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Short Communication
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The presence of adhesion factors NOX, α-enolase, TrmFO, P27, and VpmaX in *Mycoplasma bovis* wild isolates in Japan

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Abstract

Background: *Mycoplasma bovis* causes various diseases such as bronchopneumonia, otitis media, arthritis, and mastitis in cattle. *Mycoplasma bovis* is often isolated from the deep pharynges of healthy cattle and is generally considered not to cause clinical symptoms while in the upper respiratory tract. In mycoplasma infections, adhesion to the host cells is a crucial step. In recent years, five new adhesins, NOX, α-enolase, TrmFO, P27, and VpmaX, have been reported in *M. bovis* strains from pneumonia cases. However, the presence of these adhesins in wild isolates has not been established.

Aim: This study aimed to investigate the presence of these adhesin genes in wild isolates isolated from cattle nasal cavities and lesion sites (pneumonia, otitis media, arthritis, and mastitis) in various regions in Japan and clarify the relationship between adhesion and the symptoms caused by *M. bovis* infection.

Methods: A total of 141 *M. bovis* wild isolates isolated from nasal cavities (healthy or sick cattle), lungs with pneumonia, ears with otitis media, joint fluids of arthritic animals, and milk of mastitic animals. *Mycoplasma bovis* type strain PG45 was also used. Specific polymerase chain reaction reactions were performed to detect *nox*, *α-enolase*, *trmFO*, *P27*, and *vpmaX*, which are adhesins of *M. bovis*.

Results: This study reports 139 *M. bovis* wild isolates were positive for *nox*, *α-enolase*, *trmFO*, *P27*, and *vpmaX*, while two isolates each lacked *α-enolase* or *P27* genes. *Mycoplasma bovis* PG45 also had all five adherens genes.

Conclusion: Almost all *M. bovis* wild isolates possessed all *nox*, *α-enolase*, *trmFO*, *P27*, and *vpmaX* genes regardless of the lesion site or region of origin. This means no relationship was found between the presence of the five adhesins and lesion sites in *M. bovis* and *M. bovis* isolated from the nasal cavities of asymptomatic cattle have the same numbers and types of adhesins as isolates from symptomatic lesion sites (pneumonia, otitis media, arthritis, and mastitis). This suggests that not only *M. bovis* isolates from pulmonary lesions, but also *M. bovis* existing in the nasal cavity has the potential to causes symptoms in the host.

Keywords: Adhesin, Cattle, *Mycoplasma bovis*.

Introduction

Mycoplasma bovis belongs to the Mollicutes and lacks a cell wall, and it can cause bronchopneumonia, otitis media, arthritis, and mastitis in cattle (Bürki *et al*., 2015). However, *M. bovis* is often isolated from the deep pharynges of healthy cattle (Lima *et al*., 2016) and is generally considered not to cause clinical symptoms while in the upper respiratory tract (Hananeh *et al*., 2018). When *M. bovis* reaches the lung airways though it causes pneumonia. Otitis media is caused following pneumonia and arthritis, and pneumonia or mastitis is often observed during arthritis onset (Maunsell *et al*., 2011). Clinical signs of arthritis and pneumonia are often present at the same time (Caswell and Archambault, 2007). *Mycoplasma bovis* antigen has also been detected in the liver and kidney by immunohistochemistry (Maeda *et al*., 2003). In addition, it was reported that *M. bovis* is involved in bovine endocarditis (Kanda *et al*., 2019). These studies strongly suggest that *M. bovis* can hematogenously spread to the several organs and tissues and cause lesions at various sites.

One of the first steps in mycoplasma infection is adhesion to host cells (Bürki *et al*., 2015). Adhesins expressed on the cell membrane of *M. bovis*, which lacks a cell wall, are very important factors for the microorganism. P26 and variable surface lipoproteins (Vsps) are well known as typical *M. bovis* adhesins (Sachse *et al*, 2000; Bürki *et al*., 2015). In recent years, with progress in *M. bovis* genomics, NOX, α-enolase, TrmFO, P27, and VpmaX have been reported as *M. bovis* adhesins in strains Hubei-1 and HB0801 (Song *et al*., 2012; Zou *et al*., 2013; Guo *et al*, 2017; Zhao *et al*., 2017; Chen *et al*, 2018). NOX, α-enolase, TrmFO, and P27 are fibronectin- or plasminogen-binding proteins (Song *et al*., 2012; Guo *et al*, 2017; Zhao *et al*.,

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2017; Chen *et al*, 2018), and VpmaX is a lipoprotein expressed on the cell membrane (Zou *et al*., 2013).

