



Prevalence of respiratory bacterial pathogens and associated management factors in dairy calves in Taiwan

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ABSTRACT. This study aimed to investigate the prevalence at both farm-level and calf-level and to identify the risk factors of respiratory bacterial pathogens in dairy calves in Taiwan. The status of bovine respiratory disease (BRD) was evaluated by using the Wisconsin scoring system from a total of 400 pre-weaned calves from 32 different farms in Taiwan, then the nasopharyngeal swabs were collected. The prevalence of respiratory pathogens was 84.37% at farm-level and 45.50% at calf-level, and *Pasteurella multocida* (*P. multocida*) was the most prevalent pathogen. The presence of *Mycoplasma bovis* (*M. bovis*), *P. multocida*, *Mannheimia haemolytica* (*M. haemolytica*) and *Histophilus somni* (*H. somni*) were all higher in BRD positive calves than BRD negative calves, but only in *H. somni* was significant ($P < 0.001$). Then nine farm management risk factors were analyzed by using multivariate logistic regression models to determine the risk factors of respiratory bacterial pathogens (farm and calf-level). In the result at farm-level, only unheated colostrum was significantly associated with pathogen positive farms (Odds Ratio (OR)=1.43). At calf-level, the predominant risk factor for each pathogen, *M. bovis*, *P. multocida*, *M. haemolytica* and *H. somni*, was late first colostrum feeding (OR=272.82), unheated colostrum (OR=3.41), waste milk feeding (OR=6.59) and high pneumonia treatment cost (OR=2.52), respectively. For effective preventive measures, farmer education on milk and colostrum feeding are urgently warranted.

KEYWORDS: bovine respiratory disease, calf, management, prevalence, Taiwan

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Bovine respiratory disease (BRD) is a multi-factorial syndrome with various predisposing factors causing high mortality and morbidity in calves [14, 31, 36]. BRD is an important disease that contributes to economic loss in cattle industry, it is also related with cattle health status causing slow growth rate, decreased average daily gain, reduced reproductive performance, prolonged first parturition, diminished milk production, treatment, labor cost consuming besides the veterinary fees [9, 20, 45]. BRD can be identified by using a variety of methods, such as thoracic ultrasound, radiography, necropsy, pathogen isolation, and molecular diagnostic tests [1, 29, 33]. BRD has been reported as one of the most common diseases in pre-weaning and weaned calves, with 18.1% and 11.2% morbidity, respectively [36]. Multiple viral and bacterial agents are implicated in BRD; however, only bacterial pathogens that were considered as the predominant respiratory pathogens was focus in this study due to rare viral pathogens detected in dairy calves in Taiwan [32]. The most common bacterial pathogens are *Mannheimia haemolytica* (*M. haemolytica*), *Pasteurella multocida* (*P. multocida*), *Histophilus somni* (*H. somni*) and *Mycoplasma bovis* (*M. bovis*) [4, 18, 22, 42, 43]. Not only from calves with clinical signs, these bacteria could also be isolated from the respiratory tracts of asymptomatic calves [4, 43].

Farm management is an important affecting factor on BRD. Calves housed in group pens and born in pastures without additional bedding were found to have higher BRD prevalence [24, 28]. On the other hand, feeding calves with salable milk, pasteurized milk, heat treatment of colostrum and having paved road surfaces adjacent to the housing area can decrease BRD prevalence [24, 28].

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In Taiwan, there were more than 60,000 milking cows raised on 553 farms and almost 400,000 milk products produced in a year [12]. BRD has also been commonly found in pre-weaned calves in Taiwan and causes negative effects to dairy industry; however, no systematic investigation of respiratory bacterial pathogens and their related risk factors have been reported. In order to better understand the etiology, the pathogen distribution, and the possible causes of BRD, the objectives of this study were: to investigate the prevalence of respiratory bacterial pathogens in dairy calves in Taiwan and to identify the risk factors of respiratory bacterial pathogens. This information lead to the enlightenment of the distribution of BRD and the related risk factors in Taiwan, thus leading to better farm management in the future.

