Prevalence of *Salmonella* Dublin in dairy herds in Alberta, Canada

By

Rebecca Roos

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Abstract

Salmonella enterica subspecies *enterica* serotype Dublin (*Salmonella* Dublin) is a gram-negative facultative anaerobic bacteria, host adapted to cattle, but also a zoonotic pathogen (An et al., 2023; Harvey et al., 2017; Mohammed et al., 2017). Several governments of provinces in Canada have started a surveillance program among cattle on *Salmonella* Dublin, like British-Columbia, Quebec, Ontario and Alberta, since it affects reproduction and has a long term effect on milk yield in dairy cattle (Government of Québec, 2024; Janvier, 2023; O' Doherty et al., 201; Alberta Animal Health Centre, n.d.; Salmonella Dublin in BC, n.d.; T. D. Nielsen et al., 2012). The aim of the present study is to update the prevalence estimate of *Salmonella* Dublin in dairy herds in Alberta, Canada, as the previous estimates of herd-level prevalence are two years old (Shaukat et al., 2024).

The study population contains all dairy farms in Alberta, Canada. The dairy herds consist of different herd sizes and were spread across three regions in Alberta, defined by Alberta Milk in a north, central and south region. Bulk tank milk samples were collected from all active dairy herds of Alberta in June 2024 and tested for antibodies against *Salmonella* Dublin using an indirect ELISA. With the use of two different percent positive (PP%) cut-off values (PP% ≥ 35 and PP% ≥ 15) calculations were done. When using a PP% ≥ 35, the estimates were 10.3% for apparent prevalence, 0.54 for positive predictive value, and 0.86 for negative predictive value. With a PP% ≥ 15, the estimates were 20.5% for apparent prevalence, 0.67 for positive predictive value, and 0.79 for negative predictive value. The true prevalence was estimated to be 56.2% with the cut-off value PP% ≥ 35 and 38.2% using the cut-off values PP% ≥ 15%. Using cut-off value of PP% ≥ 35 no significance was found between herd size or geographic region, and ELISA test result using logistic regression. The combination of herd size and region did deliver significant results when PP% ≥ 35 was used. Medium and large size herds had a higher odds (odds ratio (OR) = 4.55; p-value = 0.03, respectively, and OR = 4.48; p-value 0.04, respectively) of getting a positive ELISA test result than small herds in the central region of Alberta. Significant result in region was found between the south (OR = 4.96; p-value 0.02) and central region for small herds. Another significant result (OR = 0.25; p-value = 0.04) was found between medium size herds in the central region and small herds in the north. Using PP% ≥ 15 significant results (OR = 1.95; p-value = 0.03) were found in herd size as bigger herds had higher odds of testing positive than small herds.

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Introduction

Infectious diseases in cattle pose a risk to animal health, public health and the agriculture industry (Cummings et al., 2018; Ministry of Agriculture and Irrigation, n.d.; Nielsen et al., 2007; Otto et al., 2018; Tablante & Lane, n.d.). The province of Alberta produced almost 9% of all Canadian milk at the end of 2022 (*Annual Report Alberta Milk*, n.d.). Cattle diseases have a significant effect on this industry by affecting cattle health and welfare, economic loss for the farmer and public health risks associated with zoonotic animal diseases (Chi et al., 2002; Davison et al., 2006).

Salmonella enterica subspecies *enterica* serotype Dublin (*Salmonella* Dublin) is an important disease among dairy cattle. *Salmonella* Dublin is a gram-negative facultative anaerobic bacteria, host adapted to cattle, but it is also a zoonotic pathogen, which means that the bacteria can be transmitted from animals to humans and causes sickness in humans (An et al., 2023; Harvey et al., 2017; Mohammed et al., 2017). Several governments of provinces in Canada have started a surveillance program among cattle on *Salmonella* Dublin, like British-Columbia, Quebec, Ontario and Alberta (Alberta Animal Health Centre, n.d.; Government of Québec, 2024; Janvier, 2023; Nobrega et al., 2024; *Salmonella Dublin in BC*, n.d.). *Salmonella* Dublin is a reportable disease in Alberta, there were however only six and two cases of *Salmonella* Dublin infection confirmed in cattle in 2021 and 2022, respectively (Alberta Animal Health Centre, n.d.). The prevalence of *Salmonella* Dublin in Alberta was 7% when 418 isolates of *Salmonella* spp. samples, collected between 1990 and 2001 from humans and animals, were tested (Guerin et al., 2005). In diseased cattle the prevalence of *Salmonella* Dublin differs between 19.8% in Alberta (2006-2014) and 72.1% in Great-Britain (2003-2008) (Carrique-Mas et al., 2010; Otto et al., 2018a). Based on Bulk Tank Milk (BTM) samples the prevalence of *Salmonella* Dublin differs from 5.1% in Ontario (2022), 20% in the first quarter (2022), 18% in the second quarter (2022) and 25% (2024) in British-Columbia and 15.6% in Alberta (2022) (BC Animal Health Centre, 2022a, 2022b, 2024; Nobrega et al., 2024; Shaukat et al., 2024). It can be concluded that *Salmonella* Dublin is an endemic disease in Alberta that remain challenging for the dairy industry (Guerin et al., 2005).

Salmonella Dublin can establish lifelong infections in cattle with an asymptomatic carrier status (Holschbach & Peek, 2018; Velasquez-Munoz et al., 2023). These so called latent carriers experience intermittent periods of bacteremia and shedding of *Salmonella* Dublin, which can be reactivated by stress or other factors, such as parturition (Counter & Gibson, 1980; Holschbach & Peek, 2018; L. R. Nielsen, 2013a; T. D. Nielsen et al., 2013; Poppe, 2011). The latent carriers can therefore be a major source of infection for other animals in the herds, humans and the environment (L. R. Nielsen, 2013a; Poppe, 2011; Velasquez-Munoz et al., 2023).

A lot of different host-specific factors determine how a *Salmonella* Dublin infection develops. Transmission between cattle is mostly via a fecal-oral spread (Holschbach & Peek, 2018; O' Doherty et al., 2015; Velasquez-Munoz et al., 2023). Studies have shown that *Salmonella* spp. cannot colonize the gut when normal microbiota is present. *Salmonella* Dublin however can survive within phagocytes with a virulence plasmid (Holschbach & Peek, 2018). *Salmonella* Dublin strains can penetrate the intestinal wall and barriers in lymphatic systems which enables the bacteria to spread and cause a systemic disease (L. R. Nielsen, 2013a; Velasquez-Munoz et al., 2023).

Salmonella Dublin can cause abortion in infected cows, even in the absence of other clinical signs (Hinton, 1977; Holschbach & Peek, 2018; L. R. Nielsen, 2013a; Velasquez-Munoz et al., 2023; Veling, 2004; Wray & Davies, 2000). The mechanism is thought to be through a septicemia, endotoxemia, and hyperthermia, which can lead to seeding of fetus and uterus that causes fetal infection and death. Abortion is possible in any stage of the gestation but is mostly seen between five and nine months of gestation (Holschbach & Peek, 2018).

