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Original article

Screening for *Mollicutes* microorganisms in perinatal calf mortality cases in Polish dairy herds

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Abstract

Perinatal calf mortality in dairy herds has been reported worldwide. The etiology of stillbirth is multifactorial, and can be caused by various species of bacteria and environmental factors. Among them some potential pathogens from the *Mollicutes* class such as *Mycoplasma* (*M.*) spp. and *Ureaplasma* (*U.*) *diversum* can be isolated from the bovine genital tract and other organs of the suspected cattle. The aim of this study was to evaluate if the bacteria belonging to the *Mollicutes* class i.e. *M. bovis*, *M. bovigenitalium*, *M. canadense*, *M. canis*, *M. arginini*, *M. bovirhinis*, *M. dispar*, *M. alkalescens* and *U. diversum* could have an impact on perinatal calf mortality in selected Polish dairy farms. The material was: 121 stillborn calves (SB), 21 live born calves (C) and 131 cows (dams) from 30 Polish Holstein-Friesian herds. Samples were examined from all the SB calves' and six control euthanized calves' abomasal contents and lung samples collected during necropsy, and from the dams' serum and placenta. In dams the serological ELISA, and in calves and placenta samples molecular PCR/denaturing gradient gel electrophoresis, methods were used. Screening of dams' sera for antibodies to *M. bovis* (ELISA) showed seven dams positive for *M. bovis*, whereas none of the nine examined *Mollicutes* microorganisms were detected in the placenta and calves.

Key words: perinatal mortality, stillborn calves, Mollicutes, serology, PCR/DGGE

Introduction

Worldwide perinatal mortality of dairy calves varies from 2% to 10% (Mee et al. 2008). The causes of stillbirth (SB) are multifactorial, including bacterial infection, and it can vary in different regions and countries (Berglund et al. 2003, Jawor et al. 2013, Jawor et al. 2017). Pathogens from the *Mollicutes* class - *Mycoplasma* (*M.*) spp. and *Ureaplasma* (*U.*) *diversum* can be isolated from the bovine genital tract (Doig 1981). One of the most important bovine mycoplasma species - *M. bovis* – is a versatile pathogen and has been previ-

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ously reported in bovine pneumonia cases (Nicholas et al. 2008), in cases of arthritis and meningitis (Stipkovits et al. 1993), abscesses (Kinde et al. 1993) and genital disorders (Ruhnke 1994). There are a few reports of *M. bovis* – induced abortions as well as isolation of *M. bovis* from aborted fetuses and joints from neonatal animals (Stalheim and Proctor 1976, Byrne et al. 1999). A number of other mycoplasmas isolated from the bovine urogenital tract or aborted fetuses, including: *M. bovigenitalium, U. diversum, M. bovirhinis*, *M. canadense*, *M. alkalescens* and *M. arginini. M. bovigenitalium* and *U. diversum* are thought to be causes of abortions (Doig 1981, Wittkowski et al. 1984, Gale 1987). *M. arginini* is one of the primary pneumonia pathogens, but it was rarely isolated from respiratory bovine cases (Hirose et al. 2003). *U. diversum* – closely related with mycoplasmas species, was often isolated from the bovine urogenital tract, is able to cause infection, and contributes to pneumonia cases as well (Wittkowski et al. 1984, Gale 1987). *M. alkalescens* has mostly been implicated in mastitis in cattle. It has been isolated from bulk tank milk samples, as well as from outbreaks and sporadic cases of clinical mastitis (Jasper 1980). *M. bovirhinis*, *M. canadense* and *M. dispar* are thought to play a secondary role in infection, since they are not able to induce an infection independently but only to complicate an existing infection (Gourlay et al. 1978, Muenster et al. 1979, Ross 1993, Nicholas et al. 2008). *M. canis* shows a predilection for the reproductive tract of dogs, but is also often isolated from bovine pneumonia cases (Nicholas et al. 2008, Chazel et al. 2010).

In Poland the seroprevalence of *M. bovis* in the entire cattle population was previously estimated at 76.6% (Bednarek et al. 2012) and at 64.3% in the Polish cattle population suffering from BRD (Dudek and Bednarek 2012). Moreover, based on the latest study concerning other pathogens belonging to the *Mollicutes* class, various such species were reported both in calves and adult Polish cattle (Szacawa 2015, Szacawa et al. 2016).

The aim of this study was to examine whether, and what kind of, the selected bacteria from the *Mollicutes* class could have an impact on perinatal calf mortality in selected Polish dairy herds.

