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Mycobacterium avium subsp. *paratuberculosis* excreted in cattle feces shows higher resistance to disinfectants than cultured cells

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Abstract

As Johne's disease progresses, clumps of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are identified in the feces. MAP shows resistance to disinfectants, and effective disinfection methods for MAP cells excreted in feces have not been verified. Furthermore, given that biofilm-like vesicles and spore-like morphotypes were observed in feces from infected cattle, we aimed to compare reduction rates between cultured and naturally infected MAP cells after disinfectant treatment. Although the logarithmic reduction value for all cultured MAP cells was > 3.0, four of 10 infected MAP cells showed values < 2.0 after dichloroisocyanuric acid treatment. Our findings suggest that materials contaminated with infected cattle feces should be exposed to higher concentrations of disinfectants than those reported to be effective based on cultured cells.

Key Words: disinfectant, *Mycobacterium avium* subsp. *paratuberculosis*, spore

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes Johne's disease, comprising chronic granulomatous inflammation of the distal small intestine and mesenteric lymph nodes in ruminants. After a long incubation period, the disease leads to emaciation, therapy-resistant diarrhea, and eventually, death[1)]. It has been debated over the past century that MAP contributes to Crohn's disease pathogenesis in humans[10)]. MAP cells have a thick, waxy cell wall composed of 60% lipids[8,11)]. The cell wall makes the organism acid-fast, hydrophobic, and resistant to chemical and heat destruction. In farms where Johne's disease has occurred, reducing the number of environmental MAP cells through cleaning and disinfection is crucial; however, effective disinfection methods for MAP cells in feces remain invalidated. Lamont et al.[6)] reported that MAP cells form a spore-like morphotype upon nutrient starvation and survive exposure to heat, lysozyme, and proteinase K. Furthermore, Marsollier et al.[7)] reported that extracellular matrix-coated *Mycobacterium* is resistant to some antibiotics. We previously developed a novel method for purifying acid-fast bacteria from the feces of MAP-infected cattle and observed spore-like morphotypes and biofilm-like vesicles using electron microscopy[2)]. These results suggested that MAP cells in feces are more resistant to environmental stresses, including disinfectant exposure, than cultured

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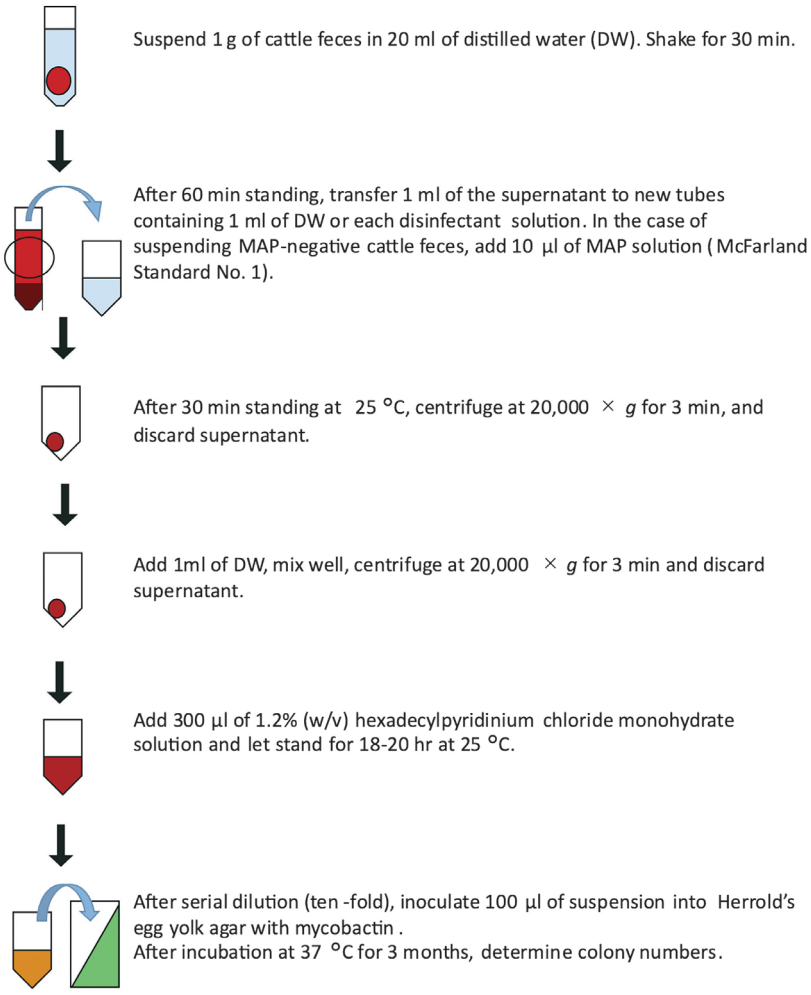

Fig. 1. Method used for the disinfection test.

cells. Therefore, in this study, we aimed to investigate the effects of disinfectants on MAP cells in cattle feces and compare MAP cell reduction between cells from natural infections and cultured cells.

