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Case Report

IMMUNOMODULATOR, *MYCOBACTERIUM* CELL WALL FRACTION, AN AID IN CONTROL OF PERSISTENT *MYCOPLASMA BOVIS* INFECTION IN DAIRY COWS

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Abstract

Mycoplasma bovis is pathogen known to cause respiratory disease, otitis media, arthritis and a variety of additional diseases in cattle. Infections caused by this pathogen have significant impact on the health, welfare and productivity of dairy and beef cattle resulting in substantial economic losses. Current preventive and treatment strategies rely on the use of antimicrobials and vaccines; however, their efficacy is limited due to difficult diagnosis and inconsistent disease expression. Here, we report results on the use of *Mycobacterium* cell wall fraction (MCWF), an immunomodulator, in dairy cows persistently infected with *M. bovis* during the period 2011-2015. Application of MCWF aided in reducing *M. bovis*-associated clinical signs, such as pneumonia, arthritis and mastitis, and improving overall reproductive performance and days in lactation of infected dairy cows. These results were compared to historical information on the health status and farm performance for period 2011-2015, and suggest that MCWF immunomodulator could be introduced as an aid in treatment protocols for persistent infections with *M. bovis* in dairy cows.

Key Words: arthritis, immunotherapy, mastitis, *M. bovis*, MCWF, pneumonia

CASE PRESENTATION

Mycoplasma bovis (*M. bovis*) is pathogen whose ability to cause respiratory disease, arthritis and mastitis has been demonstrated in numerous studies. Following natural infection, *M. bovis* can be isolated in pure culture from the mammary glands of cows with mastitis and from the joints, tendon sheaths or periarticular tissues of cattle with arthritis or chronic pneumonia and polyarthritis syndrome (CPPS) (Gagea et al., 2006). The prevalence of *Mycoplasma* infection in dairy herds in North America varies from

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7-35% and these variations are related to the either type of samples used or tests performed (Maunsell et al., 2011). In the United States of America alone, economic losses to dairy producers acquired as a result of mastitis and reduced reproductive performance are estimated to be over US$ 120 million per year (Maunsell et al., 2011).

Mycobacterium cell wall fraction (MCWF) is a biological immunomodulator derived from soil-born, non-pathogenic *Mycobacterium phlei* (NovaVive, Canada). MCWF has potential use in multiple veterinary health services, such as the treatment of infectious diseases (Rogan et al., 2007; Romanowski et al., 2017) and in anti-cancer therapy (Filion & Phillips, 2001). The mode of action of MCWF is based on the activation of innate and adaptive (humoral and cell-mediated) immunity with the purpose of the recognition, reaction to and recovery from infections (Filion & Phillips, 2001).

Case description

Farm location and management

For the purpose of this report, data on the health status, milk production and reproductive performance were collected, analyzed and described for the period from 2011 to 2015. A dairy farm populated with Simmental and Holstein cows, Orljevo, Petrovac Municipality, Serbia, was under supervision for the purpose of determining the efficacy of MCWF in reducing severity and incidence of CPPS in dairy cows. Farm management included housing of all cows in tie-stall barns and twice daily milking through vacuum pipes (De Laval). Growth of young calves and heifers was organized in separate barns from the first day after calving. The farm management system also required re-location of pregnant heifers to a barn with adult cows 2 to 3 weeks before estimated calving. This management practice was enforced due to the presence of milking equipment (milking lines and attaching claws) only in one barn. Male calves were sold at 10 days of age. Average annual milk production for the period 2012 to 2015 was 7,160 to 9,398 liters.

Case history

In order to increase average and total milk productivity on the farm, which had twenty Simmental cows, an additional sixteen pregnant Holstein heifers were imported from Germany. All sixteen cows were quarantined at Lučar Farms, Deronje, Serbia for thirty days before they were brought onto the farm in August 2011. During the quarantine period, all animals were tested for the presence of *Mycobacterium bovis* by tuberculin skin fold test. In addition, animals were tested for the presence of pathogens causing bovine brucellosis, leucosis, infectious bovine rhinothracheitis (IBR) and Bluetongue diseases. Blood samples were collected and sent to the Institute of Veterinary Medicine of Serbia, Belgrade as required by regulations laid down by the Ministry of Agriculture and Veterinary Directorate, Republic of Serbia. All submitted tests were negative, and cows did not show any clinically relevant signs, and thus, were considered healthy and released from quarantine.

