

PREVALENCE OF BOVINE MASTITIS AND THE FIRST DETECTION OF *MYCOPLASMA BOVIS* IN VIETNAM'S DAIRY FARMS

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Abstract

Mastitis still remains an economic disease in dairy farms all over the world. This study aimed to report the prevalence of this disease in large-scale farms in Vietnam, the pathogens profile, and first report the detection of *Mycoplasma bovis* (*M. bovis*) by applying conventional culture and real-time PCR (polymerase chain reaction) methods. We examined 64,802 enrolled dairy cows from six farms in Vietnam, yielding 1,874 (2.9%) total cases of clinical mastitis. The majority of the 14 pathogens were identified. *Klebsiella* spp. accounted for 21.0% of the total isolates. *Streptococcus uberis* (*S. uberis*), *Escherichia coli* (*E. coli*), other *Streptococcus* spp, and Coagulase-Negative Staphylococci (CNS), followed at 12.6, 7.6, 6.0, and 5.5 percent, respectively. The rest pathogens include *Pseudomonas* spp., *Enterococcus faecalis*, *Enterobacter* spp., *Bacillus* spp., *Citrobacter* spp., *Pasteurella* spp., *Proteus* spp., and *Staphylococcus aureus* were isolated with less than 1% each. To the best of our knowledge, this is the first report for detecting *M. bovis* in mastitis milk samples in Vietnam, where 35 out of 1,422 samples submitted in 2022 were *M. bovis* positive. Moreover, of the 35 positive cases, 11 cases had only *M. bovis*, while 24 cases had *M. bovis* and other pathogens, including 13 *Enterobacteriaceae*, 7 *Streptococcus* spp., and 4 CNS.

Keywords: Bovine mastitis, dairy cows, *E. coli*, *Klebsiella*, *Mycoplasma bovis*

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Introduction

Mastitis is defined as inflammation of mammary tissue, most often the result of infection. It is reported in numerous mammalian species, including domestic dairy cattle (Gomes and Henriques, 2016). Mastitis is an important and economically significant disease in the dairy industry worldwide, and not less so in countries like Vietnam where the dairy sector is still in a relatively early development phase. Economic effects of mastitis include decreased milk production from affected animals, changes in milk quality, discarded milk, veterinary costs, and replacement costs associated with culling chronically affected cows and mortality. Losses of milk production were 128 kg of milk per perturbation (Adriaens *et al.*, 2021). The average failure costs of mastitis were \$US131 per cow per year (Henk *et al.*, 2019). The average cost for a lactating cow with mastitis was €240 per year, with a failure cost of 50% and a prevention cost of 50% (van Soest *et al.*, 2016). The estimated milk losses associated with mastitis were 0.5, 0.8, and 1.1 kg per day in the first, second, and third lactation, respectively (Detilleux, 2018). Typical Canadian dairy farms lost 662 CAD per milking cow per year due to mastitis (Aghamohammadi *et al.*, 2018). Decreased milk production resulting from mammary tissue damage accounts for approximately 70% of the total cost of mastitis (Zhao and Lacasse, 2008). Key mastitis pathogens include a variety of gram-positive and gram-negative bacteria and can be divided into either contagious (e.g., *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp.) or environmental (e.g., *Escherichia coli* (*E. coli*), *Enterococcus* spp., Coagulase-Negative Staphylococci (CNS), *Streptococcus uberis* (*S. uberis*)) (Cheng and Han, 2020).

Most of Vietnam's high-production cows have been imported from European and American countries which were not free of *Mycoplasma bovis* (*M. bovis*). These cows are then kept in hot, high-humidity conditions using organic bedding materials (recycled manure solid), including plenty of *E. coli*, *Klebsiella* spp, and other environmental pathogens. Several studies have reported isolation rates in dairy cows of 17.2% for *M. bovis*, 62.5% for *Klebsiella* spp, 69.1% for *Staphylococci*, 22.3% for *E. coli*, and 16.8% for *Streptococci*. (Tolboom *et al.*, 2008; Timonen *et al.*, 2017; Dufour *et al.*, 2019; Salaududin *et al.*, 2019; Cheng *et al.*, 2020; Filioussis *et al.*, 2020). In Vietnam, only a few studies have reported the prevalence of mastitis and related treatment recipes; however, they did not report the pathogen's profile, which could provide

information on pathogens that researchers and farmers could use to look for solutions to manage the disease. This study aimed to analyze the proportion of mastitis in dairy cows, the prevalence of common pathogens associated with mastitis cases and to report for the first time on the detection of *M. bovis* obtained from cows farmed in north-central Vietnam.

