Short communication: Role of *Mycoplasma arginini* in mastitis caused by *Streptococcus dysgalactiae*

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ABSTRACT

We performed a comparative study on the development of mastitis induced by *Mycoplasma arginini* or *Streptococcus dysgalactiae* after challenging the cows. *Mycoplasma arginini* did not cause any clinical symptoms on its own, resulting in only a transient increase of somatic cell count (SCC; increase ranging from 0.5×10^6 to 0.8×10^6 cells/mL) and a slight decrease of milk production (10%) for 5 d. In contrast, *Strep. dysgalactiae* induced more severe clinical signs in animals and SCC increased to 1.60×10^6 to 2.11×10^6 cells/mL for 10 d. In addition, milk production decreased (22.9 to 27.0%) for 10 d. After 3 mo (2 mo after the first challenge), animals that were challenged previously with *M. arginini* were rechallenged with *Strep. dysgalactiae*. Severe clinical mastitis developed, with very high SCC (5.00×10^6 to 21.5×10^6 cells/mL), and a very significant reduction of milk production (28.6 to 68.7%), which lasted more than 4 wk, was observed. The severe clinical mastitis developed not only in cows inoculated with *Strep. dysgalactiae* and *M. arginini* in the same udder quarter but also in cows infected in the quarter previously not challenged with mycoplasma. Cows challenged first with *Strep. dysgalactiae* and rechallenged with *M. arginini* 2 mo later developed only slight changes in both SCC and milk production, similar to those when the cows were challenged with *M. arginini* alone. We conclude that *M. arginini* infection does not cause remarkable mastitis (characterized by decrease in milk production and increase of SCC) but it significantly predisposes animals to infection with *Strep. dysgalactiae*, leading to severe clinical mastitis.

Key words: challenge, *Mycoplasma arginini*, *Streptococcus dysgalactiae*, mastitis

Short Communication

The prevalence of mammary gland inflammation is relatively high in dairy cattle. Mastitis causes considerable economic losses through decreased milk yield, impaired milk quality, and increased cost of treatment and veterinary services (Seegers et al., 2003). Mastitis occurs in clinical and subclinical forms. Several bacterial species including *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Corynebacterium bovis*, and *Staphylococcus aureus* (Lee et al., 2008) are responsible for causing mastitis (Zhao and Lacasse, 2008). Among the *Mycoplasma* species, *Mycoplasma bovis* is reported very frequently as a causative agent of mastitis worldwide (Nicholas et al., 2007). Other *Mycoplasma* species, such as *Mycoplasma alkalescens* (Hirose et al., 2001), *Mycoplasma leachii* (Alexander et al., 1985), *Mycoplasma bovis* (Roy et al., 2008), *Mycoplasma californicum* (Mackie et al., 1982), *Mycoplasma canadense* (Infante-Martínez et al., 1999), and *Mycoplasma verecundum* (Higuchi et al., 2011) less frequently cause mastitis. Other *Mycoplasma* species (e.g., *Mycoplasma arginini*) are not regarded as etiological agents of mastitis; nevertheless, they are frequently recovered from bulk tank milk (Fox et al., 2005). Therefore, it is important to study the direct effect of *M. arginini* on the udders of cows and the influence of *M. arginini* infection on subsequent *Strep. dysgalactiae* challenge.

The experimental cows originated from a herd consisting of 500 Holstein-Friesian cows. All animals were examined serologically for the presence of *M. bovis* antibodies using an *M. bovis* ELISA kit (Chekit-*M. bovis* sero; Bommeli Diagnostics, Liebefeld-Bern, Switzerland). In addition to this, nasal swabs, milk samples collected from all quarters of the udder, and vaginal swabs taken from 20 cows were cultured in medium B (Erno and Stipkovits, 1973) for the presence of mycoplasmas. Tubes containing 2 mL of broth inoculated with samples were incubated for 10 d at 37°C. On d 3, 7, and 10, 10 µL of broth culture was spread on agar plates that were also incubated for 10 d at 37°C. Agar

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plates were regularly examined under the microscope for the presence of mycoplasma colonies. Inoculated broth samples were tested after 24 h of incubation by PCR using primers specific for mycoplasma and primers specific for *M. bovis* (Tenk et al., 2006).