Seven days after the imported Holstein cows were housed in the new barn, the first clinical signs were noted. Ten cows showed signs of fever (39.4 to 39.8°C), apnea, lethargy and inappetence with two cows dying within several hours. Necropsies were performed and pathomorphology examination revealed acute pericarditis and fibrinous bronchopneumonia. *Mannheimia haemolytica* was the only pathogen isolated from lung tissue. Blood samples from all remaining animals were collected and fifty-four tested positive for bovine parainfluenza 3 virus (BPI3V), six were positive for bovine respiratory syncytial virus (BRSV) and nine positive for IBR. All cows were negative for bovine virus diarrhea virus (BVDV). Calving of the imported Holsteins started within two weeks of their arrival in the new barn, with two calvings and one abortion at 256 at days of pregnancy. All remaining Holstein heifers calved the following month. Two months after calving, an outbreak of severe bronchopneumonia accompanied with joint swelling, pain and heat on palpation occurred. Seven imported Holstein cows and eleven of the resident Simmental cows died (total 18) within 60 days post calving. Necropsies were performed on each animal and tissue samples from lungs and joints were collected and sent to SVIS for further diagnostics. At necropsy, severe changes observed in lungs were consistent with the previous pathology report. Furthermore, *M. haemolitica* was confirmed by isolation from lung samples, and the presence of *Mycoplasma bovis* was confirmed by PCR technique from both lungs and joint samples. In addition, serology results revealed the presence of BPI3V. Overall, these clinical findings were considered to be a severe clinical case of bovine respiratory disease complex (BRDC). A BRDC outbreak had occurred in 2011 on the farm, and the loss of 18 cows within a two month period induced direct and indirect losses of more than € 70,000.

Similarly to the BRDC outbreak in 2011, in 2012, signs of joint swelling in large rotator joints (hip, stifle, hock, shoulder, elbow and carpal joints and periarticular soft tissue) were observed in all cows in the same barn, but not in the heifers and cows that were housed separately in a neighboring barn 300 meters away. These clinical conditions ceased spontaneously and a period of herd health was stabilized. Health status on the farm during 2012 to 2013 was stable, with occasional mortality (1 and 0 cases, with replacement rate 25% and 26.3% in 2012 and 2013, respectively Table 1). The forced-unplanned culling rates due to arthritis and pneumonia were 15% and 8%, respectively (Table 2) in naive heifers and first lactating cows after they had been housed in the same barn with older cows during a transition period.

In spring 2014, the farm faced a new outbreak of clinical conditions, predominantly affecting only heifers prior to calving, (becoming first lactating cows). Approximately fourteen days following parturition, clinical signs including cough, fever, mastitis and swelling in booth metatarsal and metacarpal joints were observed (Figure 1) in a group of eighteen pregnant heifers prior to calving (12 daughters of imported cows raised at the farm and six Simental heifers from resident cows, born in 2012). In addition, mastitis with visible changes in the milk was noted (pale to bluish, watery, reduced volume to complete drying-off). Laboratory testing excluded bacterial (*Escherichia*

coli, Staphylococcus aureus, Streptococcus uberis) and algae (*Prothoteca*) sub-clinical mastitis. Treatments applied included the antimicrobials, tylosine (Tilozin 200, VZ Subotica, 5 mg/kg) and enrofloxacin (Baytril Max. Bayer, Germany, 15mg/kg) and supportive therapy: corticosteroids, non-steriodal anti-inflammatory drugs and high doses of vitamin C.