Unlike Hubei-1 and HB0801 strains, which were isolated from pneumonia cases (Li *et al*., 2011; Qi *et al*., 2012), it is unknown whether isolates isolated from other lesion sites such as those of otitis media, arthritis, and mastitis possess these adhesins. In addition, the number of isolates whose genomes have been analyzed is very few, and the presence of these adhesins in wild isolates has not been established. Therefore, we investigated the presence of NOX, α-enolase, TrmFO, P27, and VpmaX genes by polymerase chain reaction (PCR) in wild *M. bovis* isolates from various lesion sites in various regions of Japan. This will clarify the relationship between adhesion and the symptoms caused by *M. bovis* infection and help elucidate its pathology.

Materials and Methods

Sample used in this study

A total of 141 *M. bovis* wild isolates from 134 cattle in 10 prefectures in Japan from 2008 to 2013 were used in this study. The number of isolates from nasal cavities (healthy or sick cattle), lungs with pneumonia, ears with otitis media, joint fluids of arthritic animals, and milk of mastitic animals was 86, 35, 6, 3, and 11, respectively. To confirm the identity of the isolates, PCR with specific primers for the OppD/F gene (Hotzel *et al*., 1996) or loop-mediated isothermal amplification (Higa *et al*., 2016) of *M. bovis* were performed. *Mycoplasma bovis* type strain PG45 was also used, and all isolates were stored at −80°C until use. DNA of each isolate was extracted by the hot-shot method after incubation for 4 or 5 days at 37 °C in NK broth (Kanto Chemistry, Tokyo, Japan) and stored at −20°C until use.

PCR to detect adhesins

Specific PCR reactions were performed to detect *nox*, *α-enolase*, *trmFO*, *P27*, and *vpmaX*, which are adhesins of *M. bovis*. Primers for *α-enolase* and *trmFO* were designed according to the conserved sequences of *M. bovis* provided by GeneBank (accession numbers: CP002513 and CP002058). Primers for *nox*, *P27*, and *vpmaX* were designed based on previous reports (Zou *et al*., 2013; Zhao *et al*., 2017; Chen *et al*., 2018). The primer information is shown in Table 1. Amplification of these genes was performed using KAPA2G Fast HS ReadyMix + dye (2×) (Nippon Genetics, Tokyo, Japan). Each primer was added at 0.5 μM final concentration, and the template DNA was added at 1.0 l. The reaction mixture was subjected to the following conditions: 2 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 44°C–53°C, and 1–2 minutes at 72°C, with a final cycle of 4 minutes at 72°C, followed by a hold at 4°C (Table 2). Samples were subjected to horizontal gel electrophoresis in 1.5% agarose and photographed using ChemiDoc™ Touch (Bio-Rad, Hercules, CA).

Ethical approval

Not required for this study.

Results and Discussion

The results are shown in Table 3. Of the *M. bovis* wild isolates, 139 were positive for *nox*, *α-enolase*, *trmFO*, *P27*, and *vpmaX*, while two isolates each lacked *α-enolase* or *P27* genes. *Mycoplasma bovis* PG45 also had all five adherens genes.

Mycoplasma bovis tested in this study were wild isolates from nasal cavities and infection sites with pneumonia (lung), otitis media (ear), arthritis (joint fluid), and mastitis (milk) of beef cattle and dairy cows from various regions in Japan. We found that 139 wild isolates possessed all *nox*, *α-enolase*, *trmFO*, *P27*, and *vpmaX* genes regardless of the lesion site or region of origin. This suggests that the *M. bovis* in Japan may have these genes universally. Mycoplasma is a host-dependent parasite with a small genome and poor metabolic systems (Caswell *et al*., 2007; Wise *et al*, 2011). Therefore, adhesion to the host cells is considered to be very important for survival. Although *M. bovis* has one of the smallest genomes among bacteria, at approximately 1,080 kb (Wise *et al*, 2011), it has abundant adhesins.