Materials and Methods

Herd profile and samples

This study was conducted from several large dairy farms in Nghe An province, Viet Nam in 2022. The animal population consisted of Holstein-Friesian dairy cows from intensively managed, fully housed, and TMR-fed, ranging in average size from 1,800 to 4,500 animals. All milking cows are kept in free-stall barns with manure recycled solid as bedding and milked 2-3 times daily. A total of 64,802 enrolled dairy cows from six Holstein-Friesian dairy farms in north-central Vietnam were examined, and 1,872 cases were mastitis diagnosed (by veterinarians at the farms with abnormalities in udders such as swelling, heat, hardness, redness, pain, and milk appearance i.e. watery, flakes, clots, or pus). From those animals and 2,020 samples were collected (under aseptic conditions (sterilized sample vials, clean udder(s), sampling, labeling, refrigeration post-collection, and transport to the lab in the icebox) and cultured on the day of arrival.

Aerobic pathogens isolation

The bacteria were isolated according to the procedures described by Adkins and Middleton (2017). A loopful of each milk sample (0.01 mL) was streaked on blood agar plates and then incubated aerobically at 37°C for 24 hours. Bacterial growth and morphologic characteristics such as colony size, shape, color, and hemolytic status from the cultured plates were examined and recorded. After that, bacteria were Gram-stained (or KOH 3% tested), catalase tested, and then transferred to new series of media/ mediums as follow: all Gram-positive (KOH 3% negative) and catalase-positive cocci then be transferred to Brain heart infusion broth with 6.5% NaCl (to differentiate the enterococci from the nonenterococcal species) and Esculin blood agar (for detecting the esculin hydrolysis ability). Gram-negative (KOH 3% positive) isolates then be transferred to MacConkey agar (for lactose fermentation test), Simon citrate agar (Oxoid, UK) (for citrate utilization test), Triple sugar iron agar (Oxoid, UK) (for glucose/ lactose/ sucrose fermentation, gas and H₂S production tests),

MR-VP broth (Oxoid, UK) (for MR, VP tests), and Tryptone broth (Oxoid, UK) (for indole production test) and confirmed the results on the second day. Leave one more day in the incubator if the plate has no colony. A sample was considered “contaminated” if three or more bacterial species were isolated Adkins and Middleton (2017). The term “unknown” indicates that the pathogen could not be identified using the above mentioned procedures. The absence of bacteria colonies on the culture plates after 48 hours of incubation was reported as “no growth”.

***Mycoplasma bovis* isolation and confirmation**

M. bovis isolation process follows the protocol described by OIE (2018); Alysia *et al.* (2018): 100µl of the milk samples were inoculated in Pleuro pneumonia-like organisms (PPLO) broth (BD, New Jersey, US) at 37°C in 5% CO₂ for three to five days. Then, a loopful of a mixture from PPLO broth was further cultured on PPLO agar (BD, New Jersey, US) at 37°C in 5% CO₂ for three to five days. Specific colonies on PPLO agar (fried - egg shape) were checked under the stereomicroscope and collected. These colonies then be extracted DNA using MagMAX CORE nucleic acid purification kit (Applied Biosystems / Thermo Fisher Scientific, Texas, US) according to the manufacturer's instruction. The DNA was placed in an Eppendorf tube and kept under -20°C until the PCR reaction was carried out. The PCR reactions were conducted using a VetMAX *M. bovis* kit from Applied Biosystems / Thermo Fisher Scientific (Lyon, France) according to the manufacturer's instructions for confirmation.

Statistics analysis

This study used spreadsheets as software tools for data entry, storage, analysis, and visualization.

A single sample t-test (or one sample t-test) compares the differences in the mean of *M. bovis* results of a single farm to all farms' mean.

Results and Discussion

The mastitis prevalence of the studied farms.

The prevalence of bovine mastitis from six different dairy farms in the north central of Vietnam in 2022 was collected and presented in Table 1.