In addition to abortion, other symptoms observed in adult cows can vary from fever, diarrhoea and decreased milk yield (T. D. Nielsen et al., 2010, 2013; O' Doherty et al., 2015; Velasquez-Munoz et al., 2023). Diarrhoea can however also lead to fever and cause dehydration, lethargy and death (Field, 1948; T. D. Nielsen et al., 2010; O' Doherty et al., 2015; Veling, 2004; Wray & Davies, 2000). In calves, respiratory infections, polyarthritis and dry gangrene of extremities due to cold agglutination can be observed in addition to the signs associated to the *Salmonella* Dublin infection in adult cows (Holschbach & Peek, 2018; T. D. Nielsen et al., 2010; Veling, 2004).

Therapy for calves and cows consists of administrating fluid, either orally or intravenous, depending on the dehydration state, and antimicrobial therapy (Holschbach & Peek, 2018; Velasquez-Munoz et al., 2023). Antimicrobial therapy is controversial due to concerns about the potential development of antibiotic-resistant strains, as reports of multidrug resistant (MDR) strains of *Salmonella* Dublin are increasing (Holschbach & Peek, 2018; Otto et al., 2018a).

When adult cows and calves are mostly affected by *Salmonella* Dublin is unclear. Some studies report that they are most severely affect up to six to eight weeks after calving old, but T. D. Nielsen et al. (2010) report that calves up to six months old are primarily affected (L. R. Nielsen, 2013a; Poppe, 2011; Veling et al., 2002; Wray & Davies, 2000).

Economic losses

A significant part of the Canadian dairy industry is located in Alberta. A representation of all the losses that are being described can be found in *Table 1*. When cattle contract infectious diseases, such as *Salmonella* Dublin, it leads to economic loss for the farmer due to death, abortion and decreased milk yield (L. R. Nielsen & Dohoo, 2012; T. D. Nielsen et al., 2010; O' Doherty et al., 2015).

When *Salmonella* Dublin is newly introduced in a herd there is a long-term effect on milk yield production. When *Salmonella* Dublin does not cause clinical symptoms, it was estimated that parity one cows had a decrease of 1.4 kg milk per day, parity two cows did not have a significant decrease milk yield, and parity three cows or more had a decrease of 3.0 kg per day. The largest reduction in milk production varied by parity: for parity one cows it was between ten and fifteen months; for parity two cows it was between thirteen and fifteen months; for parity three cows or more it was between seven and fifteen months (T. D. Nielsen et al., 2012, 2013). The difference between the parity and the yield loss illustrates that the effect of a *Salmonella* Dublin infection differs between cows of different ages. The total estimated loss of milk yield in a 100-cow herd is 40,000 kg in the first year after a new, non-clinical *Salmonella* Dublin infection (T. D. Nielsen et al., 2012). A clinical *Salmonella* spp. outbreak in a dairy herd of 100 cows leads to a loss of 19,430 liter milk production over two months after the outbreak, which was estimated to cost £35.97 (CA $60) per cow. Loss of aborted fetuses was estimated to be £600 (CA $1030) for a dairy herd of 100 cows (Bazeley, 2006). There are studies that report that the milk yield loss varies between herds, which makes an estimate for the milk yield loss by *Salmonella* Dublin over all herds in Alberta unreliable. This does not mean that milk yield loss should not be considered when looking at the effects of *Salmonella* Dublin in Alberta, as it does affect the income of the farmer (T. D. Nielsen et al., 2012).

A *Salmonella* Dublin infection also leads to a loss in reproductive performance in cows. A study by O' Doherty et al. (2015) reports that unvaccinated herds, positive for exposure to *Salmonella* spp., produces 3% fewer calves per cow per year when compared with unvaccinated negative herds. There is furthermore a 1.5% greater rate of calf mortality, leading up to an estimated annual loss of €94 (CA $140) per cow when unvaccinated herds are exposed to *Salmonella* spp. (O' Doherty et al., 2015).

Lastly the costs for veterinary service are considered. A median cost for a veterinary visit, excluding drugs, is estimated to be CA $100 (Aghamohammadi et al., 2018). There is however no

information known about the frequency of need for veterinary service in regard to a *Salmonella* Dublin infection.

Financial loss can also be related to management and herd size. According to T. D. Nielsen et al. (2013) financial losses are largest in the first year after infection and when management is graded as poorer and herd size gets bigger. For a herd of 200 cows with good management the loss is estimated to be €49 (CA $73) for the first year and €8 (CA $12) annually over ten years after a herd infection. For a herd of 200 cows with poor management the average loss is estimated to be €326 (CA $490) during the first year and €188 (CA $282) annually over ten years after a herd infection (T. D. Nielsen et al., 2013). Another estimate of the financial loss associated with a *Salmonella* outbreak in an unvaccinated herd of 100 dairy cows is a loss of income of €633 (CA $950) over 305 days compared to an unvaccinated, negative herds. When comparing an unvaccinated herd of 100 dairy cows experiencing a new *Salmonella* outbreak to a vaccinated negative herd of the same size, the former generates generates €1,376 (CA $2,050) less income over a period of 305 days (O' Doherty et al., 2015). The financial loss of a *Salmonella* outbreak is contingent on different factors and a general income loss estimation for *Salmonella* Dublin for dairy herds in Alberta is unreliable based on current studies.

Table 1: overview of estimated costs associated with *Salmonella* Dublin (Aghamohammadi et al., 2018; Bazeley, 2006; T. D. Nielsen et al., 2012, 2013; O' Doherty et al., 2015)

Economic Loss	**Dependence**	**Estimated amount**
Milk production reduction	Milk price Herd	CA $60/cow
Aborted Fetus	Price per calf	CA $1,030/100 cows
Less calf production	Price per calf Vaccination	3% less
Calf mortality	Vaccination	1.5% more
Veterinary Service	How often farmers call for veterinary service in regard to *Salmonella* Dublin infection Prevalence and severity of *Salmonella* Dublin	CA $100
General income loss	Management Herd Size Vaccination	Between CA $73/200 cows and CA $2050/100 cows in the first year

Public health risk

Salmonella Dublin poses a threat to public health causing severe bloodstream infections. It rarely causes clinical disease in humans, but when it does it is a severe disease that requires antimicrobial drug therapy (An et al., 2023; Harvey et al., 2017; Velasquez-Munoz et al., 2023). A study done in the United States by Harvey et al. (2017) reported that the incidence rate of *Salmonella* Dublin in humans has been steadily rising since 1986. The research, conducted up until 2013, found that there was one *Salmonella* Dublin infection in 1,2 million people in 2013, marking a 7.6-fold increase compared to 1968, when the rate was one infection in 18,2 million people. Incidence rates for other *Salmonella* spp. infections have remained relatively stable, with one infection in 10,5 thousand people in 1968 compared to one infection in 8,9 thousand people in 2013 (Harvey et al., 2017). In Denmark *Salmonella* Dublin is the fourth most common serotype to be isolated from diseased humans (T. D. Nielsen et al., 2012). People at or over 65 years of age are more susceptible to *Salmonella* Dublin infections, as 38% of the infections occur in this age group. Only 7% of infections occur in children younger than 5 years of age. *Salmonella* Dublin is isolated from blood in 60% of

affected individuals and 75% of people infected with *Salmonella* Dublin have to be hospitalized for a median of six days, while 4% of the people infected with *Salmonella* Dublin die (Harvey et al., 2017). A study in Denmark claims that *Salmonella* Dublin has led to higher mortality rates in humans than other *Salmonella* serotypes (T. D. Nielsen et al., 2012).