MATERIALS AND METHODS

Farm and animal enrollment

Between January, 2018 and January, 2019, a cross-sectional study based on questionnaire and convenience sampling was applied to conduct a survey for the understanding on BRD in dairy calves in Taiwan. There were 32 dairy farms (5.8% of dairy farms in Taiwan) enrolled in the study after consent of the farmers, including two in the north, 12 in the central, 15 in the south, and three in the east region. The distribution of these enrolled farms was similar to the distribution of dairy farms in Taiwan [11]. Each pre-weaning calf in these farms was sampled and the information of location, identification (ID) number, and age of each sampled animal were recorded.

BRD scoring and sample collection

BRD status of each calf was evaluated by the same trained veterinarian using the Wisconsin (WI) scoring system by assessing 5 clinical signs including: ocular discharge, nasal discharge, rectal temperature, coughing, ears and head positions. Each clinical sign was assigned 0 points for normal presentation, and an abnormal presentation was assigned 1, 2, or 3 points. Calves that had a sum of the points equal to or exceeding 5 were BRD positive. BRD negative calves were calves that had a score lower than 5 [31]. After the WI scores were evaluated and recorded, nasopharyngeal swab (NPS) sample was collected from each calf. An unguarded nylon swab (19.5 cm total length, 2 cm tip length, 0.7 cm tip max width; Iron Will Biomedical Technology Inc., New Taipei City, Taiwan) was inserted into the nostril to the full length of the swab. After rotating several times, the swab was withdrawn and soaked in 2 ml sterile phosphate-buffered saline (PBS), and then stored at 4°C for further analysis. All procedures performed in this study involving animal participants were in accordance with the ethical standards of Institutional Animal Care and Use Committee (IACUC), National Pingtung University of Science and Technology.

DNA extraction and pathogen detection

NPS samples were used to extract DNA using Tissue Genomic DNA extraction mini kit (Favorgen Biotech Corp., Taiwan), according to manufacturer's protocol. The DNA preparation was stored at -20°C until used for PCR or nested PCR analysis. The detection of three pathogens, *M. haemolytica*, *H. somni* and *P. multocida*, were performed by PCR as previously described [3, 4, 43]. *M. bovis* detection was performed by nested PCR [35]. Four samples containing target template bacterial DNA from previous studies in our laboratory were used as positive controls and distilled water was used as a negative control. The positive controls were verified and compared with the database using BLAST program. After PCR amplification, each PCR product was electrophoresed by 1.5% agarose gel at 100 V to identify the size of products.

Questionnaire

A questionnaire that focused on farm information, management of pneumonia calves and calf feeding management was designed. Then filled out by each farm owner through google form. The section of farm information includes the name, location and size of the farm, number of milking cows, and number of calves. The management of pneumonia calves includes previous occurrence of pneumonia in calves in this year, pneumonia treatment result, pneumonia recovery time, pneumonia treatment cost, and if the sick calves were moved to separated pen. Calf feeding management includes source of milk feeding (milk powder or waste milk), heat treatment (pasteurization) of colostrum, and first colostrum feeding time. Calves in the same farm are considered to have the same farm managements.

Statistical analysis

Data were analyzed by the SAS software version 9.4 (SAS Institute Inc.). Descriptive analysis was performed to determine frequencies for prevalence of pathogens at farm-level and calf-level. Prevalence of each pathogen at calf-level was calculated as the number of animals detected positive by using PCR divided by the total number of calves sampled. Farms with at least one animal detected with any of the four pathogens were defined as a pathogen positive farm, in contrary, farms with no animals detected for any of the four pathogens were defined as a pathogen negative farm. Farm prevalence was then calculated as the number of pathogen positive farms divided by the total number of farms sampled.