Table 1. Incidence of clinical signs associated with *Mycoplasma* infections (2012-2015)

	2012	2013	2014	2015
Number of cows/heifers	32/18	38/42	41/41	44/32
Abortus	2	1	1	3
Dead	1	0	2	1
Culling Reason				
Reproductive		5	3	4
Arthritis (joint swelling)	3	2	8	6
Pneumonia	2	1	4	1
Metabolic diseases	2		1	-
Mastitis		1	-	
Other		1	-	2
Total № of lost cows:	8	10	18	14
% of cows:	25.0	26.3	43.9	31.8

Table 2. CPPS mortality rate and milk production (2012-2015)

	2012	2013	2014	2015
Average DIM* of culled cows	52.8±37.84	121.00±140.31	120.33±119.52	250.29±131.57
Average lactation (L)	1.33±1.89	1.67±0,82	2.17±1.40	2.78±1.56
№ of cows with arthritis (joint swelling)	3	2	8	6
№ of cows with pneumonia	2	1	4	1
Percent of the herd with CPPS	15.6	7.9	29.3	15.9

*DIM – Days in milk

The treated heifers were not able to stand or move for 14 to 21 days, showing signs of severe inappetence and rapid body weight loss. Despite the therapy, seven first lactating cows lost up to 200 kg within the first thirty days following parturition. Application of antimicrobial and supportive therapy showed minimal efficacy and conditions progressed with continuous joint swelling, doubling in size, abscessation and serous-mucous exudate. In addition, milk from these freshly calved cows was discarded due to the changes in milk as a result of mastitis and antibiotic treatments. All seven first lactating cows were removed during the first 30 to 60 days in milk (DIM) due to the

complications caused by CPPS. Overall at the end of 2014, eighteen cows in total were culled (43.9%), and 12 cows (29.3%) were lost due to arthritis (8) or pneumonia (4). A vaccination program for the entire herd was established and included application of Cattle Master® vaccine (Zoetis, USA). The vaccination program was followed for three consecutive cycles and was suspended in 2015 as no visible benefits were observed in terms of the reduction in respiratory clinical signs or culling rate. At this point, *Mycoplasma* infections or CPPS were considered as the main underlying causative agent for all health problems observed on the farm.

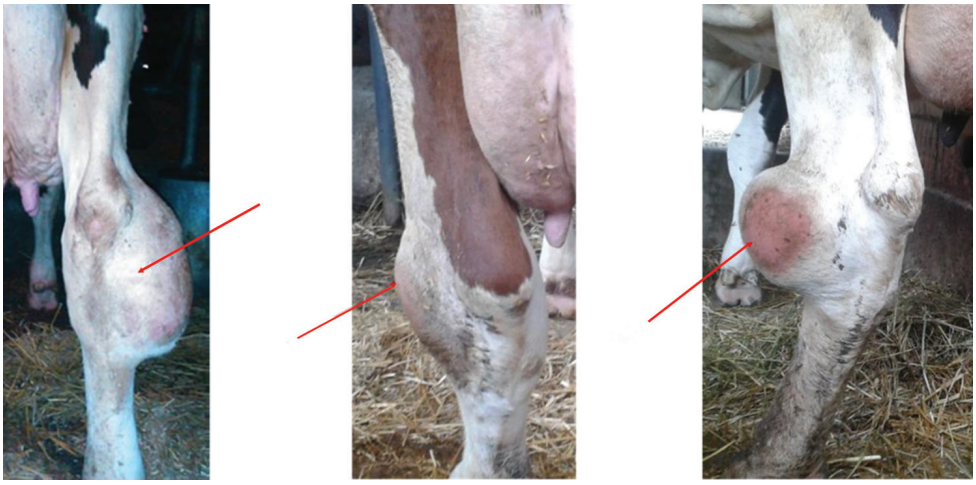

Figure 1. Chronic arthritis in heifers from 2014; before MCWF application

Application of MCWF and results

MCWF is currently registered as an immunotherapeutic for treatment of neonatal diarrhea induced by *E. coli* K99+ in calves (Romanowski et al., 2017). Treatment with MCWF started on July 1, 2014, and all 84 cows and heifers on the farm received 500ug of MCWF given subcutaneously in the neck in front of the shoulder. A second treatment was administered after 14 days, and then MCWF was given once monthly for the next two months (a total of four administrations). Prior to application, the hide area was trimmed with scissors and disinfected with 70% ethyl alcohol. Following MCWF administration, all cows were closely monitored for 2 h for the occurrence of any systemic or local adverse reactions and then the injected area was examined once daily by palpation for an additional 3 days. Ten animals

