–	–
5.	4551	–	–
6.	4144	–	–
7.	4221	–	–
8.	4331	–	–
9.	4330	–	–
10.	4347	+	+
11.	4333	+	+
12.	4335	+	+
13.	4012	+	+
14.	4112	+	+
15.	4552	–	–
16.	4669	–	–
17.	4889	–	–
18.	4998	–	–
19.	4775	–	–
20.	4772	–	–
21.	4336	–	–
22.	4116	–	–
23.	4992	–	–
24.	4339	–	–
25.	4779	+	+
26.	5002	+	+
27.	5219	+	+
28.	4998	+	+
29.	4555	–	–
30.	4963	–	–
31.	4665	–	–
32.	4888	+	+
33.	4369	–	–
34.	4999	–	–
35.	5555	–	–
36.	5558	–	–
37.	5444	+	+
38.	5596	–	–
39.	4883	–	–
40.	5446	–	–
41.	5897	–	–
42.	5852	–	–
43.	5789	–	–
44.	5824	–	–
45.	5669	–	–

Table 2. **PCR products results from amplification of DNA extracted from *Mycoplasma bovis* field strains**

No. of samples	No of calves	Detection of *Mycoplasma bovis* by PCR
1.	dist. water	control
2.	4246	+
3.	4125	–
4.	4245	+
5.	4347	+
6.	4333	+
7.	4335	+
8.	4012	–
9.	4112	+
10.	5063 DNA	positive control reference strain of *Mycoplasma bovis*
11.	DNA ladder	1626 bp
12.	5219	+
13.	4998	+
14.	4888	+
15.	5444	–
16.	4779	+
17.	5002	+

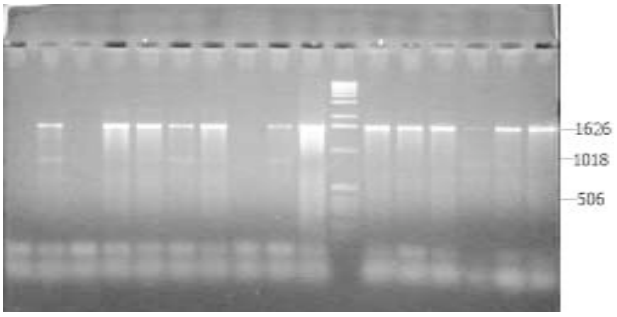

Figure. Agarose gel electrophoresis of PCR products that resulted from amplification of DNA extracted from *Mycoplasma bovis* field strains (isolated from nasal swabs of calves without clinical symptoms of pneumonia). Lane 1 – negative control (distilled water), lanes 2, 4–7, 9, 12–14, 16, and 17 – expected 1626 bp band fragments of *Mycoplasma bovis* field strains. Lane 10 – positive control Reference strain 5036, lane – 11 DNA ladder, lanes 3, 8, 15 – bands of DNA products less than 1626 bp

appropriate molecular weight [1, 2, 11]. In many diagnostic laboratories the diagnosis of mycoplasma is based on clinical symptoms and cultural characteristics [4, 5]. The objective of this study was to evaluate the PCR procedure for detection of *Mycoplasma bovis*. According to the present data PCR correlated well with cultural and biochemical features characteristic of *Mycoplasma bovis* (78.6%). The results demonstrated that the PCR is a valuable adjunct in the detection of *Mycoplasma bovis* [1, 2, 11].

Immunological speciation done with conventional antisera often causes a cross-reactivity with other mycoplama species [8].

The data on nasal mycoplasmal flora are important for the epidemiology of mycoplasma species isolated from calves without clinical symptoms [6, 7].

CONCLUSIONS

1. *Mycoplasma bovis* was isolated from calve nasal swab samples in 14 of 45 cases (31.1%).

2. The PCR procedure correlated well with cultural and biochemical characteristics of *Mycoplasma bovis*. The expected 1626 bp fragment was obtained in 11 from 14 Mycoplasma *bovis* field strains tested (78.6%).