Pearson's χ^2 or Fisher's Exact test and odds ratios (OR) with 95% confidence interval (CI) were used to compare the prevalence of respiratory bacterial pathogens (farm and calf-level) in different regions in Taiwan, and the association between the presence of respiratory bacterial pathogens (calf-level) and animal health status (BRD positive/negative). Stepwise logistic regression analysis with inclusion and exclusion criteria of $P \leq 0.10$ and $P \geq 0.15$ respectively was used to determine the farm management risk factors of respiratory bacterial pathogens (farm and calf-level). These nine variables include farm size, previous occurrence of pneumonia

in calves, pneumonia treatment result, pneumonia recovery time, pneumonia treatment cost, and if the sick calves were moved to separated pen, source of milk feeding, heat treatment of colostrum, and first colostrum feeding time. $P < 0.05$ was considered significant for statistical analyses.

RESULTS

Sample collection and BRD scoring

Between January, 2018 and January, 2019, a total of 400 pre-weaned female Holstein Friesian calves with ($n=27$) and without BRD ($n=373$) from 32 different farms in Taiwan were sampled by NPS. The farm size ranges from 137 to 2,000 cattle with 4 to 130 pre-weaned calves. The age of pre-weaned calves was between 1 to 135 days.

Prevalence of respiratory bacterial pathogens

The prevalence of bacterial pathogens at farm-level was 84.37% (27/32) and that at calf-level was 45.50% (182/400) in Taiwan. In farm level, the most prevalent bacterial pathogen was *P. multocida*, detected from 21 farms (65.62%), followed by *M. bovis* from 12 farms (37.50%), *H. somni* from 11 farms (34.38%) and *M. haemolytica* from 8 farms (25.00%). The most prevalent bacterial pathogen in calves was *P. multocida* (34.75%), detected in 139 calves, followed by *M. bovis* (16.50%) in 66 calves, *H. somni* (13.25%) in 53 calves and *M. haemolytica* (3.50%) in 14 calves. (Table 1). Two or more bacterial were detected in 15.25% of calves, with *P. multocida* + *H. somni* (42.62%) and *M. bovis* + *P. multocida* + *H. somni* (27.87%) being the most frequent combinations (Supplementary Table 1).

Table 1. Prevalence of respiratory bacterial at both farm-level and calf-level in Taiwan

Pathogens	Farm-level		Calf-level	
	Prevalence% (N/T) ¹	95% CI	Prevalence% (N/T) ²	95% CI
<i>P. multocida</i>	65.62 (21/32)	49.169–82.081	34.75 (139/400)	30.084–39.416
<i>M. bovis</i>	37.50 (12/32)	20.726–54.274	16.50 (66/400)	12.862–20.138
<i>H. somni</i>	34.38 (11/32)	17.919–50.831	13.25 (53/400)	9.928–16.572
<i>M. haemolytica</i>	25.0 (8/32)	9.997–40.003	3.50 (14/400)	1.699–5.301
Total ³	84.37 (27/32)	71.795–96.955	45.50 (182/400)	40.620–50.380

Mannheimia haemolytica (*M. haemolytica*), *Pasteurella multocida* (*P. multocida*), *Histophilus somni* (*H. somni*) and *Mycoplasma bovis* (*M. bovis*), Confidence interval (CI). ¹ Number of positive farms/total farms, ² Number of positive calves/total calves, ³ At least one of these four pathogens detected.

Table 2. The association between the prevalence of respiratory bacterial pathogens and Taiwanese regions at both farm-level and calf-level

Pathogens	Level	Regions				P-value
		North % (N/T)	Central % (N/T)	South % (N/T)	East % (N/T)	
<i>M. bovis</i>	Farm	0 (0/2)	41.66 (5/12)	40 (6/15)	33.33 (1/3)	0.871
	Calves	0 (0/24) ^a	19.87 (32/161) ^c	17.83 (33/185) ^{bc}	3.33 (1/30) ^{ab}	0.008**
<i>P. multocida</i>	Farm	100 (2/2)	66.67 (8/12)	60 (9/15)	66.67 (2/3)	0.931
	Calves	66.67 (16/24) ^b	37.26 (60/161) ^{ab}	27.02 (50/185) ^a	43.33 (13/30) ^{ab}	0.001**
<i>M. haemolytica</i>	Farm	0 (0/2)	41.66 (5/12)	20 (3/15)	0 (0/3)	0.42
	Calves	0 (0/24)	5.59 (9/161)	2.7 (5/185)	0 (0/30)	0.252
<i>H. somni</i>	Farm	50 (1/2)	41.66 (5/12)	26.66 (4/15)	33.33 (1/3)	0.879
	Calves	12.5 (3/24)	17.39 (28/161)	9.72 (18/185)	13.33 (4/30)	0.214