Mycoplasma bovis NOX is an enzyme that is similar to *Streptococcus pneumoniae* and *Streptococcus pyogenes* NADH oxidase and catalyzes the reduction of oxygen to hydrogen peroxide (Zhao *et al*., 2017). That is, *M. bovis* NOX may be able to adapt to oxygen to allow pathogen growth under oxidative stress and promote growth in the oxygen-rich bovine respiratory tract (Zhao *et al*., 2017). Hydrogen peroxide is a major virulence factor in mycoplasma, in that it has cytotoxic effects and inhibits ciliary clearance (Bürki *et al*., 2015). Therefore, *M. bovis* NOX is considered to be an important virulence factor not only for adhesion but also for adaptability to oxygen. α-Enolase is expressed on the cell surfaces of various bacteria such as *Streptococcus pnemoniae*, *Streptococcus mutans*, and *Mycoplasma fermentas*, and it binds to plasminogen (Bergmann *et al*., 2003; Jones and Holt, 2007; Yavlovich *et al*., 2007). Because plasminogen produced from the liver is in systemic circulation, the binding of those pathogens to plasminogen may facilitate dissemination of the infection in the host (Song *et al*., 2012). *Streptococcus pneumoniae* is the most common causative pathogen of community-acquired pneumonia and causes otitis media and sinusitis in humans (Bergmann *et al*., 2003). However, sometimes the bacteria cause fatal diseases such as bacteremia and meningitis, leading to high mortality (Bergmann *et al*., 2003). *Streptococcus mutans* enters the bloodstream and may be associated with infective endocarditis (Jones and Holt, 2007), and *M. bovis* has also been reported to be involved in endocarditis (Kanda *et al*., 2019). Arthritis caused by *M. bovis* is secondary to pneumonia and mastitis, and the clinical symptoms of arthritis and pneumonia are often present at the same time (Caswell and Archambault, 2007). In addition, *M. bovis* antigen has been detected in the liver and kidney (Maeda *et al*.,

Table 1. Primer set for each adhesin gene specific PCR used in this study.

Gene	Sequences (5'-3')	Product size	Reference
Nox	(F)-CCG GAA TTC ATG AAG ATT ATT TTA GTG GGA GCA AA	1,365 bp	(Zhao *et al*., 2017)
	(R)-GAC AAG CTT TTA ATA TTT GTA GTC AAT TCC TAA TGC C		
α-enolase	(F)-ATC AGC CTA CTA TAT GTT GCC TTT	1,596 bp	This study
	(R)-AGT CCG TTC AGT ACA TCG CC		
TrmFO	(F)-CTA ATC CCC AGT TGG CTT CC	996 bp	This study
	(R)-CCA AAG GGG TGC TAA AAC AA		
P27	(F)-CGG GGT ACC ATG AAA AAG ATA CAT AAA	744 bp	(Chen *et al*., 2018)
	(R)-GCG GGA TCC TTA TTT TTT TTC AAA AAT TAT TTG		
VpmaX	(F)-CAG GGA TCC ATC AAT AAA TTG CTA ATA TCT GCT GT	690 bp	(Zou *et al*., 2013)
	(R)-CAG GTC GAC TTA AAT TTT CTC AAA TAT TGG TCT AAG		

Table 2. PCR conditions for each adhesin gene in this study.

Gene	Initial denature	Denature	Anneal	Extension	Last extension
Nox	94°C 2 minutes	94°C 30 seconds	45°C 30 seconds	72°C 1.5 minutes	72°C 4 minutes
α-enolase			47°C 30 seconds[a]	72°C 2 minutes	
TrmFO			50°C 30 seconds	72°C 1 minutes	
P27			47°C 30 seconds	72°C 1 minutes	
VpmaX			53°C 30 seconds	72°C 1 minutes	
			(×30 cycles)		

[a]Only one isolate is 44°C.

Table 3. Possession rate (%) of each adhesin genes in Mycoplasma bovis wild isolates from each site, addition to M. bovis PG45.