Materials and Methods

The experimental design was approved by the II Local Ethics Committee in Wroclaw (permission numbers 23/2012, 58/2014, 60/2014).

Samples

The study was carried out on 142 calves with a gestation of ≥260 days and 131 of their dams. One hundred and twenty-one stillborn calves (SB) were born by 110 dams (11 cows birthed twin SB calves each), and 21 control calves (C) were born after normal assisted calvings by 21 control dams. The calves were born between November 2013 and June 2015 in 30 Polish Holstein-Friesian herds. After calving, the fetal part of the placenta and serum samples from dams were collected. From the C group, six male calves were euthanized and necropsied according to the same project-specific protocol as the SB calves. During necropsy the abomasal content and lung samples were collected aseptically. All samples were aliquoted and frozen at -80°C until analysed.

Anti-*M. bovis* specific antibody detection

Anti-*M. bovis* specific antibodies in the cow sera were analysed using a commercial indirect ELISA kit (Bio-X Diagnostics, Jemelle, Belgium) according to the manufacturer's instructions. Optical densities were measured at λ=450 nm. The sample was considered positive if its value as Val (the signal read for each sample well divided by the corresponding positive control serum signal and multiplied by 100) was greater or equal to 37%. Each serum's degree of positivity was then determined as one plus (1+) by 37%<Val<=60%, two plus (2+) by 60%<Val<=83% and three plus (3+) by 83%<Val<=106%. Results were considered negative when Val was under 37%.

DNA extraction

DNA from *post-mortem* lung tissue samples, abomasal content and placenta samples was examined. Approximately 24.0±1.0 mg of collected tissue were homogenized and placed in 20 ml of PBS (pH 7.4±0.2). DNA was extracted from 100 μl of the homogenate. DNA extraction from tissue samples was performed using the QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's procedure.

Mycoplasma spp. and *U. diversum* identification

PCR/denaturing gradient gel electrophoresis (PCR/DGGE) was performed using a DCode Universal Mutation Detection System (Bio-Rad, USA) for the detection of *Mycoplasma* spp. and *U. diversum*. The primers were described by McAuliffe et al. (2005) and the DGGE method by McAuliffe et al. (2005) with modifications of PCR conditions (Dudek et al. 2016) and gel composition (Szacawa et al. 2015). DNA from the following strains of the bacteria was used as controls: reference strain of *M. bovis* (ATCC 25523), type strains of *M. bovigenitalium* (ATCC 19852), and NCTC

Table 1. Results of anti-*M. bovis* specific antibody detection in dam sera with degree of positivity.

Dams	Negative	Degree of positivity of anti-*M. bovis* antibody detection (1+ – 3+)		
		1+	2+	3+
control	19	0	1	1
with PM	105	3	2	0

PM – perinatal mortality
(1+ – 3+) – degree of positivity from one to three pluses

type strains of: *M. canadense*, *M. canis*, *M. arginini*, *M. bovirhinis*, *M. dispar*, *M. alkalescens* and *U. diversum* obtained from the Animal Plant and Health Agency, Weybridge, UK.

Results

The results of serological examinations in the cow sera samples (Table 1) indicated that seven animals were positive for *M. bovis*. Among 110 examined anti-*M. bovis* specific antibodies were detected in five dams (4.6%) of SB calves derived from four herds. Among 21 of C calves two (9.5%) were positive and originated from two herds (the same herds where SB dams were positive for *M. bovis*). The PCR/DGGE did not confirm the presence of genetic material for any of the nine examined bacteria from the *Mollicutes* class in lung and abomasal samples collected from the SB calves, six control calves and in the placenta samples.