A human infection with *Salmonella* Dublin is often caused by indirect contact through foodborne exposure, for example by the consumption of raw milk or raw beef (Fossler et al., 2005; Harvey et al., 2017; Holschbach & Peek, 2018; Mangat et al., 2019; Velasquez-Munoz et al., 2023). Raw milk can contain *Salmonella* as the bacteria is often shed by milk, fecal contamination, or both (Holschbach & Peek, 2018; Veling, 2004; Wray & Davies, 2000). It is reported that in the United States 99% of *Salmonella* Dublin outbreaks can be traced back to dairy sources (Mangat et al., 2019). Farmers and veterinarians can also get infected with *Salmonella* Dublin after direct contact with diseased cattle (Velasquez-Munoz et al., 2023; Veling, 2004; Wray & Davies, 2000). It is suggested that veterinary surgeons can act as a vector of *Salmonella* Dublin if they do not wear polyethylene gloves (Williams, 1980). These reasons highlight the importance of both proper food handling and good hygiene practices for humans to mitigate the risk of *Salmonella* Dublin transmission.

The biggest threat for human health is the development of multi-drug resistant (MDR) strains of *Salmonella* Dublin. MDR strains of *Salmonella* Dublin are found in Canada, USA and the Netherlands (Harvey et al., 2017; Mangat et al., 2019; Veldman et al., 2023). Transmission of MDR can be associated with direct handling of infected animals or environmental exposure on farms. It is also reported that *Salmonella* Dublin has IS*26* activities in plasmid, which can facilitate a host range expansion (Mangat et al., 2019). The MDR development in *Salmonella* Dublin poses a risk to both cattle and human health, as treatment options for both humans and cattle are getting limited. Most importantly, the resistance to critically important beta-lactam antimicrobials in *Salmonella* Dublin have been found to be moderate to high (Otto et al., 2018b). MDR is associated with more severe illness, bloodstream infection, hospitalization and death in humans. The proportion of resistant isolates is higher in *Salmonella* Dublin than in other *Salmonella* serotypes (Harvey et al., 2017).

Control of endemic diseases

Endemic diseases, like *Salmonella* Dublin in Alberta, have a great impact on animals and farmers. The general way to control endemic diseases is by setting up a surveillance program, usually with bulk tank milk (BTM) samples, together with an eradication or control program. Control programs have been successful in controlling several diseases in Northern Europe, including leptospirosis, bovine leukosis and tuberculosis (Barkema et al., 2015a; Leblanc et al., n.d.). The goal of a control program for *Salmonella* Dublin in Alberta should be to follow trends in the prevalence, which can be done based on previous control programs from Denmark, the Netherlands and Sweden (Ågren et al., 2016; L. R. Nielsen et al., 2021; Santman-Berends et al., 2021; Van Schaik et al., 2002). A control program will not make Alberta free of *Salmonella* Dublin, since the bacteria can survive in the environment, humans can act as a vector and latent carriers can lead to self-containing herds (Counter & Gibson, 1980; L. R. Nielsen & Dohoo, 2012; Velasquez-Munoz et al., 2023; Veling, 2004; Williams, 1980). Control programs are successful when a large number of the milk producers participate in programs, which is usually when both individual producers and the whole dairy industry gets benefits in return for the time and effort it takes. Support of these programs by veterinarians is critical to implement the program successfully (Barkema et al., 2015). In the Netherlands a control program is set up for *Salmonella* Dublin to follow the prevalence in cattle herds. Initially the prevalence went up based on surveys, but the monitoring program since 2012 showed a downward trend. The prevalence of *Salmonella* Dublin is currently estimated to be less than 5% in Dutch dairy herds (Santman-Berends et al., 2021). A national surveillance and eradication program for *Salmonella* Dublin was set up for dairy herds in Denmark, where the prevalence was high (26%). The program

also proved to be successful as the prevalence was drastically reduced to 10%. Previously infected herds have a higher chance of getting new infection events of *Salmonella* Dublin, since the bacteria survives well in the environment (L. R. Nielsen & Dohoo, 2012). Therefore, ensuring sufficient biosecurity measures and identifying persistently infected carrier animals are crucial when establishing a *Salmonella* Dublin control program in Alberta (L. R. Nielsen & Dohoo, 2012). An effective disease control program for *Salmonella* Dublin in Alberta for dairy cows can be set up using the models that have been used in, among others, Denmark and the Netherlands (Santman-Berends et al., 2021; Stockmarr et al., 2013; Veling, 2004)

Knowledge gap

The aim of the present study is to update the prevalence estimate of *Salmonella* Dublin in dairy herds in Alberta, Canada, and also to relate testing positive to geographic region and herd size, as the previous estimates are two years old (Shaukat et al., 2024). The study, completed in 2022, looked at the prevalence of *Salmonella* Dublin in BTM samples of all Alberta farmers. BTM samples were collected at four time points: December 2021, April 2022, July 2022 and October 2022 (Shaukat et al., 2024). A logical continuation of this work, to set up a control program, is to estimate the within herd prevalence of *Salmonella* Dublin in infected dairy herd. As the previous estimates of infection state are two years old, it is crucial that a fresh round of BTM testing is performed to update the herd status in terms of *Salmonella* Dublin positivity (Kent et al., 2021; L. R. Nielsen, 2013b; Veling, 2004). This is furthermore important due to the continuously changing population of dairy herds in Alberta with some farms moving out of business and new producers starting up dairy farms. Not much is known about the infection dynamics of the within-herd seroprevalence of *Salmonella* Dublin and as L. R. Nielsen, et al. (2007) point out, it can be reasonable to assume that in endemically infected herds, small outbreaks occur over time intermittently. By assessing the current herd status for *Salmonella* Dublin, the study of Shaukat et al. can continue with farms that test positive and start looking at the within-herd seroprevalence of *Salmonella* Dublin and associated risk factors.

Material and Methods

This study was approved by the Animal Care Committee (AC21-0070) of the University of Calgary in Alberta, Canada. Literature search can be found in *Attachment 2*.