Gene	Possession rate of each adhesin gene (%)						PG45
	Nasal	Pneumonia	Otitis	Arthritis	Mastitis	Total	
	(*n* = 86)	(*n* = 35)	(*n* = 6)	(*n* = 3)	(*n* = 11)	(*n* = 141)	
Nox	100	100	100	100	100	100	Positive
α-enolase	100	100	100	100	90.9	99.3	Positive
TrmFO	100	100	100	100	100	100	Positive
P27	98.8	100	100	100	100	99.3	Positive
VpmaX	100	100	100	100	100	100	Positive

2003). These findings suggest that *M. bovis* also enters the bloodstream and is disseminated throughout the whole body. In this study, almost all isolates possessed *α-enolase*. This suggests that the presence of *M. bovis* α-enolase may be involved in dissemination to the whole body. TrmFO and P27 bind to fibronectin (Guo *et al*., 2017; Chen *et al*., 2018), which exists in soluble form and in the extracellular matrix in various bodily fluids and tissues (Guo *et al*., 2017; Chen *et al*., 2018), so *M. bovis* TrmFO and P27 may be associated with its spread to various host tissues. VpmaX is a lipoprotein expressed on the cell membrane surface and is thought to play an important role in *M. bovis* infection (Zou *et al*., 2013), but the details are unknown.

In this study, no relationship was found between the presence of the five adhesins and lesion sites in *M. bovis*. However, this means that *M. bovis* isolated from the nasal cavities of asymptomatic cattle have the same numbers and types of adhesins as isolates from symptomatic lesion sites (pneumonia, otitis media, arthritis, and mastitis). *Mycoplasma bovis* has also been isolated from the deep pharynges of healthy cows (Lima *et al*., 2016), and it may not cause clinical symptoms as long as it is in the upper respiratory tract (Hananeh *et al*., 2018). However, it is thought that suppression of the host immune system caused by stress or viral infection results in the rapid growth of pathogenic bacteria in the upper respiratory tract and reduced clearance in the

lower respiratory tract, and these pathogenic bacteria reach the lungs (Griffin *et al*., 2010). After reaching the lung airways, this microorganism causes pneumonia, spreads throughout the body via the bloodstream, and causes various symptoms in infected hosts. This suggests that even *M. bovis* existing in the nasal cavity of healthy cows may colonize various tissues and result in lesions. The period during which *M. bovis* exists in the bloodstream in the host is short (Caswell and Archambault, 2007). Nevertheless, during this time, it spreads to various tissues and causes myriad symptoms. This may be due to the abundance of adhesins as shown in this study.

In conclusion, we found that *M. bovis* wild isolates in Japan universally possess *nox*, *α-enolase*, *trmFO*, *P27*, and *vpmaX*. The presence of this variety of adhesins may be involved in the pathogenicity and pathology of *M. bovis*.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

Conceptualization, F. Shitamori, T. Kanda, and R. Uemura; methodology, F. Shitamori and T. Kanda; validation, F. Shitamori and R. Uemura; investigation, F. Shitamori and T. Kanda; resources, R. Uemura; writing—original draft preparation, F. Shitamori; writing—review and editing, F. Shitamori, R. Uemura and M. Sueyoshi; supervision, R. Uemura and M. Sueyoshi; project administration, R. Uemura; funding acquisition, R. Uemura. All authors have read and agreed to the published version of the manuscript.

References

Bergmann, S., Wild, D., Diekmann, O., Frank, R., Bracht, D., Chhatwal, G.S. and Hammerschmidt, S. 2003. Identification of a novel plasmin (ogen)-binding motif in surface displayed α-enolase of *Streptococcus pneumoniae*. Mol. Microbiol. 49, 411–423.

Bürki, S., Frey, J. and Pilo, P. 2015. Virulence, persistence and dissemination of *Mycoplasma bovis*. Vet. Microbiol. 179, 15–22.

Caswell, J. L. and Archambault, M. 2007. *Mycoplasma bovis* pneumonia in cattle. *Anim.* Health Res. Rev. 8, 161–186.

Chen, X., Huang, J., Zhu, H., Guo, Y., Khan, F.A., Menghwar, H., Zhao, G. and Guo, A. 2018. P27 (MBOV_RS03440) is a novel fibronectin binding adhesin of *Mycoplasma bovis*. Int. J. Med. Microbiol. 308, 848–857.

Griffin, D., Chengappa, M.M., Kuszak, J. and McVey, D.S. 2010. Bacterial pathogens of the bovine respiratory disease complex. Vet Clin North Am Food Anim Pract. 26, 381–394.

Guo, Y., Zhu, H., Wang, J., Huang, J., Khan, F.A., Zhang, J., Guo, A. and Chen, X. 2017. TrmFO, a fibronectin-binding adhesin of *Mycoplasma bovis*. Int. J. Mol. Sci. 18, 1732.