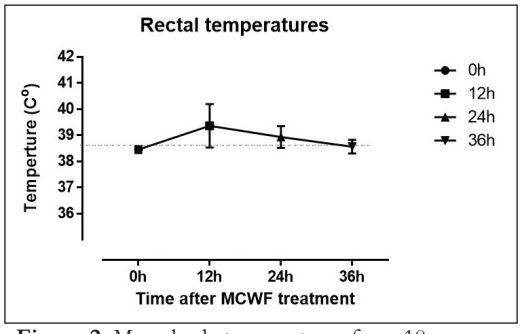

Figure 2. Mean body temperatures from 10 cows following MCWF application

out of 84 reacted with elevated temperatures (Figure 2) within 24 h following MCWF administration.

The mild fever observed was transient and lasted for 24 to 36 h. During that period, the same animals showed signs of lethargy, inappetence and 15% drop in milk production. This type of reaction is usually considered as normal in other animal species, as it indicates strong immunomodulatory reaction. There were no other clinically significant changes observed at the site of injection during the examination period. MCWF was applied to pregnant heifers five days prior their relocation to milking/parturition barn. All heifers that received MCWF five days prior to relocation to milking/parturition barn, and ten days before their estimated parturition, they did not show any clinical signs or these were minimal and transient and mostly presented as mild joint swelling. In addition, heifers that were already in the barn at the time of MCWF application did not show any clinical signs associated with CPPS. Consequently, there was no forced culling within the first two months post parturition (the first 60 DIM). In cows that were already affected with CPPS and had significant swelling in their joints, MCWF application resulted in initial slight increases of affected joint sizes within five days post administration, followed by the gradual regression and complete remission within 30 to 45 days post MCWF administration (Figures 3 and 4).