Study Population

The study population consists of all dairy farms in Alberta, Canada. Since all herds were sampled, a sample size calculation was not warranted. Dairy herds were located all over Alberta and consisted of different herd sizes. Difference in region was defined by Alberta Milk in a north, central and south region (Annual Report Alberta Milk, n.d.).

Sample Collection

A 40 milliliter BTM sample was collected by milk collectors as an extra sample during routine milk collection from active dairy producers in Alberta in June 2024 with collaboration of Alberta Milk. Samples were well mixed and carefully obtained from each tank, following standard operating procedure, advised by Alberta Milk for routine BTM sample collection. Samples were kept at Alberta Milk in Edmonton at 4°C and collected by researchers and shipped at 4°C to the laboratory at the Faculty of Veterinary Medicine of the University of Calgary. Samples were stored in the freezer at -20°C until further sorting and testing.

Salmonella Dublin ELISA

The frozen whole milk samples were thawed in the fridge overnight and tested using an indirect ELISA for detection of antibodies against *Salmonella* Dublin in milk. The sensitivity for the test, using the cut-off value from the manufacturer (PP%≥ 35) is 16.3% and the specificity is 97.5% (Um et al., 2022). The sensitivity for the test, using cut-off value PP%≥ 15 is 40.6% and the specificity is 91.9%. The ELISA test was performed according to the manual of the manufacturer, which can be found in *Attachment 1* (Fisher Scientific, n.d.). Briefly, the indirect ELISA tests for the presence of antibodies against *Salmonella* Dublin lipopolysaccharide (LPS) O-antigen (L. R. Nielsen & Ersbøll, 2004; Um et al., 2022). Plates were coated with purified LPS, to detect LPS O-antigen 1, 9 and 12. The optical density (OD) was measured at 450 nm in 15 minutes (Hoorfar et al., 1994; Shaukat et al., 2024). The corrected OD per sample was calculated by subtracting the average negative control OD of that plate off the sample OD_{450} value. The percentage positivity (PP%) was calculated as follows:

$$PP\% = \left(\frac{Corrected\ OD\ samples}{Average\ corrected\ OD\ positive\ Controls} \times 100\right) - 10 \quad (1)$$

Data Management

Data on geographical region (north, central, south) and herd size were obtained from Alberta Milk. Herd size was calculated as described by Shaukat et al. (2024) since no information about the herd size in animal numbers was available from Alberta Milk, but information about milk delivered was available. Herds were categorized into three herd size categories: small (≤3600 L/day milk delivered), medium (3600-7200 L/day milk delivered), and large (>7200 L/day milk delivered) (Shaukat et al., 2024).

Statistical Analyses

All data was first put into Microsoft Excel and then imported into R-studio. Statistical analyses were performed with a p-value <0.05 considered as statistically significant.

First the apparent prevalence (AP) of *Salmonella* Dublin on all dairy herds was calculated by dividing the number of farms that tested positive by the total number of farms tested. As the sensitivity and specificity of the test with the cut-off value PP% ≥ 35 and PP% ≥ 15 were known, this could be used to make an estimate of the true prevalence (TP) using the Rogan-Gladen estimation of True Prevalence (Rogan & Gladen, 1978). This was done using the formula:

$$TP = \frac{(AP + Sp - 1)}{(Se + Sp - 1)} \quad (2)$$

where *Sp* is the specificity of the test used and *Se* is the sensitivity of the test used. The standard error of TP (SE(TP)) was calculated with the following formula:

$$SE(TP) = \sqrt{\frac{AP(1 - AP)}{nJ^2}} \quad (3)$$

where *n* is the total amount of animals tested and in which J is the Youden's index which is calculated as follows:

$$J = Se + Sp - 1 \quad (4)$$

These formulas were used in Excel Sheet to calculate the true prevalence and standard error.

Using the calculated prevalence with cut-off value of PP% ≥ 35 (15.5%) and PP% ≥ 15 (28.3%) as tested by Shaukat et al. (2024), the positive predictive value (PPV) and negative predictive value (NPV) were estimated in Excel, using the formulas (Thrusfield, 2008):

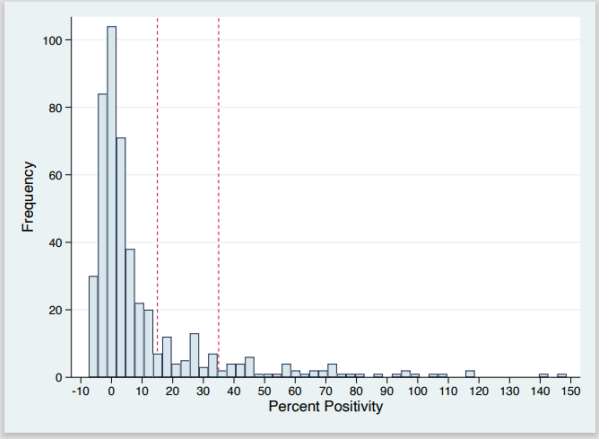
$$PPV = \frac{Se \times prevalence}{(Se \times prevalence) + [(1 - Sp) \times (1 - prevalence)]} \quad (5)$$

$$NPV = \frac{Sp \times (1 - prevalence)}{[(Sp \times (1 - prevalence)] + [(1 - Se) \times prevalence]} \quad (6)$$

Logistic regression was done in R-studio to determine the association of *Salmonella* Dublin with the geographic region and the herd size for the cut-off value of PP% ≥ 35 and PP% ≥ 15. First a reduced model was used where the Chi-square test is performed to determine if there is a difference between observed and expected frequencies. After that an ANOVA test was performed to test the likelihood ratio between the reduced model and the full model. Then the p-value was extracted from the result for the comparison between full model and reduced model. If the p-value was significant (<0.05) it meant that the full model had to be used. The full model was a logistic regression model that predicts the probability of the binary outcomes (test results) based on one or two predictor variable (geographic region and herd size). The model coefficients were than transformed from log scale to odds ratio scale and then 95% confidence intervals for the coefficients and odds ratio were calculated. The code that was used in R-studio can be found in *Attachment 5*.

Results

The PP% value ranges from -7.40 to 148.01, this range is visualized in *Histogram 1* using Stata. Both the cut-off value of PP%≥ 35 and PP%≥ 15 are visualized in the histogram.



Histogram 1: visualization of the PP%-values in the data set. The red dotted lines show the cut-off value of PP% ≥ 15 and PP% ≥ 35.

The apparent prevalence is estimated to be 10.3% with a cut-off value of PP% ≥ 35 and the true prevalence is estimated to be 56.2% with a standard error of 0.10. With the cut-off value of PP% ≥ 15 the apparent prevalence is estimated to be 20.5% and the true prevalence is estimated to be 38.2% with a standard error of 0.06. Using cut-off value PP% ≥ 35, the apparent prevalence in the north, central and south regions is estimated to be 6.7%, 11.7% and 12.2% respectively, while the true prevalence is estimated to be 30.2%, 66.6% and 70.6% , respectively. On the other hand, using the cut-off value of PP% ≥ 15, the apparent prevalence in the north, central and south regions is estimated to be 17.5%, 19.9% and 25.2%, respectively, while the true prevalence is estimated to be 28.9%, 36.3% and 52.5%, respectively.