Overall, the prevalence of bovine mastitis in the study was 2.9%, the same as 1,874 positive cases out of 64,802 enrolled dairy cows. In descending order, the rates of mastitis cases on the six farms were 3.4, 3.2, 3.0, 2.8, 2.5, and 2.5%. However, there were no significant differences between the six dairy farms in the study period, and the proportion of bovine mastitis in this study may be the common rate of bovine mastitis in Vietnam. In addition, our prevalence results were slightly different from other reports in Pakistan (1.67%) (Ali *et al.*, 2021), Uruguay (1.2%) (Giannechini *et al.*, 2002), Canada (1.4%) (Olde Riekerink *et al.*, 2008), and slightly lower as compared to Finland (3.1%) (Pitkälä *et al.*, 2004), South Ethiopia (3.9%) (Abebe *et al.* 2016), China (3.3%) (Gao *et al.*, 2017). The variations in the results may link to the difference in climate, geography, the origin of cows, husbandry technology, and health care protocols between the countries.

The mastitis pathogens profile.

2020 samples were tested during the study time, and the pathogens profile was collected and summarized in Table 2.

Table 1. Prevalence of dairy cow mastitis in north-central Vietnam in 2022

	Farm						Total
	A	B	C	D	E	F	
No. of cows	11,313	10,919	7,613	11,711	12,000	11,246	64,802
No. of Mastitis cases	357	268	214	288	362	386	1,874
(%)	3.2	2.5	2.8	2.5	3.0	3.4	2.9

Table 2. Bacteria profile isolated from mastitis milk samples

Bacteria species	No. of samples	% of samples
<i>Klebsiella</i> spp.	425	21.0
<i>Streptococcus uberis</i>	254	12.6
<i>E. coli</i>	154	7.6
Other <i>Streptococcus</i>	121	6.0
Coagulase-negative staphylococci - CNS	111	5.5
<i>Pseudomonas</i> spp.	17	0.8
<i>Enterococcus faecalis</i>	16	0.8
<i>Enterobacter</i> spp.	14	0.7
<i>Bacillus</i> spp.	11	0.5
<i>Citrobacter</i> spp.	7	0.3
<i>Pasteurella</i> spp.	6	0.3
<i>Proteus</i> spp.	4	0.2
<i>Staphylococcus aureus</i>	3	0.1
Total of bacteria positive	1,143	56.7

* Note: the isolates pathogens include 768 “no growth”, 66 fungi, 42 contamination, and 1 unknown pathogen.

Overall, 1,143 samples (56.7%) were considered bacteriologically positive with *Klebsiella* spp.; *S. uberis*, other *Streptococcus*, *E. coli*, *Pseudomonas*, and *Enterobacter* being the main isolated environment pathogens. Among them, *Klebsiella* spp. isolated was the most prevalent at 21.0% of all tested milk samples, followed by *S. uberis* at 12.6%, *E. coli*, Other *Streptococcus*, and CNS at 7.6%, 6.0%, and 5.5%, respectively. Other bacteria species isolated at lower than one percent were *Pseudomonas* spp., *Bacillus* spp., *Citrobacter* spp., *Pasteurella* spp., *S. aureus*, and *Proteus* spp. Along with bacteria isolated, we found samples contaminated were 2.1% (42/2020), fungi 3.3% (66/2020), and the rate of ‘no growth’ was 38.0% (768/2020) of total samples during the study period. Our results indicated that *Klebsiella* spp. was the most common pathogen detected from mastitis cases in Vietnam. In this study, *Streptococcus* spp., which includes *S. uberis* and other *Streptococcus* spp., accounted for 18.6% of mastitis-causing bacteria, which is closer to the results of 12.8% in Egypt (Abd El-Razik *et al.*, 2021) and 18.2% in Belgium (Verbeke *et al.*, 2014). However, it differed considerably from the results in Poland, which ranged from 22% to over 49% (Kaczorek *et al.*, 2021). In addition, *E. coli* accounted for 7.6% of the current study, which is lower as compared to other studies, which reported *E. coli* isolation to 13.6% in Ethiopia (Asmare and Kassa, 2017), and 50% in Egypt (Fahim *et al.*, 2019) of all samples from the study, respectively. Environmental pathogens can be present in the litter, manure, milking equipment, water, milkers’ hands, and cow skin; hence not surprising to isolate the named bacteria species in the current study. There is a need

for dairy farmers to adhere to good hygiene practices in their dairy environment to reduce the risk of bacterial infection in cows and farms.