Mannheimia haemolytica (*M. haemolytica*), *Pasteurella multocida* (*P. multocida*), *Histophilus somni* (*H. somni*), *Mycoplasma bovis* (*M. bovis*). In each column different superscript letters indicate significant different ($P < 0.05$). **: Significant different between groups ($P < 0.01$).

Table 3. The association between the prevalence of respiratory bacterial pathogens and outcomes

Pathogens	Healthy status	Prevalence% (N/T) ¹	OR (95% CI)	P-value
<i>M. bovis</i>	BRD negative	16.10 (60/373)	Reference	0.42
	BRD positive	22.20 (6/27)	1.490 (0.577–3.847)	
<i>P. multocida</i>	BRD negative	31.25 (125/373)	Reference	0.061
	BRD positive	51.85 (14/27)	2.136 (0.9746–4.684)	
<i>M. haemolytica</i>	BRD negative	3.50 (13/373)	Reference	1
	BRD positive	3.70 (1/27)	1.065 (0.134–8.462)	
<i>H. somni</i>	BRD negative	10.90 (41/373)	Reference	<0.001**
	BRD positive	44.40 (12/27)	6.478 (2.837–14.790)	
Total ²	BRD negative	42.90 (160/373)	Reference	<0.001**
	BRD positive	81.48 (22/27)	5.857 (2.171–15.802)	

Mannheimia haemolytica (*M. haemolytica*), *Pasteurella multocida* (*P. multocida*), *Histophilus somni* (*H. somni*), *Mycoplasma bovis* (*M. bovis*), Odds ratio (OR), Confidence interval (CI). ¹ Number of positive calves/total calves. ² At least one of these four pathogens detected. **: Significant different between groups ($P<0.01$).

The prevalence of respiratory pathogens in different Taiwanese regions

The prevalence of respiratory bacterial pathogens at farm and calf-level in different Taiwanese regions were shown in Table 2. At farm-level, *M. bovis* was mostly found in the central of Taiwan (41.66%), followed by south (40.00%) and east region (33.33%), but not found in the north region. *P. multocida* was mostly found in the north of Taiwan (100.00%), followed by the central and east regions (both 66.67%), and south region (60.00%). *M. haemolytica* was mostly found in the central of Taiwan (41.66%), followed by south region (20.00%), but not found in the north and east regions. *H. somni* was mostly found in the north region (50.00%), followed by central (41.66%), east (33.33%) and south region (26.66%). No difference was observed in presence of respiratory bacterial pathogens and the Taiwanese regions for farm-level.

The prevalence of *M. bovis* in calves in the central region (19.87%) was higher than that in south region (17.83%), and significantly higher than those in north (0%) and east (3.33%) regions. The highest prevalence of *P. multocida* was found in north region (66.67%), followed by in east (43.33%) and central (37.26%) regions, and significantly higher than in south (27.02%) region. *M. haemolytica* was only detected from the calves in the central (5.59%) and south region (2.70%) of Taiwan. *H. somni* was detected in all Taiwanese regions, and no significant difference among regions was observed (Table 2).

The association between the presence of respiratory bacterial pathogens and clinical outcomes

Overall, the percentage of calves detected at least one bacterium in the respiratory tract was found to be significantly higher in BRD positive calves than in BRD negative calves ($P<0.001$). For individual pathogen detection, *H. somni* was found to be significantly higher in BRD positive calves than in BRD negative calves ($P<0.001$). *M. bovis*, *P. multocida* and *M. haemolytica* were also detected more frequently in BRD positive calves even the results were not significantly different ($P>0.05$) (Table 3).