The PPV is calculated to be 0.54 when the cut-off value of PP% ≥ 35 is chosen, this means that 54% of herds that are testing positive can be considered truly positive. The NPV is calculated to be 0.86, this means that approximately 86% of herds that test negative are truly negative, while 14% of the negative herds are likely to be false negatives. When the cut-off value of PP% ≥ 15 is used, the PPV is calculated to be 0.67, which means that 67% of the positive herds will be truly positive. The NPV is calculated to be 0.79, which means that 79% of the negatively tested herds are truly negative, while 21% of the negative herds may be false negatives.

The statistical test results with the cut-off value of PP% ≥ 35 are visualized in tables in *Attachment 3*. No significant association is found between geographic region and ELISA test result with the cut-ff value of PP% ≥ 35 (p-value = 0.20). No significant result is found between herd size and ELISA test result (p-value = 0.28). Therefore the logistic regression model is fit to combine both

geographic region and herd size. This combination provides significant results. Medium and large herd sizes have higher odds (odds ratio (OR) = 4.55; p-value = 0.03, and OR = 4.48; p-value 0.04, respectively) of testing positive on ELISA for *Salmonella* Dublin than a small herd in the central region, however there is no significant difference between small and large herds in north and south regions. The south region has higher odds (OR = 4.96; p-value = 0.03) of testing positive compared to the north region for small herds. The central small herds have higher odds (OR = 3.97; p-value = 0.04) of getting a positive ELISA test than a medium herd in the north.

Statistical test results with cut-off value of PP% ≥ 15 are visualized in tables in *Attachment 4*. When the reduced model is used for geographic region and ELISA test result, there is no significance (p-value = 0.26). The result of the reduced model for herd size and ELISA test result is significant (p-value = 0.04), meaning a full logistic regression model is indicated. Large herds have higher odds (OR = 1.95; p-value = 0.03) of testing positive than small herds.

Discussion

Prevalence

The prevalence of *Salmonella* Dublin based on BTM samples differs across different countries and regions. The observed prevalence in the current study will be compared with other data on the prevalence found in other countries and regions. In Ireland a true prevalence, based on the Rogan-Gladen estimator, of 49% was found in 2009 and 32.3% between 2018 and 2020 in dairy herds. Both studies used a different cut-off value of PP% ≥ 70 and PP% ≥ 34 respectively. Using cut-off value PP% ≥ 35 this study finds a true prevalence of 56.2%, which is higher than the other studies found. This can be attributed to the widespread vaccination of dairy herds in Ireland against *Salmonella* Dublin. In 2009, 49% of tested dairy herds were vaccinated for *Salmonella* Dublin and this increased to 83.4% between 2018 and 2020 (McCarthy et al., 2021; O'Doherty et al., 2013). In Great-Brittain a study found an apparent prevalence of 25% based on BTM samples between 2016 and 2020 using cut-off value PP% ≥ 25. This prevalence estimate is higher than the apparent prevalence estimates in the current study using cut-off value PP% ≥ 15 (20.5%) or PP% ≥ 35 (10.3%). This difference in prevalence may be explained by an average bigger herd size of 163 cows, which can influence the spread of *Salmonella* Dublin within a herd. The sensitivity of the ELISA test of Henderson et al. is higher than the current study, since BTM samples were collected on four different timepoints. An estimation of the prevalence in Estonia between 2019 and 2020, using cut-off value PP% ≥ 35 is 24.2%, which is higher than this study found (10.3%). This disparity may be explained by the fact that the Estonian study included only herds with a minimum size of 100 cows, while this study included all dairy herds in the province. Exclusion of dairy herds can influence the prevalence of *Salmonella* Dublin observed in the study (Mõtus et al., 2021).

Several studies on *Salmonella* Dublin have been conducted in different provinces in Canada. The apparent prevalence in Ontario, using cut-off value PP% ≥ 15 in 2021 is 25% to 7.5%. The apparent prevalence of 25% is based on 100 dairy herds and not all dairy herds, which is true for the apparent prevalence estimate of 7.5%. The apparent prevalence found in the current study with cut-off value PP% ≥ 15 based on all dairy herds, is 20.5%, which is a lot higher than the estimate found in Ontario. The true prevalence in Ontario is estimated to be 5.1%, while it is estimated to be 38.2% in Alberta in the current study. The prevalence in Alberta is expected to be similar to the prevalence measured in Ontario, however the prevalence in Ontario is lower. This may be explained by repetitive sampling in Ontario, which increases the overall sensitivity of the test (Nobrega et al., 2024). The apparent prevalence in British-Columbia, using cut-off value PP% ≥ 35 is estimated to be 20.3% in the first quarter of 2022, 18% in the second quarter of 2022 and 25% in 2024 based on BTM samples (BC Animal Health Centre, 2022b, 2022a, 2024). The apparent prevalence that is found in the current

study, using cut-off value PP% ≥ 35, is 10.3%, which is lower than the results found in British-Columbia. When the cut-off value is lowered to PP% ≥ 15, the apparent prevalence in the first quarter of 2022 is estimated to be 34.9%, which is higher than this study found with PP% ≥ 15 (20.5%). The cause for this difference can be based on several factors, such as time of BTM collection, difference in herd size and difference in climate between the provinces, however this information is not known. The overall apparent prevalence in Alberta, using cut-off value PP% ≥ 35 is estimated to be 15.6% based on four BTM samples, which is higher than the current study estimates (10.3%). If the cut-off is changed to PP% ≥ 15, the apparent prevalence changes to 28.2% for Shaukat et al. (2024) compared to 20.5% in the current study. This may be explained by an overall enhanced sensitivity for Shaukat et al. since BTM samples were collected on four different occasions.

Region

The current study finds no association between geographic region and ELISA test result even though Shaukat et al. (2024) report that in South Alberta a higher prevalence of *Salmonella* Dublin is found when compared to the north region. This observation is consistent with the increased number of positive ELISA test results reported in South Ontario (Nobrega et al., 2024). In the Netherlands an association is found between geographic region and higher prevalence of *Salmonella* Dublin in the north (Van Schaik et al., 2002). This is suggested to be linked to issues with paratyphoid infections in cattle from 1919 until 1992, since the study was done in 1992 the influence of paratyphoid infection on the current prevalence is unknown (Visser et al., 1992). The lack of a significant association between region and positive ELISA test results in the current study may be due to the fact that BTM samples are collected only once during the summer, while other studies collected BTM samples more than once and in different months (Nobrega et al., 2024; Shaukat et al., 2024).