The present study also reported a low prevalence of important infectious mastitis pathogens (*Pasteurella* spp. and *S. aureus*). Despite that, data on *Pasteurella* mastitis is not well reported. A prevalence of 0.5% for *Pasteurella* spp. has been reported in this study which is higher than 0.1% in the study from the United States of America (USA) (Wilson *et al.*, 1997). The source of infection for *Pasteurella* spp. is the upper respiratory tract of mammals, but the route of spread from cow to cow is unknown (Adkins and Middleton, 2017). *Staphylococcus aureus* is notable worldwide as one of the major pathogens of intramammary infection in dairy cows transmitted between cows from an infected cow and between glands from infected glands or teats due to the unhygienic dairy environment such as unhygienic milking process (Adkins and Middleton, 2017). The reported *S. aureus* infection rates are higher than the 0.2% in the current study, such as 9.9% (62/624) in China (Liu *et al.*, 2020) and 15.2% (29/191) in Nepal (Shrestha *et al.*, 2021).

The miscellaneous organisms (Fungi & *Bacillus* spp.) are often considered minor or opportunistic mastitis pathogens. Fungi (yeasts & molds) are mycotic agents that live freely in the environment. *Bacillus* spp. reservoir for the infection is the environment, including soil, water, feces, feedstuff, and wounds, and likely to account for less than 1% of all mastitis cases (Adkins and Middleton, 2017). An increase in the proportion of fungal-isolated samples from less than 1% (2008) to more than 3.2% (2017) has been reported in Canada (Acharya *et al.*, 2021). Compared to this survey, our results (3.8%) are very similar and trend upward as well. A report of mastitis pathogens in China showed that the prevalence of *Bacillus* spp. was 8.2% (Song *et al.*, 2020), whereas in Ecuador, the proportion was up to 7.5% (Amer *et al.*, 2018). Our findings for *Bacillus* spp. are considerably lower when compared to these results. This group of pathogens is often related to poor hygiene during milking and sampling and may transmit between cows through the milker’s hands and treatment devices. Thus, farm owners and their workers need to focus on general hygiene not only for the cow but also for all the materials used in dairy farms to reduce the risk.

‘No growth’ means no observed bacteria colony during the basic culture process. This can be caused by various reasons, including intermittent shedding of the causative pathogens, fastidious bacteria dying during transport, and incorrect sampling times/techniques. Our ‘no growth’ results

showed 38.0% of all herds, very high compared to 19.9% in Canada to 22.4% in Belgium (Verbeke *et al.* 2014; Levison *et al.* 2016), but the values were similar to another study in Canada 43.9 % (Olde Riekerink *et al.*, 2008). This result was lower than a report from Bangladesh, with 62.5% of “no growth” (Salaudin *et al.*, 2019). ‘No growth’ results are problematic not only for the testing laboratory but also for clinical veterinarians and farm owners, and as a result, it can be confusing when making decisions. Therefore, sampling on time and using proper technique before sending to the laboratory are critical to making good decisions based on test results.

***Mycoplasma bovis* isolation results.**

The result of *M. bovis* isolation through the process described above and then confirmed by Realtime PCR from clinical mastitis cases during the study was summarized in Table 3.

Table 3. The number and percentage of *M. bovis* isolated from the farms

Samples	Farm						Total
	A	B	C	D	E	F	
Total samples	211	249	198	115	216	433	1,422
No. of <i>M. bovis</i>	3	6	3	2	4	17	35
(%)	1.4	2.4	1.5	1.7	1.9	3.9	2.5