Discussion

The SB and C calves were previously examined for the potential infections of *Neospora caninum*, *bovine herpesvirus* type 1, *bovine viral diarrhea virus*, *Schmallenberg virus*, *Leptospira hardjo* and *Leptospira pomona* and culture for other bacteria in aerobic, anaerobic and microaerobic conditions (Jawor et al. 2017). In that study it was shown that 21.5% of SB calves were infected in utero and *Neospora caninum* was the most commonly detected pathogen. Despite evidence of the presence of infection *in utero* in Polish dairy herds, in none of the examined samples were *Mollicutes* microorganisms detected. However, the presence of antibodies against *M. bovis* in 4.6-9.5% of examined dams of stillborn and control calves respectively, indicates that this bacteria was present in the examined herd and therefore could not be certainly excluded as a possible cause of perinatal calf mortality. Mycoplasmas occur in the genital tract of cattle with clinical manifestation of genital problems and sometimes as a subclinical infection. Usually, *M. bovis* is a main cause of pneumonia and arthritis in calves and mastitis in cows (Pfützner and Sachse 1996), but it can also be associated with endometritis and abortion; however, a typical genital disease with consequences such as abortion was found only in a few individuals (Byrne et al. 1999). Hermeyer et al. (2012) reported the etiological role of *M. bovis* in one aborted bovine fetus and in one neonatal weak calf with uncommon pneumonic lesions and a disseminated infection. Moreover, there are few experimental studies which demonstrated that *M. bovis* and ureaplasmas are capable of causing abortion and SB in cattle (Doig 1981, Wittkowski et al. 1984). It should be noted that, in consequence of *M. bovis* derived pneumonia and arthritis, mastitis can occur and this may cause transmission of the pathogen to the uterus or fetus (González and Wilson 2003, Houlihan et al. 2007). As compared to abortion, the *Mycoplasma* sp. infection as a cause of stillbirth is less commonly detected (Syrjala et al. 2007, Waldner et al. 2010). This might be a reason why, even if the *M. bovis* is able to cause abortion, no SB calf in our study was positive for *Mollicutes*. Absence of clinical symptoms of mycoplasma infections in the dams probably indicated an adequate stimulation of humoral immune response which lead to the effective elimination of the pathogen from the host in the early stage of infection, and this might explain the lack of detection of *M. bovis* in calves. Positive results for *M. bovis* in the control cows could suggest the wide spread of the bacterium in dairy herds, which does not always result in reproductive disorders.

Although there are studies which show an association between positive antibody reaction to *M. bovis* and abortion (Byrne et al. 1999), the results of our study do not confirm that similar association may be seen in cases of stillbirth. In the study quoted, cows that aborted fetuses did not have typical characteristics of mycoplasmal infection, but only anti-*M. bovis* antibodies were detected in the dams' sera. Lack of isolation of the *Mollicutes* class bacteria in our study indicates that this bacteria should be considered as an unlikely cause of perinatal mortality in dairy cattle.

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References

Bednarek D, Ayling RD, Nicholas RA, Dudek K, Szymańska-Czerwińska M (**2012**) Serological survey to determine the occurrence of respiratory *Mycoplasma* infections in the Polish cattle population. Vet Rec 171: 45.

Berglund B, Steinbock L, Elvander M (**2003**) Causes of Stillbirth and Time of Death in Swedish Holstein Calves Examined Post Mortem. Acta Vet Scand 44: 111-120.

Byrne WJ, Brennan P, McCormack R, Ball HJ (**1999**) Isolation of *Mycoplasma bovis* from the abomasal contents of an aborted bovine fetus. Vet Rec 144: 211-212.

Chazel M, Tardy F, Le Grand D, Calavas D, Poumarat F (**2010**) Mycoplasmoses of ruminants in France: recent data from the national surveillance network. BMC Vet Res 6: 32.

Doig PA (**1981**) Bovine genital mycoplasmosis. Can Vet J 22: 339-343.

Dudek K, Bednarek D, Ayling RD, Kycko A, Szacawa E, Karpińska TA (**2016**) An experimental vaccine composed of two adjuvants gives protection against *Mycoplasma bovis* in calves. Vaccine 34: 3051-3058.

Dudek K, Bednarek D (**2012**) Last survey of *Mycoplasma bovis* prevalence in Polish cattle affected with respiratory syndrome. Bull Vet Inst Pulawy 56: 447-451.

Gale SP (**1987**) The effects of two *Ureaplasma diversum* strains on early pregnancy in heifers. Can J Vet Res 51: 536-538.

González RN, Wilson DJ (**2003**) Mycoplasmal mastitis in dairy herds. Vet Clin North Am Food Anim Pract 19: 199-221.

Gourlay RN, Wyld SG, Burke NF, Edmonds MJ (**1978**) Isolation of *Mycoplasma canadense* from an outbreak of bovine mastitis in England. Vet Rec 103: 74-75.

Hermeyer K, Peters M, Brügmann M, Jacobsen B, Hewicker-Trautwein M (**2012**) Demonstration of *Mycoplasma bovis* by immunohistochemistry and in situ hybridization in an aborted bovine fetus and neonatal calf. J Vet Diagn Invest 24: 364-369.

