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Intrauterine infection with *Mycobacterium avium* subsp. *paratuberculosis* in pregnant cattle diagnosed with Johne's disease

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Abstract

Johne's disease (JD) is caused by infection with *Mycobacterium avium* subsp. *paratuberulosis* (MAP). We confirmed the intrauterine infection of MAP in 22 pregnant cattle diagnosed with JD in Hokkaido, Japan. MAP was isolated from the umbilical cord (3/22: 13.6%) or caruncle (6/22: 27.3%) derived from the pregnant dams. Furthermore, dams with MAP from which MAP was isolated were also found to have a high mount of MAP or detected bacterial load in their feces. Fetuses of the tested dams indicated positive polymerase chain reaction (PCR) results for the *MAP* gene (17/22:77.3%) in several tissues. MAP was isolated from the PCR-positive sites of dams detected with high levels of bacteria (6/22: 27.3%). These results indicate that MAP infection in pregnant cattle must be prevented as it is important for JD control.

Key Words: *Mycobacterium avium* subsp. *paratuberulosis*, intrauterine infection, vertical transmission

Johne's disease (JD) is caused by infection with *Mycobacterium avium* subsp. *paratuberulosis* (MAP). JD causes chronic enteritis, resulting in decreased milk production, wasting, and eventual death in ruminants, including cattle[6)]. Recently, the number of cattle with JD has been increasing in Japan, especially in Hokkaido (https://www.maff.go.jp/j/syouan/douei/kansi_densen/kansi_densen.html). Basically, MAP is transmitted orally among the cattle herd via contaminated materials, such as feces and milk from infected animals. Therefore, based on the MAP transmission route and because there is no effective vaccine for MAP infection[5)], recommended disease control measures include prompt removal of infected animals and pasteurization of milk that is fed to calves.

In cases of MAP-infected pregnant dams, MAP is vertically transmitted to newborn calves and fetuses in the dam[11)]. To date, two possible routes of vertical MAP transmission have been recognized. One is oral bacterial acquisition in

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the newborn via the maternal colostrum or milk containing MAP-infected cells, and another is intrauterine infection of the fetus in the MAP-infected pregnant dam[3]. Among reports on the vertical transmission of MAP, fetal infection was first reported by Alexejeff-Goloff[2]. The presence of MAP was confirmed in fetal membranes, blood, liver, and other fetal tissues from a clinically affected cow with JD[2]. Similar findings concerning fetal infection have been reported since the first report[11].

Thus, in addition to the prevention of oral MAP transmission, the recognition of risk of intrauterine MAP infection in pregnant cattle also is important to control the incidence of JD. We investigated 22 fetuses from infected pregnant dams with a high bacterial load in Hokkaido to gather further evidences concerning the intrauterine transmission of MAP.

In the Tokachi District of Hokkaido, between July 2016 and March 2017, 22 pregnant cattle (14 Japanese Black cattle and 8 Holstein Friesian cattle) that were legislatively diagnosed with JD (>0.001 pg/well [2.5 μL] of MAP DNA) were investigated. The rectal stool, umbilical cord, caruncle, umbilical blood, and amniotic fluid were collected as maternal specimens. The fetal specimens were carefully collected from the heart, lung, kidney, liver, spleen, ilea, jejunum, colon, and meconium. All specimens except the maternal rectal feces were collected immediately at rendering plants after euthanasia. The collected maternal and fetal samples were used for MAP detection and isolation.

Briefly, the bacterial load of MAP was confirmed using real-time polymerase chain reaction (PCR), Johne-spin (FASMAC, Atsugi, Japan) for DNA extraction, and Johne's Gene test kit "KS" (Kyoritsu Seiyaku Co., Tokyo, Japan) targeting the MAP-specific gene *IS900* according to the manufacturer's instructions. MAP was further confirmed via bacterial isolation using a Harold's medium with mycobactin (Kyoritsu Seiyaku Co.) according to the manufacturer's instructions.