Twelve cows in middle of second lactation were selected for the experiment. Before the study, all cows were retested for the presence of mycoplasmas as described earlier; no mycoplasmas were detected. Cows were divided into 3 groups of 4 animals each in such a way that the average milk production of a group did not differ statistically from that of the other groups during the 5 d before challenge. The cows were labeled A, B, C, and D in group 1; E, F, G, and H in group 2; and I, J, K, and L in group 3.

The cows of group 1 were challenged with 5 mL of a 48-h-old *M. arginini* culture (strain 65), which was isolated from the serum of a 1-d-old calf (Stipkovits et al., 1975). The strain was cloned 3 times, lyophilized, and stored at -70°C ; it was cultured every 4 to 5 yr. Before challenge, the broth culture was diluted 1:100 with PBS. The concentration of the inoculum was 1.3×10^6 cfu/mL, and cows were inoculated in the milk cistern of the left rear (LR) quarter of the udder after the morning milking using a 20-cm-long catheter 3 mm in diameter. After inoculation, the udder was massaged gently. The protocol of the animal experiment was approved by the Animal Research and Care Committee of the Carlsbad Research Organization Ltd. (Mosonmagyaróvár, Hungary), and animal care and experimentation were carried out in accordance with the institutional and national guidelines. Cows of group 2 were inoculated in the cistern of the LR quarter with 5.0 mL of a 24-h-old broth culture of *Strep. dysgalactiae* diluted 1:100 in PBS (5.3×10^6 cfu/mL). Animals in group 3 (control group) were inoculated with 5 mL of sterile PBS into the LR quarter. The groups were kept in separate air spaces. Feeding and maintenance were the same in all 3 groups, but care and milking of cows in different groups were performed by different personnel.

For 5 d before and 15 d after challenge, the cows were examined clinically every day after the morning milking. The presence of clinical signs was scored as follows: 1 = udder was swollen or painful or the local temperature on the udder surface was increased. Milk production of cows was measured in the morning and evening. The SCC of milk samples obtained from each quarter at the morning milking was checked. Milk quality was recorded every morning and scored as follows: score 1 = color of the milk had become yellowish or brownish; score 2 = milk contained flakes, and score 3 = milk coagulated. In group 1, the mycoplasma counts of milk samples obtained during the morning milking

from each quarter were checked. Similarly, in group 2 the bacterial count of milk samples was tested. Milk samples from cows of group 3 were also tested for the presence of mycoplasma and bacteria.

Two months after the end of the first experiment (first challenge), the cows were rechallenged as follows: In group 1, cows A and B were challenged in the LR quarter and cows C and D were challenged in the right rear (RR) quarter with 5 mL of 1:100 diluted 24-h-old broth culture of *Strep. dysgalactiae* (6.5×10^6 cfu/mL). In group 2, cows E and F were challenged in the LR quarter and cows G and H in the RR quarter with a 1:100 diluted 49-h-old *M. arginini* broth culture (3.9×10^6 cfu/mL) in the same way as in the previous experiment. Clinical examination, milk production recording, and the checking of milk SCC and milk quality were performed as described previously.

Two months after the start of the rechallenge experiment, the animals were euthanized and examined for pathological lesions in the internal organs and udder. Lung lesions were scored as follows: score 1 = area of lesions was $<50\%$ of the lobe, and score 2 = lesions extended to $>50\%$ of the lobe. The udder quarters were scored in a similar manner. Attempts were made to isolate *M. arginini* and *Strep. dysgalactiae* from all lobes of the lung, from the liver, spleen, and kidney, and from all udder quarters.

The clinical scores, scores of milk changes, and the isolation of mycoplasmas from the organs of animals in the different groups were compared by using the Chi-squared test. The quantities of milk produced in the groups during 5-d periods were compared by Student's *t*-test.

The cows used for the studies originated from a herd that was serologically negative for *M. bovis* by ELISA test and negative for *M. bovis* and *M. arginini* by culture and PCR. Nasal swabs, milk samples collected from all quarters of the udder, and vaginal swabs taken from 20 cows selected for studies proved to be negative by culturing and by PCR using primers specific for mycoplasmas and primers specific for *M. bovis*.