Risk factors analyses

Multivariate logistic regression analysis was conducted to determine if the farm managements are statistically associated with respiratory bacterial pathogens in both farm and calf-levels (Table 4). At farm-level, a statistical association was found between unheated colostrum and pathogen positivity. Compare with those farms in which the calves were not fed by unheated colostrum, the farms with calves fed by unheated colostrum are 11.43 times more likely to be pathogen positive farms. At calf-level, the risk factors for *M. bovis* presence included late first colostrum feeding (OR=272.82, $P<0.001$), large farm size (OR=22.09, $P<0.001$), farms that have high occurrence of pneumonia in calves in this year (OR=7.83, $P=0.001$), farms that have poor response for pneumonia treatment (OR=6.48, $P=0.017$), high pneumonia treatment cost (OR=2.49, $P=0.085$), short pneumonia recovery period (OR=0.26, $P=0.041$) and heat treatment of colostrum (OR=0.26, $P<0.001$). The risk factors of *P. multocida* presence included unheated colostrum (OR=3.41, $P<0.001$), waste milk feeding (OR=3.35, $P<0.001$), small farm size (OR=0.33, $P=0.003$), farms that have low occurrence of pneumonia in calves in this year (OR=0.31, $P=0.051$) and farms that have good responded for pneumonia treatment (OR=0.31, $P=0.006$). The risk factor of *M. haemolytica* presence was only waste milk feeding (OR=6.59, $P=0.001$). The calves fed by waste milk are 6.59 times more likely to harbour *M. haemolytica* than those fed by milk powder. The risk factors for *H. somni* included high pneumonia treatment cost (OR=2.52, $P=0.001$) and late first colostrum feeding (OR=2.00, $P=0.034$).

DISCUSSION

To the authors' knowledge, this is the first report on prevalence and farm management factors to bovine respiratory bacterial pathogens in dairy calves in Asia. In this study, convenience sampling was used rather than other sampling methods due to the limitation of farm consents. However, the lack of representative to the farm population and possible bias could be reduced because the distribution of enrolled farms is similar to the distribution of dairy farms in Taiwan. The prevalence of respiratory bacterial pathogens in dairy calves in the current study in Taiwan (45.50%) was lower than those detected in feedlot and/or dairy

Table 4. Multivariate analysis of farm management risk factors related to respiratory bacterial pathogens at both farm and calf-levels in dairy farms in Taiwan