Herd Size

It is suggested that herd size is an important risk factor with regards to infection with *Salmonella* Dublin on dairy farms and can therefore influence the results found in the current study (Fossler et al., 2005; Vaessen et al., 1998). A surveillance program in Denmark looked at the effect of herd size on *Salmonella* Dublin test results, herds were classified based on the number of cows. It is reported that if the herd is bigger, that the chance of testing positive for *Salmonella* Dublin after testing negative for *Salmonella* Dublin, is higher (L. R. Nielsen, Warnick, et al., 2007). The current study does not find a significance between herd size and testing positive using cut-off value PP% ≥ 35. When the cut-off value PP% ≥ 15 is used, a significance is found between herd size and ELISA test result. The association that is found in the current study with the cut-off value of PP% ≥ 15 is similar to what L. R. Nielsen, Warnick, et al. (2007) report, bigger herd size have a higher change of testing positive for *Salmonella* Dublin. Another study in Alberta, by Shaukat et al., also found an association between large herd size and testing positive for *Salmonella* Dublin on ELISA using the same definition for herd size. It therefore seems that herd size is indeed an important risk factor in regards to an infection with *Salmonella* Dublin on dairy farms.

Something that is not considered in the current study since no information was collected in this stage of the study but is reported by Warnick et al. (2006), is that there can be an association between herd size and management. This can influence the biosecurity and therefore the exposure risk of a dairy herd and have an influence on the prevalence that is found in the current study (L. R. Nielsen, Warnick, et al., 2007). The influence of management can explain why there is no significant association between herd size and ELISA test result using cut-off value PP% ≥ 35, while if management can be considered, a significant association perhaps can be found.

Time of collection

BTM samples from all the farms were collected in June 2024. The month in which BTM is collected, might be associated with a higher prevalence of *Salmonella* Dublin as some studies report a clear seasonal presentation, especially in late summer and autumn (Carrique-Mas et al., 2010; Davison et al., 2006; Fossler et al., 2005a; L. R. Nielsen & Dohoo, 2012, 2013; O' Doherty et al., 2015). Other studies report that there is no seasonal presentation and that there is no distinct pattern between testing positive for *Salmonella* and calving season or recovery from a *Salmonella* Dublin infection (Guerin et al., 2005; O'Doherty et al., 2013). Studies reporting that there is no seasonal influence give different reasons for why there is no seasonal influence, often based on under-reporting (Guerin et al., 2005).

The apparent prevalence that is found in this study is considered as an underestimation of the true prevalence of *Salmonella* Dublin based on time collection. When the prevalence on BTM samples in Alberta is followed over the period of a year, the apparent prevalence, using cut-off value PP% ≥ 15 is 28.8%, which is higher than found in the current study (20.5%) (Shaukat et al., 2024). The apparent prevalence estimated is therefore an underestimation of the actual prevalence of *Salmonella* Dublin on dairy farms in Alberta, since no calculation can correct for the influence and no test is done to test the influence of seasonality in the current study.

Test error

Clinical and pathology signs are often not specific enough to diagnose current *Salmonella* infections as they can be latent, which is why antibodies in milk can be used to determine the infection state (Ågren et al., 2018; Kent et al., 2021). In herds and regions where probability of infection is higher, for example with a history of confirmed *Salmonella* infection which is true in Alberta, the positive predictive value (PPV) of test results is crucial to determine the reliability of the test (Ågren et al., 2018;Um et al., 2022). The PPV on ELISA BTM sample testing is calculated to be 0.67 with the cut-off value of PP% ≥ 15, this is higher than with the cut-off value of PP% ≥ 35. The cut-off value to determine *Salmonella* Dublin infection in herds in Alberta for this study is therefore chosen to be PP% ≥ 15.

In endemic settings, like in Alberta, BTM testing can point out the true infection state as it has a high specificity and it is easy to obtain (Henderson et al., 2022; Nobrega et al., 2023; Veling et al., 2002). As BTM samples can be collected during routine milk collection, it is a time and cost efficient method, which is why ELISA on BTM is done in this study (Veling et al., 2002). There are however some sidenotes to be made. The sensitivity of ELISA testing for *Salmonella* Dublin is low, meaning that BTM at a single timepoint underestimates the true prevalence since it will only come back positive if one or more lactating cows have a serologic response to *Salmonella* Dublin that is strong enough to be detected (Cummings et al., 2018b; Nobrega et al., 2023, 2024; Perry et al., 2023; Veling et al., 2002). BTM testing will furthermore underestimate the true prevalence of the entire herd as nonlactating cattle, like heifers, dry cows and calves, are not sampled (Nobrega et al., 2023). It is also important to note that some cross-reactivity with other *Salmonella* strains, such as *Salmonella* Typhimurium, can happen as they have the same O-antigen factors. The prevalence estimate in this study, based on a single BTM sample, is an underestimation of the true prevalence. The estimated true prevalence is expected to be a closer representation of the actual prevalence of *Salmonella* Dublin in dairy herds in Alberta.

It is suggested that BTM ELISA in combination with serology of four to six months old calves has a high sensitivity (99%), which is beneficial, since the sensitivity of BTM ELISA is 16.3% (with PP% ≥ 35) or 40.6% (with PP% ≥ 15). Culture methods on BTM is another alternative, but the downside to this method is that it has a lower sensitivity when it is compared with serological methods and

therefore not preferred. Fecal sample testing is also a way to test for the prevalence of *Salmonella* Dublin as they have an almost perfect specificity, but the sensitivity is lower than ELISA on BTM samples (Ågren et al., 2018; L. R. Nielsen, 2013a). Another difficulty with fecal samples testing is that *Salmonella* Dublin is shed intermittent, which lowers the sensitivity even more (Holschbach & Peek, 2018; L. R. Nielsen, 2013a; T. D. Nielsen et al., 2013; Poppe, 2011). Additionally, two to twelve weeks after infection, fecal culture results can be negative, even though the animal still carries the bacteria (Veling, 2004). Therefore, fecal sampling is not done in this study, but will be combined with individual blood and milk samples later as a continuation of this study.

Statistical test

Working with binary outcomes and independent data a different model than logistic regression can be used. A Poisson regression with robust variance can be used to calculate the prevalence ratios. This model assumes that the outcome has a Poisson distribution and is more appropriate when there is a time interval over the collection of BTM samples (Bielefeldt et al., 2011; Fonseca Martinez et al., 2017). As the BTM samples collected in this study do not meet these requirements, the Poisson regression is not used. The Rodan-Gladen estimation of true prevalence is appropriate for this study as it uses the known specificity and sensitivity (Habibzadeh et al., 2022; McV Messam et al., 2008; Nobrega et al., 2024).

To calculate the correlation between the ELISA test result, and region and herd size, a logistic regression test is used. One of the conditions is that when a table is made, at least five records are in each cell. With the broader model of logistic regression for the combination between geographic region and herd size, this is not true. When tables are made for each variable separately however, this condition is met and the result of the logistic regression can be used and interpreted (Thrusfield, 2008). An alternative model to use for the correlation is the chi-square test of independence, which is used to compare the full and reduced model (McHugh, 2013). Another model that can be used is the Fisher's exact test, but it is best to use it when more than 20% of the cells have an expected frequency of less than five, which is not true in this study and therefore this model was not used (Kim, 2017; McHugh, 2013).