In group 1, we observed no swelling or pain of the udder or increased local temperature of the udder surface during the 15-d observation period after challenge with *M. arginini*. In group 2, 3 animals showed slight swelling of the udder with mild pain and increased local temperature of the udder surface for 2 to 3 d between d 6 and 10; scores varied between 5 and 9 (Table 1). No clinical symptoms were present in animals of group 3.

Milk production of cows was assessed from d -5 to d -1 (prechallenge milk production), and no differences were recorded between groups 1, 2, and 3 based on Student's *t*-test. Following challenge, however, milk production in group 1 was 3.6 and 8.4% lower from d 1 to

Table 1. Clinical signs and milk production on d 1 to 15 postchallenge of cows challenged in the udder with *Mycoplasma arginini* or *Streptococcus dysgalactiae*

Group and challenge agent	Clinical sign	Days postchallenge		
		1 to 5	6 to 10	11 to 15
	Clinical sign score			
Group 1: <i>M. arginini</i>	Udder swelling	0	0	0
	Udder pain	0	0	0
	Increased udder temperature	0	0	0
Group 2: <i>Strep. dysgalactiae</i>	Udder swelling	0	6	0
	Udder pain	0	9	0
	Increased udder temperature	0	5	0
Group 3: control	Udder swelling	0	0	0
	Udder pain	0	0	0
	Increased udder temperature	0	0	0
Prechallenge milk production ¹ (L)		Postchallenge milk production (L)		
Group 1: <i>M. arginini</i>	221.60	212.6 (95.5%) ² (95.6%) ³	200.4 (90.4%) (90.4%)	216.3 (98.0%) (98.0%)
Group 2: <i>Strep. dysgalactiae</i>	224.60	177.7 (79.1%) (79.1%)	164.0 (73.0%) (73.0%)	216.6 (96.4%) (95.8%)
Group 3: control	224.4	222.5 (99.2%) ⁴	221.6 (98.8%) ⁴	220.8 (98.4%) ⁴

¹Total milk produced (L) during 5 d before challenge.²Milk production compared with that prechallenge.³Milk production compared with that of control group.⁴No effect on milk production.

5 and from d 6 to 10, respectively, compared with that in the control group (group 3). The quantity of milk obtained in group 2 was 19.3 and 24.8% lower from d 1 to 5 and from d 6 to 10, respectively, compared with that in group 3 (Table 1).

The SCC of milk before challenge ranged between 0.06×10^6 and 0.39×10^6 cells/mL. On d 3 after challenge, a slight increase of SCC was recorded in the inoculated LR quarters of group 1 cows, reaching an average of 1.25×10^6 and 1.30×10^6 cells/mL from d 1 to 5 and from d 6 to 10, respectively. In the period from d 11 to 15, SCC decreased to 0.55×10^6 cells/mL. No increase of SCC was observed in milk samples from other quarters—left fore (LF), RR, and right fore (RF). In group 2, on d 3 after challenge, SCC started to increase and reached values of 1.60×10^6 , 2.11×10^6 , and 0.75×10^6 cells/mL between d 1 and 5, d 6 and 10, and d 11 and 15, respectively. Somatic cell counts exceeding 0.37×10^6 cells/mL were not recorded in milk samples from the uninoculated LF, RR, or RF quarters of this group in any of the postchallenge observation periods (Table 2). In the control group (group 3), the highest SCC was only 0.36×10^6 cells/mL.

The quality of all milk samples obtained in group 1 during the 15-d postchallenge observation period was normal (same as in the control group). In group 2, the

color of milk produced in the period from d 6 to 10 was yellowish and flakes were sometimes observed in it; the appearance of milk from cows in group 2 later returned to normal.

After rechallenge of cows A and B with *Strep. dysgalactiae* in the same quarter (LR) as the first challenge (*M. arginini*), severe clinical signs were observed. The inoculated quarters became swollen and painful, and the temperature of the udder surface increased. Scores of these signs ranged between 3 and 10 in the observation period (Table 3). These signs were also present between 25 and 30 d postchallenge. The animals became depressed and their appetite decreased. Similar symptoms were observed in cows C and D (inoculated with *Strep. dysgalactiae* in the RR quarter, a quarter not previously inoculated with *M. arginini*; Table 3). No or only very mild clinical signs were noticed in cows E, F, G, and H of group 2 (challenged first with *Strep. dysgalactiae* and rechallenged with *M. arginini*) and in cows of the control group (group 3).