MAP was isolated from six (27.3%) maternal specimens of caruncle and umbilical cord (Dam IDs 1, 3, 4) or either caruncle or umbilical cord (Dam IDs 2, 10, 11) among the 22 tested pregnant dams (Table 1). All MAP infections isolated from the maternal specimens of pregnant dams showed extremely high bacterial loads in the feces. Detection was easier and the detected colony-forming units (cfu) were higher in the caruncle than in the umbilical cord. However, 17 (77.3%) of 22 fetuses derived from the pregnant dams showed positive PCR results for the *MAP* gene in any tested tissues, and MAP was isolated in six (27.3%) fetuses. All MAP-isolated fetal tissues were from PCR-positive sites. Interestingly, three of six MAP-isolated fetuses were derived from dams (Dam IDs 1, 3, 4) with high bacterial loads and MAP isolation from the caruncle plus umbilical cord. For the youngest fetus (4 months, from pregnant dam ID 3), MAP was isolated from three tissues (liver, spleen, and kidney). The remaining 3 MAP-isolated fetuses were obtained from dams (Dam IDs 7, 8, 9) with high bacterial loads in the feces; however, MAP was not isolated from the caruncle or umbilical cord. On the other hand, MAP was not detected and isolated in the umbilical blood and amniotic fluid derived from pregnant dams and meconium in this study (data not shown).

To date, there are many reports on the intrauterine transmission of MAP in cattle with JD. However, it is likely that these reports varied on the rarity and risk of intrauterine transmission of MAP in the dam[11]. Indeed, Whittington *et al.* (2009)[11] reported the prevalence of fetal infection with MAP to be as high as 39% of cows with clinical signs of JD, which is higher than 8.6% of fetuses in cows without signs of the disease. Seitz *et al.* (1989) reported a high rate (26.4%) of intrauterine infection in a dairy herd[7], whereas Adaska *et al.* (2012) reported a low rate (4.3%)[1].

In addition to the prevalence of MAP-infected animals in the herd[1], the difference in findings may be attributable to different bacterial loads or disease progression in the infected cattle[11]. Indeed,

Table 1. Detection of *M. paratuberculosis* in the infected dams and fetus.

Dam ID	Breed	Age (years)	Bacterial load (pg/well)	Bacteria isolation (+(cfu)/-)			Fetus								
										Details of the detection (Tested tissue (Bacteria isolation(+(cfu)/-) / PCR(+/-))					
			Feces	Feces	Umbilical cord	Caruncle	Fetal age (months)	Bacteria isolation (+/-)	PCR (+/-)	Lung	Heart	Liver	Spleen	Kidney	Intestines
1	J.B	11	185.000	+ (UC)	+ (1)	+ (60)	5	+	+	-/+	-/+	+(1)/+	-/+	-/+	-/+
2	J.B	11	119.000	+ (UC)	-	+ (12)	8	-	+	-/-	-/+	-/-	-/-	-/-	-/+
3	J.B	4	26.900	+ (UC)	+ (7)	+ (24)	4	+	+	-/+	-/-	+(1)/+	+(1)/+	+(1)/+	-/+
4	J.B	5	8.010	+ (UC)	+ (1)	+ (2)	9	+	+	-/+	-/-	+(1)/+	-/-	-/+	-/+
5	J.B	9	7.980	NT	-	-	6	-	-	-/-	-/-	-/-	-/-	-/-	-/-
6	J.B	11	3.740	+ (UC)	-	-	8	-	+	-/-	-/-	-/+	-/-	-/-	-/+
7	Hol	4	0.110	+ (UC)	-	-	7	+	+	-/-	-/-	+(1)/+	-/-	-/-	-/+
8	J.B	3	0.027	+ (UC)	-	-	8	+	+	-/+	+(1)/+	-/-	-/-	-/+	-/+
9	J.B	6	0.017	+ (7)	-	-	6	+	+	-/-	/-	-/-	-/-	-/-	+(4)/+
10	Hol	5	0.013	+ (UC)	-	+ (1)	7.5	-	+	-/+	-/+	-/+	-/-	-/+	-/+
11	J.B	10	0.013	+ (30)	-	+ (4)	9	-	+	-/-	-/+	-/-	-/-	-/-	-/+
12	Hol	4	0.010	+ (13)	-	-	4	-	+	-/-	-/-	-/-	-/-	-/-	-/+
13	J.B	3	0.008	+ (8)	-	-	6	-	-	-/-	-/-	-/-	-/-	-/-	-/-
14	Hol	4	0.007	NT	-	-	7	-	+	-/-	-/-	-/-	-/+	-/-	-/-
15	Hol	1	0.007	-	-	-	6	-	+	-/-	-/-	-/-	-/+	-/+	-/-
16	J.B	9	0.005	-	-	-	4.5	-	+	-/-	-/+	-/-	-/-	-/-	-/+
17	Hol	8	0.004	+ (6)	-	-	5	-	+	-/-	-/-	-/-	-/-	-/+	-/-
18	Hol	5	0.002	+ (16)	-	-	6	-	-	-/-	-/-	-/-	-/-	-/-	-/+
19	J.B	13	0.001	NT	-	-	7	-	+	-/+	-/-	-/-	-/-	-/-	-/-
20	J.B	9	0.001	NT	-	-	4.5	-	-	-/-	-/-	-/-	-/-	-/-	-/-
21	Hol	6	0.001	-	-	-	5	-	+	-/+	-/+	-/-	-/+	-/-	-/-
22	J.B	8	0.001	NT	-	-	7	-	-	-/-	-/-	-/-	-/-	-/-	-/-
Total					3/22	6/22		6/22	17/22						
n=22					(13.6%)	(27.3%)		(27.3%)	(77.3%)						