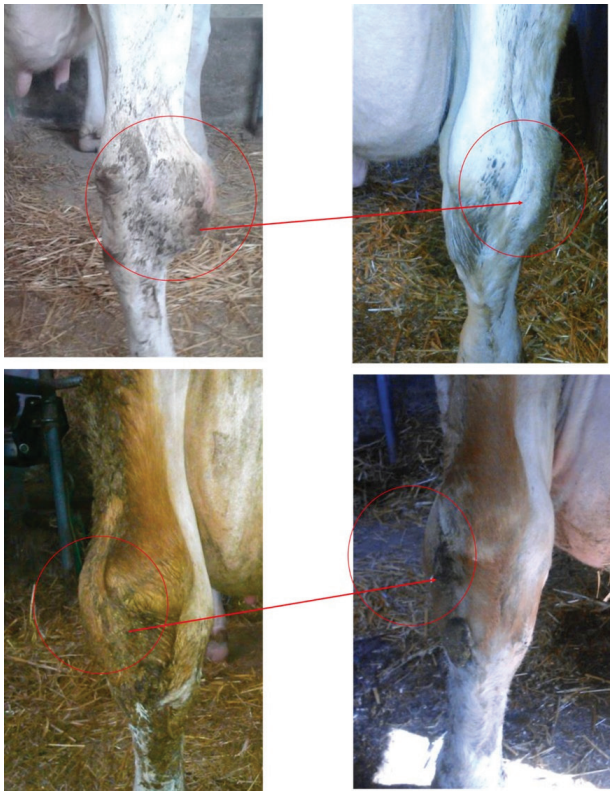

Figure 3. Joints of affected heifers before and after MCWF application

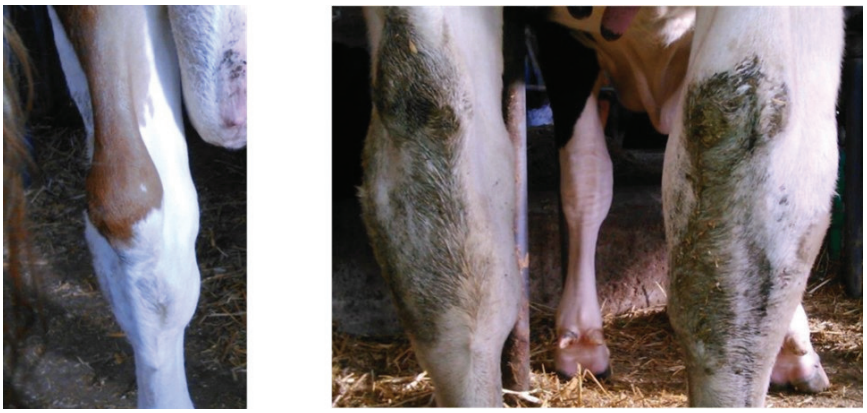

Figure 4. Current status of cows on the farm (arthritis)

Following application of MCWF, the culling rate due to the CPPS was decreased in 2015 compared to 2014 (from 29.3% to 15.9%, respectively). In addition, average DIM of culled cows was increased (from 120.33±119.52 to 250.29±131.57), as well as average lactation (from 2.17±1.40 to 2.78±1.56) Table 2.

Three months after the last MCWF administration, sporadic clinical signs of CPPS were observed, accompanied with decreases in reproductive performance (abortions and embryo mortality were noted and identified as the main problem for cows and heifers in their 4th to 6th month of pregnancy and rebreeds at 30 to 35 days. Aborted fetuses were sent to the Scientific Veterinary Institute Novi Sad for necropsies and microbiological testing, and *M. bovis* was the only pathogen detected. The repeated occurrence of clinical signs related to CPPS can be attributed to the persistent presence of *M. bovis* on the farm and lack of additional MCWF administrations that would likely increase immune capacity of the infected animals in order to effectively control the disease.

DISCUSSION

M. bovis has characteristics that enable it to colonize and persist on mucosal surfaces, to invade tissues, and to persist at sites of disease despite an aggressive immune response. Molecules involved in adherence, antigenic variation, invasion, immunomodulation, biofilm formation and production of toxic metabolites are important in pathogenesis, but how exactly *M. bovis* interacts with the host is poorly defined (Maunsell et al., 2011). Innate immune responses are crucial in the early stage of *Mycoplasma* infections. Attraction and activation of alveolar macrophages are important in the early clearance of *Mycoplasma* from the lungs. Inappropriate activation of alveolar macrophages by mycoplasmas can promote an excessive cytokine production by alveolar macrophages (Jungi et al., 1996) and lead to tissue damage. Activation of macrophages results in the recruitment of neutrophils to sites of inflammation, and neutrophils are prominent cell type found in the lungs, middle ear and joints in calves infected with *M. bovis*

(Gagea et al., 2006). Excessive neutrophil recruitment with the subsequent release of large amounts of inflammatory mediators may occur, and the extent of neutrophil recruitment is directly correlated with the severity of *Mycoplasma* disease. Although bovine neutrophils are able to kill opsonized *M. bovis*, unopsonized *M. bovis* can adhere to neutrophils and inhibit respiratory burst activity (Thomas et al., 1991).