Milk production of cows A and B (group 1) from d 1 to 5 was 29.4% lower than that of group 3 ($P < 0.001$). In the periods from d 6 to 10 and from d 11 to 15, milk production decreased even more, with reductions of 37.1% ($P < 0.001$) and 51.4% ($P > 0.005$) compared with that of cows of group 3. Similar reductions of milk

Table 2. Somatic cell counts ($\times 10^6$ cells/mL) in milk of cows challenged in the udder with *Mycoplasma arginini* or *Streptococcus dysgalactiae*

Group and challenge agent	Udder quarter	Days postchallenge		
		1 to 5	6 to 10	11 to 15
Group 1: <i>M. arginini</i>	Left fore	0.30	0.35	0.35
	Left rear ¹	1.25	1.30	0.55
	Right fore	0.32	0.33	0.34
	Right rear	0.35	0.36	0.34
Group 2: <i>Strep. dysgalactiae</i>	Left fore	0.36	0.33	0.34
	Left rear ¹	1.60	2.11	0.55
	Right fore	0.36	0.35	0.37
	Right rear	0.33	0.33	0.34
Group 3: PBS (control)	Left fore	0.35	0.36	0.32
	Left rear ¹	0.33	0.34	0.34
	Right fore	0.33	0.34	0.34
	Right rear	0.34	0.36	0.34

¹Left rear quarter challenged with *M. arginini*, *Strep. dysgalactiae*, or PBS, depending on group.

production were recorded in cows C and D (26.7, 36.3, and 47.4% in d 1 to 5, 6 to 10, and 11 to 15, respectively). No statistically significant decrease in milk production was noted between cows rechallenged with *Strep. dysgalactiae* into quarters previously inoculated (or not) with *M. arginini*. Milk production of cows E, F, G, and H decreased only slightly, by 2.5 to 5.8% (E and F) or 0.9 to 1.5% (G and H) in the different periods of observation compared with that of the control group (Table 4).

From d 2 after *Strep. dysgalactiae* rechallenge of cows A and B into the LR quarter first inoculated with *M. arginini*, we recorded a significant increase of SCC, reaching 3.25×10^6 to 21.50×10^6 cells/mL. Increased SCC was recorded in the milk of the LF, RF, and RR

quarters as well. A significant increase of SCC was recorded in all udder quarters of cows C and D (challenged with *Strep. dysgalactiae* into the udder quarter not inoculated with *M. arginini* at first challenge). In group 2, a slight increase was noticed only in the udder quarters inoculated with *M. arginini*, regardless of whether or not they were previously challenged with *Strep. dysgalactiae*. In milk from the udder quarters of the control cows, the SCC never exceeded 0.40×10^6 cells/mL (Table 5).

The quality of all milk samples obtained from the cows of group 1 changed very significantly 4 d postchallenge. Milk color became yellowish and then yellow, and many flakes were seen in the milk, which sedimented within a few minutes. From d 6 to 10 and from d 11

Table 3. Scores of clinical signs of cows rechallenged in the udder with *Mycoplasma arginini* or *Streptococcus dysgalactiae*

Group and cows	First challenge	Rechallenge	Parameter	Days postchallenge		
				1 to 5	6 to 10	11 to 15
Group 1: Cows A and B	<i>M. arginini</i>	<i>Strep. dysgalactiae</i>	Udder swelling	4	10	10
			Udder pain	3	10	10
			Increased udder temperature	3	9	10
Group 1: Cows C and D	<i>M. arginini</i>	<i>Strep. dysgalactiae</i>	Udder swelling	4	8	9
			Udder pain	3	8	10
			Increased udder temperature	3	9	10
Group 2: Cows E and F	<i>Strep. dysgalactiae</i>	<i>M. arginini</i>	Udder swelling	0	1	1
			Udder pain	0	0	0
			Increased udder temperature	0	0	0
Group 2: Cows G and H	<i>Strep. dysgalactiae</i>	<i>M. arginini</i>	Udder swelling	0	0	0
			Udder pain	0	0	0
			Increased udder temperature	0	0	0
Group 3: Cows I and J	PBS	PBS	Udder swelling	0	0	0
			Udder pain	0	0	0
			Increased udder temperature	0	0	0