UC: uncountable, NT: not tested,

Sweeney *et al.* (1992) reported that MAP was isolated from fetal tissues in 5 of 58 (17.8%) cattle and all 5 positive fetuses were from dams with heavy fecal shedding[9]. In our study, intrauterine infection was confirmed in pregnant cattle with different bacterial loads. As expected, the risk of MAP intrauterine transmission tended to be higher in infected dams with high bacterial loads as shown in previous reports[9,11]. Sonawane *et al.* (2009) reported that the isolation rate of MAP from blood increased in infected cattle with severe lesions[8]. Therefore, MAP might migrate to the blood of infected cattle with the progression of the disease state, infecting the fetus hematogenously through the umbilical cord[4]. Indeed, in our study, MAP was isolated from the umbilical cord or caruncle as noted in previous reports[11]. However, although hematogenous infection of MAP through the umbilical cord is possible, the bovine placenta is classified histologically as a syndesmochorial placenta with a strong junction. Therefore, the possibility of hematogenous infection of MAP through the umbilical cord must be clarified. In addition to this issue, although a high level of MAP DNA was detected (77.3%) in the fetal sample, the intrauterine infection was established when MAP was isolated from the fetal specimen because the positive PCR results included those derived from dead cells and gene fragments. However, it is possible for the pregnant cattle to have an intrauterine infection with a low bacteria load. Further clarification is required to determine the accurate risk of intrauterine infection in MAP-infected cattle.

In conclusion, our study presents direct evidence of intrauterine MAP infection. In

Hokkaido, Japan, the number of cattle with JD is increasing because there is no effective treatment or vaccine for MAP infection. Recently, we reported that the shedding level of MAP DNA in feces was related to the histopathologic classification of disease progression in cattle with JD[10]. As mentioned, such cattle with high MAP shedding or clinical signs could be at a high risk for intrauterine infection[9,11]. Furthermore, it is notable that fetal infection with MAP in utero tended to demonstrate high shedding with clinical signs[11]. Thus, Whittington and Windsor explained the possibility of intrauterine infection with a high risk of progressive MAP infection because the uncontrolled infection could retard the success of disease control programs for JD based on pasteurization of milk and removal of infected cattle[11]. According to a report, the incidence of intrauterine transmission depends on within-herd prevalence of MAP infection[1]. Although intrauterine transmission of MAP could not be prevented, the removal of suspected cattle with JD is the most important issue to prevent vertical transmission of MAP. These results indicated that prevention of MAP in the pregnant cattle is important for JD control.

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