Ten fecal samples from cattle (Holstein Friesian) naturally infected with MAP in Hokkaido Prefecture in 2023 and 2024 were used in this study. To confirm the diagnosis, Johne-spin (FASMAC, Atsugi, Japan) was used for DNA extraction from the feces, and the MAP IS900 gene was quantified using Johne's Gene Test Kit "KS" (Kyoritsu Seiyaku Co., Tokyo, Japan) and the LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland), as previously described[2]. As a control, eight MAP-negative fecal samples were spiked with cultured MAP strain K13-R5-1 (incubated for 3 months at 37°C on Herrold's egg yolk agar with mycobactin), which was isolated from cattle feces in Hokkaido Prefecture in 2024. The tested disinfectants and methods are presented in Table 1 and Fig. 1, respectively. Briefly, 1.0 ml of the fecal suspension was treated with an equal volume of each disinfectant or distilled water for 30 min. After one wash with distilled water, the fecal sediments were treated with 1.2% (w/v) hexadecylpyridinium chloride monohydrate solution overnight. The suspensions were then inoculated on Herrold's egg yolk agar with mycobactin (Kyoritsu Seiyaku Co.), and colony numbers were determined after 3 months of incubation at 37°C.

The results are summarized in Table 2. Slaked lime treatment resulted in a logarithmic reduction

Table 1. Disinfectants used in this study

Disinfectants	Product name and manufacturers	Concentration of active component after adjustment*
Slaked lime	Slaked lime; Hokkaido Lime Co., Ltd. (Tomakomai, Japan)	Supernatant of 100 mg/ml solution
Peracetic acid	Perasan MP2-J; EnviroTech (Tokyo, Japan)	1,000 ppm of peracetic acid
Dichloroisocyanuric acid	Crente; Meiji Seika Pharma (Tokyo, Japan)	1.7 mg/ml (available chlorine 0.1%)

* The concentrations described here were applied to equal amounts of the samples as shown in Fig. 1. (the final concentration was halved)

Table 2. MAP cell counts in infected and contaminated feces

	Feces	Farm	MAP gene (pg/well)	Log CFUs (LRV) after treatment					
				Distilled water	Slaked lime		Peracetic acid		Dichloroisocyanuric acid	
Infected MAP	A	A	6.86E+01	4.38	4.25	(0.13)	3.67	(0.72)	≥ 3.30	(< 1.08)
	B	A	5.13E+01	4.06	3.92	(0.14)	< 0.00	(> 4.06)	2.15	(1.91)
	C	B	3.69E+01	4.62	4.23	(0.39)	3.18	(1.44)	≥ 3.30	(< 1.32)
	D	C	3.43E+01	4.25	4.08	(0.17)	2.43	(1.82)	< 0.00	(> 4.25)
	E	D	6.45E+00	3.65	3.67	(-0.02)	< 0.00	(> 3.65)	< 0.00	(> 3.65)
	F	E	5.15E+00	3.78	3.39	(0.39)	1.42	(2.36)	1.77	(2.01)
	G	F	2.86E+00	3.73	3.75	(-0.02)	3.05	(0.68)	< 0.00	(> 3.73)
	H	B	2.37E+00	3.06	3.04	(0.02)	0.00	(3.06)	< 0.00	(> 3.06)
	I	G	1.61E+00	3.34	2.96	(0.38)	1.84	(1.49)	2.83	(0.51)
	J	H	8.30E-01	2.90	2.86	(0.04)	1.81	(1.09)	< 0.00	(> 2.90)
Cultured MAP	K	I	ND*	5.79	4.88	(0.91)	3.64	(2.15)	< 0.00	(> 5.79)
	L	C	ND	5.63	4.55	(1.08)	0.00	(5.63)	< 0.00	(> 5.63)
	M	H	ND	5.60	4.82	(0.78)	3.19	(2.41)	0.92	(4.68)
	N	D	ND	5.60	4.77	(0.83)	< 0.00	(> 5.60)	< 0.00	(> 5.60)
	O	H	ND	5.40	4.19	(1.21)	2.63	(2.77)	0.00	(5.40)
	P	B	ND	5.31	4.60	(0.71)	< 0.00	(> 5.31)	1.44	(3.87)
	Q	D	ND	5.06	4.02	(1.03)	1.15	(3.91)	< 0.00	(> 5.06)
	R	J	ND	4.72	4.15	(0.57)	1.01	(3.71)	< 0.00	(> 4.72)