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Jūratė Šiugždaitė

Mycoplasma bovis NUSTATYMAS HAYFLIKO AGARE NAUDOJANT POLIMERAZĖS GRANDININĘ REAKCIJĄ

S a n t r a u k a

Iš 45 tirtų veršelių nosies išskyrų mėginių keturiolikoje rastos mikoplazmų kultūros (31,1%). Mėginiai imti iš veršelių iki 3 mėnesių amžiaus be klinikinių pneumonijos simptomų. Hayfliko agare augančios mikoplazmų kolonijos pastebėtos 3–7 parą. Kolonijos išaugo apvalios nuo 50 µm iki 500 µm dydžio. Jos savo forma priminė nesuplaktą kiaušinį. Ištyrus biochemines savybes, nustatyta, kad Hayfliko agare visos išskirtos mikoplazmų kultūros sudarė taškelius ir plėveles. Tai būdinga *Mycoplasma bovis* rūšiai. *Mycoplasma bovis* rūšiai patvirtinti buvo pritaikytas polimerazės grandininės reakcijos (PGR) metodas. Išsifrakcionavę *Mycoplasma bovis* DNR fragmentai 1,2% agarozės gelyje buvo vertinti UV šviesoje, prieš tai juos nudažius etidžio bromido dažais. Norėdami nustatyti mikoplazmų DNR fragmentų dydį, panaudojome *Mycoplasma bovis* žymeklį, turintį 1626 bazių poras. Ištyrus keturiolika *Mycoplasma bovis* kamienų, nustatyta, kad DNR fragmentai vienuolikoje mėginių (78,6%) buvo 1626 bazių poros. Mikoplazmų rūšiai identifikuoti taikomi įvairūs serologiniai ir imunologiniai metodai. Kadangi mikoplazmos neturi ląstelės sienelės, jų identifikavimą apsunkina kryžminės reakcijos su kitomis mikoplazmų rūšimis. Polimerazės grandininės reakcijos metodas jautrus, specifiškas ir tinkamas nustatyti *Mycoplasma bovis* rūšį. Mūsų tirti veršeliai klinikinių pneumonijos požymių neturėjo, todėl jie galėjo būti slaptas *Mycoplasma bovis* infekcijos sukėlėjo šaltinis.

Raktažodžiai: *Mycoplasma bovis*, kultūros, biocheminės savybės, polimerazės grandininės reakcijos metodas

Юрате Шюгждайте

УСТАНОВЛЕНИЕ *Mycoplasma bovis* С АГАРА ГАЙФЛИКА С ПОМОЩЬЮ РЕАКЦИИ ПОЛИМЕРАЗНОЙ ЦЕПИ

Р е з ю м е

Из 45 исследованных телят без клинических симптомов пневмонии было выделено 14 микоплазмовых культур (31,1%).

Рост колоний был отмечен на 3–7 сутки культивирования микоплазм на агаре Гайфлика. Выросли круглые колонии, диаметром от 50 µм до 500 µм. По своей форме колонии напоминают феномен „взбитого яйца“. В результате исследования биохимических свойств установлено, что все выделенные культуры микоплазм на агаре Гайфлика образовали точки и пленки. Это характерно для вида *Mycoplasma bovis.*

Для подтверждения вида *Mycoplasma bovis* была применена реакция полимеразной цепи (РПЦ). Выфракционировавшиеся фрагменты ДНК *Mycoplasma bovis* на 1,2%-ной гели были исследованы на фоне УВ света, перед этим покрасив их краской этидиум бромида.

Для установления величины фрагментов ДНК микоплазм был использован маркер ДНК *Mycoplasma bovis* (состоящий из 1626 базовых пар). При исследовании 14 штаммов *Mycoplasma bovis* установлено, что в 11 случаях (78,6%) были ДНК фрагменты с 1626 базовыми парами, а в 3 случаях – менее 1626 базовых пар.

Для идентификации микоплазм до вида применяются разные серологические и иммунологические методы. Поскольку у микоплазмы нет клеточной стенки, микоплазмы часто вызывают крестную реакцию с другими видами микоплазм. Это осложняет их идентификацию.

По нашим данным, полимеразная цепочная реакция для определения вида *Mycoplasma bovis* чувствительна и специфична. Хотя у исследуемых телят не было клинических симптомов пневмонии, они могли являться носителями возбудителя микоплазменной инфекции.

Ключевые слова: *Mycoplasma bovis*, культуры, биохимические свойства, реакция полимеразной цепи