Table 4. Milk production of cows rechallenged in the udder with *Mycoplasma arginini* or *Streptococcus dysgalactiae*

Group	First challenge	Rechallenge	Prechallenge milk production ¹ (L)	Days postchallenge		
				1 to 5	6 to 10	11 to 15
Group 1: Cows A and B	<i>M. arginini</i>	<i>Strep. dysgalactiae</i>	88.6	60.8 (68.6%) ² 68.7% ³	52.8 (59.6%)	37.2 (42.0%)
Group 1: Cows C and D	<i>M. arginini</i>	<i>Strep. dysgalactiae</i>	89.6	63.2 (70.5%)	54.1 (60.4%)	40.7 (45.4%)
Group 2: Cows E and F	<i>Strep. dysgalactiae</i>	<i>M. arginini</i>	89.9	71.4% 85.5 (95.3%)	61.4% 81.8 (91.3%)	47.5% 76.3 (84.9%)
Group 2: Cows G and H	<i>Strep. dysgalactiae</i>	<i>M. arginini</i>	89.2	96.6% 85.5 (95.9%)	92.8% 85.3 (95.6%)	89.1% 84.5 (94.7%)
Group 3: Cows I and J	PBS	PBS	90.2	96.6% 88.5 (98.1%)	96.8% 88.1 (97.7%)	98.7% 85.6 (94.9%)

¹Total milk produced (L) during 5 d before challenge.²Milk production compared with that prechallenge.³Milk production compared with control group.

to 15 postchallenge, some milk samples coagulated. At 1 mo postchallenge, the milk showed a similar appearance to that at d 11 to 15 postchallenge.

No lesions were present in the lung, spleen, liver, or kidney of cows A and B, although severe lesions were found in all udder quarters of these cows. The lesion score of the udders was 14. Similarly, no lesions were

seen in the inner organs of cows C and D but severe lesions were recorded in their udders. The lesion score was 12. No lesions were observed in any organs or in the udders of cows of groups 2 and 3.

Mycoplasmas were detected in the lungs of all cows of group 1, and the spleen of cows A and B and the kidney of cow D were also positive for mycoplasmas.

Table 5. Somatic cell counts ($\times 10^6$ cells/mL) in the milk of cows rechallenged in the udder with *Mycoplasma arginini* or *Streptococcus dysgalactiae*

Group	First challenge	Rechallenge	Udder quarter	Days postchallenge		
				1 to 5	6 to 10	11 to 15
Group 1: Cows A and B	<i>M. arginini</i>	<i>Strep. dysgalactiae</i>	Left fore	1.02	3.76	5.12
			Left rear ¹	5.00	13.67	21.50
			Right fore	2.31	13.44	3.54
			Right rear	3.58	14.57	6.59
Group 1: Cows C and D	<i>M. arginini</i>	<i>Strep. dysgalactiae</i>	Left fore	1.56	2.98	3.21
			Left rear ²	1.25	4.88	7.65
			Right fore	1.22	2.34	2.33
			Right rear ³	1.98	5.66	5.99
Group 2: Cows E and F	<i>Strep. dysgalactiae</i>	<i>M. arginini</i>	Left fore	0.35	0.40	0.36
			Left rear ⁴	1.39	1.46	0.68
			Right fore	0.35	0.33	0.39
			Right rear	0.40	0.36	0.38
Group 2: Cows G and H	<i>Strep. dysgalactiae</i>	<i>M. arginini</i>	Left fore	0.39	0.39	0.97
			Left rear ⁵	0.38	0.39	0.41
			Right fore	0.40	0.39	0.37
			Right rear ⁶	1.45	1.58	0.86
Group 3: Cows I and J	PBS	Control	Left fore	0.33	0.35	0.36
			Left rear	0.34	0.37	0.40
			Right fore	0.38	0.37	0.38
			Right rear	0.34	0.36	0.37