The recommendation for cut off-value of the manufacturer is PP% ≥ 35, which means that a PP% ≥ 35 is considered positive and PP% < 35 is considered negative (Shaukat et al., 2024; Um et al., 2020). With the cut-off value of PP% ≥ 15 the sensitivity changes from 16.3% to 40.6% and the specificity changes from 97.5% to 91.9%. The PPV increases as well from 54.5% to 67.0%, the NPV drops down from 86.4% to 79.3%. The enhance in sensitivity and PPV is significant and as the goal of this test is to find out which farms have to be included in further studies, the cut-off value PP% ≥ 15 is recommended to be used in studies similar to this one.

Latent carriers

There are three types of carriers of *Salmonella* Dublin after an infection: active carriers, latent carriers and passive carriers (Veling, 2004). Active carriers shed *Salmonella* Dublin permanent for years, if not their whole life (Sojka et al., 1974; Wray & Davies, 2000). Latent carriers shed the bacteria periodically, especially after stress, like parturition, and can be the origin of outbreaks in self-contained herds (Counter & Gibson, 1980; Velasquez-Munoz et al., 2023; Veling, 2004). Passive carriers excrete the bacteria if there is an active carrier present (Veling, 2004; Wray & Davies, 2000). Both active and latent carriers of *Salmonella* Dublin may produce congenitally infected calves, most of these will be stillborn, but some may survive to infect other animals or become carriers (Wray & Davies, 2000). Latent carriers and passive carriers can influence the outcome of ELISA testing on one time collected BTM samples for the prevalence, as is done in this study. It is therefore important that

the within-herd prevalence of *Salmonella* Dublin is determined in dairy herds in Alberta to determine this influence on the current estimated prevalence.

Other pathogens

It is shown that young cattle that are infected with liver fluke, i.e. *Fasciola hepatica* are more susceptible to an infection with *Salmonella* Dublin. It is also stated that infected animals excrete *Salmonella* Dublin for a longer period and that the bacterium is more common on fluke-infected farms (Carrique-Mas et al., 2010). The association between sicknesses can be explained by common risk factors and mutual control methods that can be effective in reducing overall risk of infection (Mõtus et al., 2021). Another study however reports no association between a positive result for one pathogen and a positive result for another pathogen (O'Doherty et al., 2013). As this study does not test for other sicknesses in the herds it cannot be excluded that other infections might have had an influence on the estimated prevalence.

Further Research

More research needs to be done to set up a good surveillance and control program for *Salmonella* Dublin for dairy cows in Alberta, Canada. The combination between BTM ELISA and serology on specific age groups needs to be tested to determine the *Salmonella* Dublin state of a herd and the within-herd prevalence (Veling et al., 2002). As stated by Kent et al. (2021) culling decisions and enhanced biosecurity efforts are best described in terms of controlling and eliminating *Salmonella* Dublin from persistently infected herds. As is stated in the introduction the milk yield loss and economic effect of an infection in a herd with *Salmonella* Dublin is unreliable (T. D. Nielsen et al., 2012). Further research should be focused on assessing the economic losses of a *Salmonella* Dublin infection in a herd.

Conclusion

The goal of this study is to determine the up to date herd-level true prevalence of *Salmonella* Dublin on dairy farms in Alberta and relate the ELISA test result to geographic region and herd size. This study concludes that the true prevalence of *Salmonella* Dublin on dairy farms in Alberta is 56.2% with a PP% ≥ 35 and 38.2% with a PP% ≥ 15. For cut-off value PP% ≥ 35 the NPV is estimated to be 86.4% and the PPV to be 54.5%. For cut-off value PP% ≥ 15 the NPV is estimated to be 79.3% and the PPV to be 67.0%. Bigger herds have higher odds for testing positive for *Salmonella* Dublin on BTM sample testing using cut-off value PP% ≥ 15. When using cut-off value PP% ≥ 35 geographic region and herd size should be combined as a factor of influence on the BTM ELISA test results. The cut-off value of PP% ≥ 15 therefore highlights a difference in herd size between herds in Alberta, that was not found when the cut-off value of PP% ≥ 35 is used.

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Attachments

Attachment 1 ELISA-Test

appliedbiosystems INSTRUCT

PrioCHECK™ Salmonella Dublin Ab Strip Kit

ELISA for *in vitro* detection of antibodies against *Salmonella* in milk of cattle

Catalog Number 7610640 Pub. No. MAN0013903 Rev. A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support.**

WARNING! POTENTIAL BIOHAZARD. Read the biological hazard safety information at this product's page at **thermofisher.com.** Wear appropriate protective eyewear, clothing, and gloves.

Introduction

Salmonella infections in cattle can cause serious economical and welfare losses in the cattle Industry. Infection can be transmitted to humans by the consumption of infected meat or dairy products and cause severe health problems or even death. Infections caused by *Salmonella* strains belonging to serotypes B, C1 and D are the most frequent occurring and serious infectious. *Salmonella* Dublin (serotype D) is adapted to cattle and unlike most other types of *Salmonella* bacteria, has the tendency to persist in herds for decades. Additionally *Salmonella* Dublin infections in humans are extremely invasive, and when compared to other *Salmonella* infection, mortality rate is high. In order to control the infection in infected herds it is necessary to cull the carriers and prevent production of new carriers. In Europe *Salmonella* programs to control infections in swine, poultry and eggs are already implemented or in the process of being implemented. Additionally some countries already implemented control programs for *Salmonella* in cattle. The Applied Biosystems™ PrioCHECK™ Salmonella Dublin Ab Strip Kit originates from the Danish Veterinary Institute and has been successfully applied in the control program for *Salmonella* in Denmark since 2002.

The PrioCHECK™ Salmonella Dublin Ab Strip Kit can be used to specifically detect infections caused by *Salmonella* Dublin, however cross reaction because of the O-antigen factors 1, 9 and 12 will occur. The test is suitable for large-scale screening of serum and (bulk) milk samples.

Test principle

The PrioCHECK™ Salmonella Dublin Ab Strip Kit is an indirect ELISA for the detection of *Salmonella* antibodies in cattle directed against *Salmonella* Dublin and detects antibodies against *Salmonella* polysaccharide LPS O-antigens 1, 9 and 12. Plates are coated with the purified LPS isolated from *Salmonella* Dublin. The conjugate is goat- anti bovine IgG coupled to horse radish peroxidase. Test samples are placed in the wells of the test plate and incubated at room temperature (22±3°C). Subsequently plates are washed and the HRPO conjugate is added and incubated at room temperature (22±3°C). After the plates are
washed the ready-to-use Chromogen (TMB) Substrate is dispensed to all wells of the test plate. After incubation at 22±3°C the color development is stoppedand measured at 450 nm.