Compounds capable of eliciting an immune response in the host, either upregulating or downregulating specific events, are classified as immunomodulators. MCWF contains immunomodulating compounds such as trehalose 6,6'-dimycolate (TDM) and muramyl dipeptide (MDP) (Le Garrec, 1986). MDP enhances the expression of surface markers on microorganisms that are necessary for cell adhesion and antigen presentation. This enhancement allows for an increase in phagocytosis, antimicrobial activity and antibody-mediated cytotoxicity (O'Reilly & Zak, 1992). Moreover, MDP has been shown to induce immune responses by increasing interferon gamma (IFN-γ) and other cytokine production and stimulating the production of lymphocytes (Traub et al., 2006). TDM is a glycolipid component of the mycobacterial cell wall, and was found to be a non-specific immunotherapeutic agent against cancer and infectious diseases (Azuma, 1992). TDM was also shown to stimulate macrophage activity (Madonna et al., 1989) and enhance the production of interleukin 6 (IL-6) (Nishizawa et al., 2007). It is proposed that MCWF targets the bovine innate immune and cell-mediated immune responses, providing increased resistance to early infection and reduction of associated clinical signs. It was demonstrated that MCWF has the ability to attract macrophages, neutrophils and initiate cytokine production in neonatal calves following intravenous, subcutaneous and intramuscular administration (Internal report #98-02SK). Data also suggest that single MCWF administration is sufficient to trigger strong innate and cell-mediated immune responses, as measured by IFN-γ, IL-2 and neutrophil influx and activation of naïve CD4+ and CD8+ cells. In addition, MCWF can increase phagocitic capacity and oxidative burst activity following intrauterine administration, as measured by flow cytometry (Internal report #16-06SRB). These data, accompanied by our current findings on reduced clinical signs, and lower culling rate, plus the improvement of reproductive status and lactation suggest that MCWF could be used as a therapeutic for treatment or control *Mycoplasma* infection as single agent or in combination with antibiotics. Furthermore, data suggest that MCWF application in *Mycoplasma*-infected dairy cows with CPPS should occur once a month or at least every other month in order to reduce and control clinical signs, assure normal farm production and maintain positive economic value.

One hypothesis is that MCWF's ability to attract neutrophils and increase their phagocytic activity along with the increase in an array of pro- and anti-inflammatory cytokines could be beneficial in reducing clinical signs associated with CPPS. The exact mode of action of MCWF in *Mycoplasma*-infected dairy cows remains unclear and additional *in vitro* and *in vivo* studies are required to better understand the positive effect observed in clinical settings following MCWF application in *M. bovis* infected animals.

Overall, the use of MCWF could provide a significant health and cost benefit to the farm management, particularly to organic farming operations where use of antibiotics is prohibited. Additional controlled studies are planned to further explore MCWF applications and application protocols, in order to improve the health status of both cows and calves in small and large dairy and beef operations.

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Authors' contributions

AM has developed the administration protocol, design the paper, selected reference for the presentation and wrote the manuscript. BP, JA, TB, TP and DB assisted in data collection and analysis. DB, AM performed all clinical observations. AM takes responsibilities for all aspects of the work and accuracy of the quoted data in the manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Aleksandar Masic is affiliated with NovaVive Inc., a company having licensing and marketing rights for the MCWF product used in this study

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IMUNOMODULATOR – FRAKCIJE ĆELIJSKOG ZIDA MIKOBAKTERIJA KAO POMOĆ U KONTROLI PERZISTENTNE INFEKCIJE MLEČNIH KRAVA SA *MYCOPLASMA BOVIS*

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Kratak sadržaj

Mycoplasma bovis je poznata kao uzročnik respiratornih bolesti, upale srednjeg uha, artritisa i niza drugih bolesti goveda. Infekcije izazvane ovim patogenim mikroorganizmom imaju uticaj na zdravlje, dobrobit i produktivnost mlečnih krava i goveda, što rezultira značajnim ekonomskim gubicima. Aktuelne strategije u preventivi i lečenju se oslanjaju na upotrebu antimikrobnih sredstava i vakcina, međutim, njihova efikasnost je ograničena zbog teškoća u dijagnostikovanju kao i nekonzistentnom

manifestovanju bolesti. U ovom radu iznosimo rezultate nakon upotrebe imunomodulatora poreklom od Frakcija ćelijskog zida mikobakterija (MCWF) kod mlečnih krava perzistentno inficiranih sa *M. bovis* u periodu 2011-2015. Aplikacija MCWF doprinela je redukciji kliničkih znakova povezanih sa *M. bovis* infekcijom, kao što su pneumonija, artritis, mastitis ali i poboljšanju ukupnog reproduktivnog učinka i broja dana u laktaciji inficiranih mlečnih krava. Ovi rezultati, u odnosu na prethodne informacije o zdravstvenom stanju i produktivnosti za period 2011-2015, sugerišu da bi MCWF imunomodulator mogao da se uvede kao pomoćno sredstvo u protokolu lečenja perzistentnih infekcija izazvanih *M. bovis* kod mlečnih krava.

Ključne reči: Artritis, imunoterapija, mastitis, *M. bovis*, MCWF, pneumonija