The underlined items indicate those for which disinfection is ineffective (< 2.90 LRV).
*ND; not detected

value (LRV) < 2.0 for all specimens. Results based on peracetic acid and dichloroisocyanuric acid treatments varied across fecal samples. All specimens spiked with cultured cells showed LRVs > 2.0 and > 3.0 after peracetic acid and dichloroisocyanuric acid treatments, respectively; however, MAP cells from the feces of six and four naturally infected cattle showed LRVs < 2.0. In some cases, the CFU number was reduced below the detection limit after treatment, and a statistical comparison of the LRV was not applicable. Given that the minimum log CFU in untreated feces was 2.9 in this study, when > 2.9 LRV (> 99.87% reduction in the number of bacteria) was defined as an effective disinfection, a statistical analysis using Fisher's exact test revealed a significantly lower disinfection efficacy of dichloroisocyanuric acid against MAP cells from the feces of naturally infected cattle than that against cultured MAP cells (Table 3).

We used three disinfectants that are effective against cultured MAP and other mycobacterial species[5,12,15]; however, the mechanism of reduction in effectiveness due to the fecal component is unclear. The minimum MAP gene concentration used in this study was 0.83 pg/well for the feces of infected cattle; therefore, the formation of leprosy lesions was considered to be progressing[13] and the MAP cells in feces would form clumps[2]. Thus, the colony counts do not necessarily coincide with viable cell counts; however, this applies not only to infected MAP but also to cultured MAP as shown in supplementary Fig. Based on a t-test, the cultured MAP (average: 0.89) showed a significantly higher LRV than the infected MAP (average: 0.16) after slaked lime treatment (*P*

Table 3. Statistical analysis using Fisher's exact test of the effectiveness of infected MAP and cultured MAP

	Slaked lime		Peracetic acid		Dichloroisocyanuric acid	
	LRV < 2.9	LRV > 2.9	LRV < 2.9	LRV > 2.9	LRV < 2.9	LRV > 2.9
Infected MAP	10	0	7	3	5	5
Cultured MAP	8	0	3	5	10	0
P value	1.000		0.342		0.036	

< 0.01). In a previous study, MAP clumps were wrapped with unidentified components that were removed via alkaline treatment[2]. Similarly, slaked lime treatment might remove the wrapping component, and subsequent hexadecylpyridinium chloride monohydrate treatment, which acts as a surfactant, could loosen the MAP clumps. In contrast, the difference in the minimum LRVs between cultured and infection-associated MAP was > 3.0 after dichloroisocyanuric acid treatment; the differences in disinfectant resistance are considered to have a greater effect than the fecal components or loosening of clumps.

Prior growth conditions influence stress resistance in cultured cells. Gruzdev et al.[3] reported that bacteria cultured on solid media showed greater resistance to dehydration stress than those grown in broth media. Similarly, cells in the stationary phase exhibited greater resistance than those in the logarithmic phase. Therefore, it is necessary to consider the culture conditions when conducting disinfection tests. In this study, spiked MAP cells were obtained from the solid medium after 3 months of incubation. At this stage, cells are considered to be in a stress-resistant state compared to those in the logarithmic phase in the broth medium. Only a few cells exhibited spore-like morphotypes, as spore-like morphotypes were only partially observed after 1 year of cultivation[6]. Similarly, biofilm-like vesicles were only partially observed in cultured MAP[2]. Greater resistance observed in infection-associated MAP cells is attributable to the formation of a spore-like morphotype and biofilm formation in infected MAPs. However, our results showed varying LRVs among fecal specimens, and further investigation into other factors such as fecal component and the progression state in cattle is required. In addition, the difference in disinfectant susceptibility depending on the strain should be considered; however, because MAP is a slow-growing bacterium, and all infected cattle, including the source of the K13-R5-1 strain, exist within a radius of 50 km, there is presumed to be little difference among the strains.

In conclusion, our study found that MAP cells in the feces of some naturally infected cattle showed greater resistance to disinfectants than cultured cells, although there were variations among fecal specimens. Peracetic and dichloroisocyanuric acids are effective against spore-forming bacteria; however, these require a higher concentration and longer exposure period than non-spore-forming bacteria[4,14]. Our results highlight the necessity of reviewing appropriate disinfectants for livestock hygiene and public health. Potentially contaminated materials including milk and meat[9], should be exposed to effective disinfectants at higher concentrations and for longer periods than those reported for cultured MAP cells.

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