¹Left rear quarters previously challenged with *M. arginini* and then later not challenged with *Strep. dysgalactiae*.²Left rear quarters previously challenged with *M. arginini* and then later challenged with *Strep. dysgalactiae*.³Right rear quarter previously not challenged with *M. arginini* and then later challenged with *Strep. dysgalactiae*.⁴Left rear quarters previously challenged with *Strep. dysgalactiae* and then later challenged with *M. arginini*.⁵Left rear quarters previously challenged with *Strep. dysgalactiae* and then later not challenged with *M. arginini*.⁶Right rear quarters previously not challenged with *Strep. dysgalactiae* and then later challenged with *M. arginini*.

Mycoplasma arginini was isolated from the LR quarters of cows A, B, C, and D, as well as from the LR quarters of cows E and F and the RR quarters of cows G and H. The concentration of *M. arginini* in the milk samples varied between 2.4×10^2 and 5.1×10^4 cfu/mL. No *Strep. dysgalactiae* was found in any of the inner organs of any cow. *Streptococcus dysgalactiae* was present in a high concentration in all udder quarters of cows A, B, C, and D, but not in any of the udder quarters of groups 2 and 3.

Mycoplasma arginini is frequently isolated from various animal species affected with different pathological conditions; for example, from cattle with respiratory disease (Thomas et al., 2002; Ayling et al., 2004). It has been demonstrated that *M. arginini* can persist for a long time under different environmental conditions (Nagatomo et al., 2001).

Until now, no data have been available regarding mastitis caused by *M. arginini*, although this species is frequently detected in bulk milk originating from herds affected with mastitis of different etiologies (Fox et al., 2005; Higuchi et al., 2011; Justice-Allen et al., 2011). In this study, only a mild decrease in milk production and a slight increase in SCC were noticed after challenge of cows with *M. arginini* into the udder, whereas *Strep. dysgalactiae* induced more remarkable signs of mastitis. However, if the cows were first challenged in the udder with *M. arginini*, mycoplasma infection increased their sensitivity to *Strep. dysgalactiae*. In such cases, *Strep. dysgalactiae* caused very severe mastitis (swollen and painful udder with increased local temperature on the udder surface, significant decrease of milk production, very high milk SCC, significant changes in milk characteristics) compared with that when cows were inoculated with *Strep. dysgalactiae* into the udder without previous *M. arginini* challenge. No such synergistic effect was observed when the cows were challenged with *M. arginini* after a first challenge with *Strep. dysgalactiae*. The predisposing effect of *M. arginini* to bacterial infection can be explained by its ability to inhibit the growth of T cells (Komada et al., 1997) and the development of cytotoxic T-cell activity (Claesson et al., 1990), to suppress B-cell differentiation (Foresman et al., 1989), and to induce the secretion of cytokines involved in the inflammatory response (such as tumor necrosis factor α and IL6) by blood monocytes (Herbelin et al., 1994), among other properties.

Detection of *M. arginini* in the tank milk may indicate the frequent appearance of bacterial mastitis in cow herds. Today, most antimastitis drugs generally contain penicillin or its derivatives, which have no effect on mycoplasmas. Therefore, to prevent the predisposing effect of *M. arginini* in the development of bacterial

mastitis, we recommend that antimycoplasma drugs also be applied for the control of mastitis.

According to some reports, *M. arginini* infection has been associated with severe disease in humans (Yechou-ron et al., 1992; Sillis, 1994). In previous studies in our laboratory, we isolated *M. arginini* from the blood of a 23-yr-old man who had suffered from severe eosinophilic fasciitis for about 2 yr. After appropriate antibiotic and steroid therapy, the patient recovered (Silló et al., 2012). This individual did not have direct contact with animals but he regularly consumed cow milk. Because *M. arginini* can be present in milk over long periods, contaminated milk could be a source of infection for humans, especially for immunocompromised individuals.

Based on the data obtained in the current study, we conclude that *M. arginini* infection does not produce clinical signs of mastitis but it does predispose cows to development of severe mastitis associated with *Strep. dysgalactiae*.

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