Hananeh, W.M., Momani, W., Ababneh, M.M. and Abutarbush, S.M. 2018. *Mycoplasma bovis* arthritis and pneumonia in calves in Jordan: An emerging disease. Vet. World. 11, 1663–1668.

Higa, Y., Uemura, R., Yamazaki, W., Goto, S., Goto, Y. and Sueyoshi, M. 2016. An improved loop-mediated isothermal amplification assay for the detection of *Mycoplasma bovis*. J Vet. Med. Sci. 78, 1343–1346.

Hotzel, H., Saches, K. and Pfutzner, H. 1996. Rapid detection of *Mycoplasma bovis* in milk samples and nasal swabs using the polymerase chain reaction. J. Appl. Bacteriol. 80, 505–510.

Jones, M.N. and Holt, R.G. 2007. Cloning and characterization of an α-enolase of the oral pathogen *Streptococcus mutans* that binds human plasminogen. Biochem. Biophys. Res. Commun. 364, 924–929.

Kanda, T., Tanaka, S., Suwanruengsri, M., Sukmawinata, E., Uemura, R., Yamaguchi, R. and Sueyoshi, M. 2019. Bovine endocarditis associated with *Mycoplasma bovis*. J. Comp. Pathol. 171, 53–58.

Li, Y., Zheng, H., Liu, Y., Jiang, Y., Xin, J., Chen, W. and Song, Z. 2011. The complete genome sequence of *Mycoplasma bovis* strain Hubei-1. PLoS One 6, e20999.

Lima, S.F., Teixeira, A.G., Higgins, C.H., Lima, F.S. and Bicalho, R.C. 2016. The upper respiratory tract microbiome and its potential role in bovine respiratory disease and otitis media. Sci. Rep. 6, 29050.

Maeda, T., Shibahara, T., Kimura, K., Wada, Y., Sato, K., Imada, Y., Ishikawa, Y. and Kadota, K. 2003. *Mycoplasma bovis*-associated suppurative otitis media and pneumonia in bull calves. J. Comp. Pathol. 129, 100–110.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J. and Janzen, E.D. 2011. *Mycoplasma bovis* infections in cattle. J. Vet. Intern. Med. 25, 772–783.

Qi, J., Guo, A., Cui, P., Chen, Y., Mustafa, R., Ba, X., Hu, C., Bai, Z., Chen, X., Shi, L. and Chen, H. 2012. Comparative geno-plasticity analysis of *Mycoplasma bovis* HB0801 (Chinese isolate). PLoS One 7, e38239.

Sachse, K., Helbig, J.H., Lysnyansky, I., Grajetzki, C., Müller, W., Jacobs, E. and Yogev, D. 2000. Epitope mapping of immunogenic and adhesive structures in repetitive domains of *Mycoplasma bovis* variable surface lipoproteins. Infect. Immun. 68, 680–687.

Song, Z., Li, Y., Liu, Y., Xin, J., Zou, X. and Sun, W. 2012. α-enolase, an adhesion-related factor of *Mycoplasma bovis*. PLoS One 7, e38836.

Wise, K.S., Calcutt, M.J., Foecking, M.F., Röske, K., Madupu, R. and Methé, B.A. 2011. Complete genome sequence of *Mycoplasma bovis* type strain PG45 (ATCC 25523). Infect. Immun. 79, 982–983.

Yavlovich, A., Rechnitzer, H. and Rottem, S. 2007. α-enolase resides on the cell surface of *Mycoplasma fermentans* and binds plasminogen. Infect. Immun. 75, 5716–5719.

Zhao, G., Zhang, H., Chen, X., Zhu, X., Guo, Y., He, C., Anwar Khan, F., Chen, Y., Hu, C., Chen, H. and Guo, A. 2017. *Mycoplasma bovis* NADH oxidase functions as both a NADH oxidizing and O_2 reducing enzyme and an adhesin. Sci. Rep. 7, 44.

Zou, X., Li, Y., Wang, Y., Zhou, Y., Liu, Y. and Xin, J. 2013. Molecular cloning and characterization of a surface-localized adhesion protein in *Mycoplasma bovis* Hubei-1 strain. PloS One 8, e69644.