Variables	Farm-level ¹				Calf-level										
	Positive% (N/T) ²	OR (95% CI)	P-value	M. bovis Positive% (N/T) ³	OR (95% CI)	P-value	P. multocida Positive% (N/T) ³	OR (95% CI)	P-value	M. haemolytica Positive% (N/T) ³	OR (95% CI)	P-value	H. somni Positive% (N/T) ³	OR (95% CI)	P-value
Farm size															
100–499 cattle	88.46 (23/26)	-	-	8.42 (25/297)	Reference	-	38.05 (113/297)	Reference	-	-	-	-	15.15 (45/297)	-	-
≥500 cattle	66.7 (4/6)			39.81 (41/103)	22.088 (7.890–61.834)	<0.001**	25.24 (26/103)	0.326 (0.167–0.633)	0.003**	4.85 (5/103)			7.77 (8/103)		
Previous occurrence of pneumonia in calves in this year															
Rare (less than 30% of calves)	81.48 (22/27)	-	-	15.45 (53/343)	Reference	-	35.28 (121/343)	Reference	-	-	-	-	12.54 (43/343)	-	-
Often (at least 30% of calves)	100 (5/5)			22.81 (13/57)	7.834 (2.480–24.749)	0.001**	31.58 (18/57)	0.313 (0.141–0.698)	0.051	1.75 (1/57)			17.54 (10/57)		
Pneumonia treatment result															
Better	82.75 (24/29)	-	-	18.04 (59/327)	Reference	-	36.09 (118/327)	Reference	-	-	-	-	13.76 (45/327)	-	-
Stable or worse	100 (3/3)			9.59 (7/73)	6.484 (1.554–27.04)	0.017*	28.77 (21/73)	0.313 (0.147–0.668)	0.006**	2.74 (2/73)			10.96 (8/73)		
Pneumonia recovery period															
Within 1 week	87.5 (14/16)	-	-	19.3 (44/228)	Reference	-	35.53 (81/228)	Reference	-	-	-	-	12.72 (29/228)	-	-
≥2 weeks	81.3 (13/16)			12.79 (22/172)	0.264 (0.102–0.679)	0.041*	33.72 (58/172)			2.33 (4/172)			13.95 (24/172)		
Pneumonia treatment cost															
0–500 NT	76.9 (10/13)	-	-	19.76 (33/167)	Reference	-	31.14 (52/167)	Reference	-	-	-	-	6.59 (11/167)	-	Reference
>500 NT	89.5 (17/19)			14.16 (33/233)	2.493 (0.864–7.197)	0.085	37.34 (87/233)			3 (7/233)			18.03 (42/233)	2.518 (1.219–5.199)	0.001**
If the sick calves were moved to separated pens															
Yes	8 (8/10)	-	-	21.58 (30/139)	-	-	22.3 (31/139)	-	-	-	-	-	11.51 (16/139)	-	-
No	86.4 (19/22)			13.79 (36/261)			41.38 (108/261)			3.83 (10/261)			14.18 (37/261)		
Source of milk feeding															
Milk powder	76.2 (16/21)	-	-	10.76 (27/251)	Reference	-	25.1 (63/251)	Reference	-	Reference	-	-	14.74 (37/251)	-	-
Waste milk	100 (11/11)			26.17 (39/149)	3.346 (1.879–5.957)	<0.001**	51.01 (76/149)	3.346 (1.879–5.957)	<0.001**	7.38 (11/149)	6.589 (1.808–24.021)	0.001**	10.74 (16/149)		
Heat treatment of colostrum															
Yes	63.69 (7/11)	Reference	Reference	21.67 (26/120)	Reference	Reference	17.5 (21/120)	Reference	Reference	4.17 (5/120)	Reference	Reference	7.5 (9/120)	Reference	Reference
No	95.2 (20/21)	11.429 (1.085–120.349)	0.019*	14.29 (40/280)	0.256 (0.110–0.597)	<0.001**	42.14 (118/280)	3.414 (1.904–6.124)	<0.001**	3.21 (9/280)			15.71 (44/280)		
First colostrum feeding time															
Within 2 hr	90.9 (10/11)	-	-	1.31 (2/152)	Reference	Reference	30.92 (47/152)	-	-	3.29 (5/152)	Reference	Reference	8.55 (13/152)	Reference	Reference
Within 12 hr	85.7 (18/21)			25.8 (64/248)	272.821 (20.400–3999.999)	<0.001**	37.1 (92/248)			3.63 (9/248)			16.12 (40/248)	1.999 (1.001–3.992)	0.034*

Mannheimia haemolytica (M. haemolytica), *Pasteurella multocida* (P. multocida), *Histophilus somni* (H. somni), *Mycoplasma bovis* (M. bovis), Odds ratio (OR), Confidence interval (CI). ¹ At least one of these four pathogens detected. ² Number of positive farm/total farm. ³ Number of positive calf/total calf. *: Significant different between groups (P<0.05). **: Significant different between groups (P<0.01).

calves reported in Canada, Brazil and Europe (Denmark, Scotland, Belgium), ranging from 70.90 to 90.80% [2, 4, 43]. The possible reason would be that all calves enrolled in this study were pre-weaned dairy calves, and live in individual pens without commingling and transportation until weaned. Dairy calves in Taiwan were exposed to less pathogens and stressors that would suppress their immune system [2, 42]. On the other hand, cold stress and heat stress have been demonstrated to suppress the immune system of calves, by increasing plasma cortisol levels and reduce in serum concentrations of IgG, respectively [13, 15, 37]. These variations in immune system could impact the ability of the calf to maintain normal homeostasis with the opportunistic organism [13]. Because the climate in Taiwan ranges from tropical to subtropical with less seasonal temperature variations, the stressors from extremely hot or cold temperatures had less effects on the immune system of calves.