Samples were obtained from udders suspected of having *M. bovis* infection by veterinarians, and the number of *M. bovis*-positive results is indicated. The highest positive rate (significantly different from other farms with $P < 0.05$) was on farm F at 3.9% and the lowest on farm A at 1.4%. Despite its low prevalence on all farms we studied, *M. bovis* remains on the herd owner's priority pathogen list because it is highly contagious and poorly therapeutic. Our prevalence of *M. bovis* from mastitis samples was at the same level as other studies in Prince Edward Island (Canada), with a positive rate was 1.5% (Olde Riekerink *et al.*, 2006), in Australia 0.9%, and in Belgium 1.5% (Passchyn *et al.*, 2012), in Israel from 2009 to 2014 the prevalence changed from less than 1% to 3% (Lysnyansky *et al.*, 2016). The positive of *M. bovis* in our study was much lower than the finding in Estonia, which showed a positive rate of 17.2% (Timonen *et al.*, 2017). In addition, the positive rate in Spain was 28% from 2016 through 2019 (García-Galán *et al.*, 2020). Our result by farm level is 100% (6/6 farm) which is similar to the study in Iran

(100%) (Ghazaei, 2006) but higher than the study in Mexico (55%) (Miranda-Morales *et al.*, 2008), New Zealand is probably free of *M. bovis* (McDonald *et al.*, 2009), in US the prevalence was 17.7% (157/889) from non-New York State and 57.5% (511/889) in New York State, in combination, it was 75.1% (Gioia *et al.*, 2021).

M. bovis was detected for the first time from dairy farms in Vietnam. Its infection rate was still at a low level compared to other countries. Farm owners and veterinarians should properly manage the source of imported cattle to detect the presence of *M. bovis* early and avoid infecting other cattle.

The combination of *M. bovis* with other pathogens.

To investigate the combination of *M. bovis* with other pathogens during that time, we did aerobics and *M. bovis* isolation together on the 1,422 samples. The result is summarized in Table 04 below:

Table 4. The pathogens combination between *M. bovis* and others

	Pathogens	Number of cases	Proportion (%)
		797	56.0
<i>M. bovis</i> negative	<i>Enterobacteriaceae</i>	428	29.4
	<i>Streptococcus</i> spp.	287	21.0
	<i>Staphylococcus</i> spp.	67	4.7
	<i>Pseudomonas</i> spp.	10	0.7
	<i>Pasteurella</i> spp.	2	0.1
	<i>Bacillus</i> spp.	2	0.1
	<i>Proteus</i> spp.	1	0.1
		35	2.5
<i>M. bovis</i> positive	None	11	0.8
	<i>Enterobacteriaceae</i>	13	0.9
	<i>Streptococcus</i> spp.	7	0.5
	CNS	4	0.3

*Note: 1,422 clinical mastitis samples included: 518 “no growth”, 47 fungi, 19 contamination, and 6 “unknown”.

From the result, the total number of dairy cows infected with one pathogen (mono-infection) without *M. bovis* was 797 cases, and the pathogens include *Enterobacteriaceae* (428), *Streptococcus* spp. (287), and *Staphylococcus* spp. (67), *Pseudomonas* spp. (10), *Pasteurella* and *Bacillus* spp. (2), and *Proteus* spp. (1). In 35 dairy cows positive with *M. bovis*, 11 cases had only *M. bovis*, 13 cases had *M. bovis* together with *Enterobacteriaceae*, 7 cases carried both *M. bovis* and *Streptococcus* spp., and 4 cases carried *M. bovis* together with CNS. There are not many studies on combinations of *M. bovis* with other pathogens. A study in Estonia reported that both *M. bovis*

and *Streptococcus agalactiae* were isolated simultaneously from 7.5% of the samples (Timonen *et al.*, 2017). A study in Australia reported that co-infection with mycoplasma and two or more genera/species was 52%, and infection with *M. bovis* alone was 6.2% (Al-Farha *et al.*, 2017). The results of this study suggest that mastitis in dairy cows in Vietnam is often mono-infection. By focusing on the causative agent of each case of mastitis, the optimal antibiotic for treatment can be selected based on previous publishes or carried out antimicrobial susceptibility testing, increasing the efficiency of the antimicrobial resistance management process and reducing costs

Conclusions

This study presented a prevalence of mastitis in Vietnam of 2.9% and identified 14 pathogens. *Klebsiella* spp. was leading among the isolated pathogens, followed by *S. uberis* and *E. coli*. In particular, *M. bovis* was isolated from 2.5% of mastitis samples. Hence, to the best of our knowledge, this is the first report in Vietnam of *Mycoplasma* isolation from bovine mastitis. It was suggested that *M. bovis* may be isolated alone or together with other bacterial pathogens.

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