Hirose K, Kobayashi H, Ito N, Kawasaki Y, Zako M, Kotani K, Ogawa H, Sato H (**2003**) Isolation of Mycoplasmas from nasal swabs of calves affected with respiratory diseases and antimicrobial susceptibility of their isolates. J Vet Med B Infect Dis Vet Public Health. 50: 347-351.

Houlihan MG, Veenstra B, Christian MK, Nicholas R, Ayling R (**2007**) Mastitis and arthritis in two dairy herds caused by *Mycoplasma bovis*. Vet Rec 160: 126-127.

Jasper DE (**1980**) Prevalence of mycoplasmal mastitis in the Western States. California Vet 34: 24-26.

Jawor P, Stefaniak T, Sołtysiak Z, Dzimira S, Bednarski M (**2013**) Salmonella enterica serovar Stanley intrauterine infection in a stillborn calf case report. Acta Vet Brno 82: 363-367.

Jawor P, Król D, Mee JF, Sołtysiak Z, Dzimira S, Larska M, Stefaniak T (**2017**) Infection exposure, detection and causes of death in perinatal mortalities in Polish dairy herds. Theriogenology 103: 130-136.

Kinde H, Daft BM, Walker RL, Charlton BR, Petty R (**1993**) *Mycoplasma bovis* associated with decubital abscesses in Holstein calves. J Vet Diagn Invest 5: 194-197.

McAuliffe L, Ellis RJ, Lawes JR, Ayling RD, Nicholas RA (**2005**) 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species. J Med Microbiol 54: 731-739.

Mee JF, Berry DP, Cromie AR (**2008**) Prevelence of and risk factors associated with perinatal calf mortality in pasture-based Holstein-Fresian cows. Animal 2: 613-620.

Muenster OA, Ose EE, Matsuoka T (**1979**) The incidence of *Mycoplasma dispar*, Ureaplasma and conventional Mycoplasma in the pneumonic calf lung. Can J Comp Med 43: 392-398.

Nicholas R, Ayling RD, McAuliffe L (**2008**) Mycoplasma Diseases of Ruminants. CAB International, pp 133-168.

Pfützner H, Sachse K (**1996**) *Mycoplasma bovis* as an agent of mastitis, pneumonia, arthritis, genital disorders in cattle. Rev Sci Tech 15: 1477-1994.

Ross RF (**1993**) *Mycoplasma* - animal pathogens. In: Kahane I, Adoni A (eds) Rapid diagnosis of mycoplasmas. Plenum Press, New York, pp 69-109.

Ruhnke HL (**1994**) Mycoplasmas associated with bovine genital tract infections. In: Whitford HW, Rosenbusch RF, Lauerman LH (eds) Mycoplasmosis in Animals. Laboratory Diagnosis. Iowa State University Press, Ames, Iowa, pp. 56-61.

Stalheim OH, Proctor SJ (**1976**) Experimentally induced bovine abortion with *Mycoplasma agalactia* subsp *bovis*. Am J Vet Res 37: 879-883.

Stipkovits L, Rady M, Glavits R (**1993**) Mycoplasmal arthritis and meningitis in calves. Acta Vet Hung 41: 73-88.

Syrjala P, Anttila M, Dillard K, Fossi M, Collin K, Nylund M, Autio T (**2007**) Causes of bovine abortion, stillbirth and neonatal death in Finland 1999-2006. Acta Vet Scand 49 (Suppl I): S3.

Szacawa E, Niemczuk K, Dudek K, Bednarek D, Rosales R, Ayling R (**2015**) *Mycoplasma bovis* infections and co-infections with other *Mycoplasma* spp. with different clinical manifestations in affected cattle herds in eastern region of Poland. Bull Vet Inst Pulawy 59: 331-337.

Szacawa E, Szymańska-Czerwińska M, Niemczuk K, Dudek K, Woźniakowski G, Bednarek D (**2016**) Prevalence of pathogens from *Mollicutes* class in cattle affected by respiratory diseases and molecular characteristics of *Mycoplasma bovis* field strains. J Vet Res 60: 391-397.

Waldner CL, Kennedy RI, Rosengren LB, Pollock CM, Clark ET (**2010**) Gross postmortem and histologic examination findings from abortion losses and calf mortalities in western Canadian beef herds. Can Vet J 51: 1227-1238.

Wittkowski G, Roth B, Kirchhoff H (**1984**) Demonstration of mycoplasms and ureaplasmas in the bovine genital tract. Berl Munch Tieraerztl Wochenschr 97: 189-190, 193-197.