P. multocida was the predominant pathogen detected in respiratory tract of calves in Taiwan (34.75%), similar to Denmark, Scotland and Canada, ranging from 26 to 61% [2, 4, 17, 22, 43]. *H. somni* was also detected in Taiwan (13.25%) and were similar to the range of prevalence in Canada and Denmark from 10 to 30% [2, 4, 17, 43]. *H. somni* has been found significantly less frequently isolated ($P<0.01$) from the upper respiratory tract than the lower respiratory tract [46]. Since only NPS samples were collected in the current study, the prevalence of *H. somni* in Taiwan may be underestimated. For *M. haemolytica*, the prevalence was low (3.5%) in Taiwan when compared with the reports from other countries, which ranged between 22 to 25% [2, 4, 17, 43]. The PCR method for pathogen detection applied in this study has higher sensitivity than the method of cultivation, thus false negative results would be minimized [4]. The prevalence of *M. bovis* were found higher in feedlot studies (20–45%) when compared with those in dairy farms (0–14.2%) [2, 4, 17, 44, 46]. In this study, the prevalence of *M. bovis* (16.50%) was higher than other studies in dairy calves, which may be due to different farm management practices, especially the late first colostrum feeding (OR=272.82, $P<0.001$), that was the predominant risk factor for *M. bovis* presence in calves in Taiwan. Co-infection of respiratory bacterial pathogens has been noted in previous study in Canada, in which the same four bacteria pathogens were examined. The co-infection rate in calves was 28.42%, and *P. multocida* + *H. somni* (29.63%) and *P. multocida* + *M. haemolytica* (25.93%) were the most frequent combinations detected among the pathogen co-infected calves [17]. In the present study, *P. multocida* + *H. somni* was also the most frequent bacteria combination (42.62%), followed by *M. bovis* + *P. multocida* + *H. somni* co-infection (27.88%), while relatively low co-infection rate (15.25%) in calves was found.

The prevalence of respiratory bacterial pathogens in the present study at farm-level was not significantly different among Taiwanese regions, and only those of *M. bovis* and *P. multocida* at calf-level were significant different between regions. The results also showed the evidence that respiratory bacterial pathogens were distributed widely in dairy farms in Taiwan. Taiwan is located in the East Asia, with total area 13,976 square miles, and the climate ranges from tropical in the south to subtropical in the north [10]. As a result of small island, the climate is not much different between regions, and this may explain our findings.

BRD is a multi-factorial syndrome, with various physical and physiological predisposing factors being necessary to induce disease [33, 41]. The WI scoring system was designed for detect the clinical signs of BRD in the early stage of infection in order to provide early treatment, slow the spread of the disease and decrease economic loss [28–31]. When applying WI scoring system as the predictor of BRD in this study, 42.90% of pathogen infected calves did not show clinical signs. Therefore, for subclinical cases of BRD that harbor bacterial pathogens without clinical signs may not supposed to use WI scoring system to predict bacterial pathogens in animals. In this study, *H. somni* has been found significantly more frequent in samples from sick animals than in healthy animals ($P=0.04$), and our findings also showed that *H. somni* was significantly associated with BRD positive calves ($P<0.001$), thus suggesting particular importance of this organism as likely etiology of BRD [26].

Based on our findings, unheated colostrum was firstly reported significantly associated with the increasing respiratory pathogens presence at farm-level (OR=11.43) and *P. multocida* presence (OR=3.41) at calf-level. The unheated colostrum usually contains overload bacteria that may transmit to the calves during suckling and colonized into the respiratory tract of the calves as a result of bacterial infection or BRD [5, 19, 25]. The results at calf-level in this study showed that late first colostrum feeding (OR=272.82) and large farm size (OR=22.09) were the most important risk factors for *M. bovis* presence. Generally, the late first colostrum was associated with failure of passive immunity transfer according to the limited time for passive transfer of IgG across the enterocyte, within the first 4 hr after birth and rapidly declines after 12 hr postpartum [38]. This maybe the reason for the strong association between late first colostrum feeding and *M. bovis* presence in calves. In this study, large farm size was the risk factor for *M. bovis* presence. In Taiwan, purchasing cows and heifers from other herds were frequently either to maintain or expand the existing herd. It has suggested that cows and heifers from other herds were a key risk factor for the introduction of *Mycoplasma* spp. into a dairy herd [16, 34, 47]. A screening test for *M. bovis* and quarantine the purchased animals before they are introduced to the stock could minimize *M. bovis* infection [23]. In the present study, farms that have high occurrence of pneumonia, calves have poor responses to pneumonia treatment and need to paid higher cost for the treatment were also the risk factors for *M. bovis* presence. Although the results were based on the data from questionnaires, antimicrobial resistance of *M. bovis* may be one of the considerable reasons. *M. bovis* is lack of cell wall, and refractory to β -lactams and to all antimicrobials that target to inhibit cell wall synthesis, thus the number of potentially effective antimicrobials is limited. In addition, *M. bovis* also naturally resistant to cephalosporins, polymyxins, sulfonamides, nalidixic acid, and rifampin [27, 40]. Among the few antimicrobials for treatment of *M. bovis* infection, antimicrobial resistance by *M. bovis* to tetracyclines, macrolides, lincosamides, aminoglycosides, chloramphenicols, and fluoroquinolones has been reported and appears to be increasing [6, 27]. Therefore, it is suggested that applying antimicrobial susceptibility test to the detected *M. bovis* to improve the efficacy of treatment for *M. bovis* infection in dairy calves in Taiwan. Further study on the antimicrobial resistance of *M. bovis* would also be needed.

Unheated colostrum (OR=3.41) and waste milk feeding (OR=3.35) were the main risk factors for *P. multocida* presence. The unheated colostrum usually contains overload bacteria that may interfere with passive absorption of immunoglobulin into

the calf's circulation as a result of total protein and plasma IgG deficiency [5, 19]. Failure in immunoglobulin transfer was considered responsible for reduced resistance to bacteria and virus infection and increased mortality in calves [39]. The unheated milk is a potential source of exposure to respiratory bacterial infection such as *Pasteurella* spp., *M. haemolytica*, and *M. bovis* that can transmit to the calves during suckling [7, 8, 25]. The upper respiratory tract and the tonsillar mucosa were major site of colonization following oral transmission of these bacteria subsequent development of lower respiratory tract infection [30]. That could support our result that waste milk feeding was the main risk factor for *M. haemolytica* presence (OR=6.59). In this study, the main risk factors for *H. somni* presence included high pneumonia treatment cost (OR=2.52) and late first colostrum feeding (OR=2.00). The possible reasons would be similar to those for *M. bovis* presence.

Although *M. bovis*, *P. multocida*, *M. haemolytica* and *H. somni* usually considered as the major bacteria associated with BRD, other potential bacteria in the respiratory tract such as *Mycoplasma* spp., *Arcanobacterium pyogenes*, multiple species of the *Pasteurella*, gram-positive staphylococci and streptococci should not be overlooked. These bacteria might be associated with cases of BRD as well [21]. A better understanding of the association between these bacteria and BRD, and also their farm management risk factors, may lead to improved dairy industry in Taiwan.

For effective preventive measures, farmer education on milk and colostrum feeding are urgently warranted. Antimicrobial susceptibility test for respiratory bacterial pathogens, especially *M. bovis* and *H. somni*, should be considered to find the effective treatment for respiratory bacterial pathogens in dairy calves in Taiwan.

CONFLICT OF INTEREST. The authors declare no conflict of interest

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