Kit components

5 plate kit for 450 samples. Store kit at 5±3°C until the expiry date. See kit label for actual expiry date.
The shelf life of diluted, opened or reconstituted components is notedbelow, where appropriate.

Component	Description
1: Test Plate	Five Test Plates.
2: Conjugate (30x)	30x concentrate, dilute before use. One vial containing 2.2 mL of Conjugate. Diluted Conjugate is not stable, prepare just before use.
3: Dilution Buffer (5x)	5x concentrate, dilute before use. One vial containing 60 mL of Dilution Buffer. Shelf life of dilution buffer working solution: 2 weeks at 22±3°C.
4: Washing Fluid (200x)	200x concentrate, dilute before use. One vial containing 60 mL of Washing Fluid. Shelf life of washing solution: 1 week at 22±3°C.
5: Negative Control	One vial containing 0.5 mL of Negative Control.
6: Validation Control	One vial containing 0.5 mL of Validation Control.
7: Positive Control	One vial containing 0.5 mL of Positive Control.
8: Chromogen (TMB) Substrate	Ready-to-use. One vial containing 60 mL of Chromogen (TMB) Substrate.
9: Stop Solution	Ready-to-use. One vial containing 60 mL of Stop Solution.
Additional kit contents	• Package insert • 15 plate sealers

Additional material required

Unless otherwise indicated, all materials are available through **thermofisher.com**.

Use	Description
General	Laboratory equipment according to national safety regulations.
Analysis of results	Plate Reader. The reader has to have an appropriate filter set to read the plates at 450 nm.
Optional	Plate washer.

Test procedure

Precautions

- National Safety Regulations must be strictly followed.
- The PrioCHECK™ Salmonella Dublin Ab Strip Kit must be performed in laboratories suited for this purpose.
- Samples should be considered as potentially infectious and all itemswhich contact the samples as potentially contaminated.

Notes

To achieve optimal results with the PrioCHECK™ Salmonella Dublin Ab Strip Kit,the following aspects must be considered:

- **The Test Procedure protocol must be strictly followed.**
- All reagents of the kit must be equilibrated to room temperature(22±3°C) before use.
- Pipette tips have to be changed for every pipetting step.
- Separate solution reservoirs must be used for each reagent.
- Kit components must not be used after their expiry date or if changes intheir appearance are observed.
  - Kit components of different kit lot numbers must not be used together.
  - Demineralized or water of equal quality must be used for the test.

Solutions to be made in advance

Dilution buffer working solution

The Dilution Buffer (Component 3) must be diluted 5 times in demineralized or distilled water. To perform a test with one plate, prepare 45 mL (add 9 mL Dilution Buffer (5x) to 36 mL demineralized or distilled water).

Shelf life of dilution buffer working solution: 2 weeks at 22±3°C.

Conjugate dilution

Prepare dilution of the Conjugate (Component 2) in dilution buffer workingsolution. To perform a test with one plate, prepare 12 mL (add 0.4 mL concentrated conjugate to 11.6 mL of dilution buffer working solution).

Note: **The diluted conjugate must be prepared just before use.**

Washing solution

The Washing Fluid (Component 4) must be diluted (200x) in demineralizedwater and is sufficient for a final volume of 12 liters. To perform a test with one plate, prepare 500 mL (add 2.5 mL Washing Solution (200x) to 497.5 mLdemineralized or distilled water).

Stability of washing solution: 1 week stored at 22±3°C.

Incubation of control and test milk samples

1. Label each strip of the Test Plate (Component 1) with a marker pen.
2. Dispense 100 µL of the test milk samples to wells G1–H12 of the Test Plate.
3. Dispense 90 µL of the dilution buffer working solution to the wells A1–F1 ofthe Test Plate.
4. Dispense 10 µL of the Negative Control (Component 5) to wells A1 and B1.
5. Dispense 10 µL of the Validation Control (Component 6) to wellsC1 and D1.
6. Dispense 10 µL of the Positive Control (Component 7) to wells E1 and F1.
7. Seal the test plate(s) with a plate sealer(s).
8. Shake the plate(s) during 1 minute, level 700 (for example SLT micro plate shaker EAS 2/4, rpm 1/min level 700, SLT lab instruments).
9. Incubate the Test Plate(s) for 60±5 minutes at room temperature (22±3°C).

Note: **Mixing the sample with the dilution buffer working solution is essential for the test.**

Incubation with Conjugate

1. Empty the Test Plate and wash the plate 6 times with 200 to 300 µL diluted washing fluid. Tap the plate firmly after the last wash cycle.
2. Dispense 100 µL of the working solution of the conjugate to all wells.
3. Seal the test plate with a plate sealer.
4. Incubate the plate(s) for 60±5 minutes at room temperature (22±3°C).

Incubation with Chromogen (TMB) Substrate

1. Empty the Test Plate and wash the plate 6 times with 200 to 300 µL diluted washing fluid. Tap the plate firmly after the last wash cycle.
2. Dispense 100 µL of the Chromogen (TMB) Substrate (Component 8) toall wells.
3. Incubate the plate(s) 15 minutes at room temperature (22±3°C).
4. Add 100 µL of the Stop Solution (Component 9) to all wells.
5. Mix the content of the wells of the plate(s).

Note: Start the addition of stop solution 15 minutes after the first well was filled with the Chromogen (TMB) Substrate. Add the Stop Solution in the same order and at the same pace as the Chromogen (TMB) Substrate was dispensed.

Reading of the test and calculating the results

1. Measure the optical density (OD) of the wells at 450 nm, preferably within15 minutes after color development has been stopped.
2. Calculate the mean OD_{450} value of the Negative Control (wells A1 and B1).
3. Calculate the mean OD_{450} value of the Positive Control (wells E1 and F1).
4. Calculate the corrected OD_{450} value of the Positive Control, Validation Control and all samples by subtracting the mean OD_{450} of the Negative Control (wells A1 and B1).
5. Calculate the percent positivity (PP) of all controls and of the test samples according to the formula below.

The OD_{450} of all samples is expressed as percent positivity (PP) of the OD_{450} of Positive Control (PC) (wells E1 and F1) corrected with the mean OD_{450} of the Negative Control (NC) (wells A1 and B1).

$$PP = (\text{corrected } OD_{450\ \text{test sample}} / \text{corrected } OD_{450\ \text{Positive Control}} \times 100) - 10$$

Result interpretation

Validation criteria

1. The mean OD_{450} of the Negative Control (wells A1 and B1) must be <0.4.
2. The OD_{450} of the Positive Control (not corrected) should be >1.000.
3. The percent positivity of the Validation Control must be ≥30.

Not meeting these criteria is reason to discard the results of that specifictest plate.

Note: If the OD_{450} of the Positive Control (not corrected) is below 1.000 possibly the Chromogen (TMB) Substrate is too cold. In that case pre-warmthe solution to 22±3°C or incubate up to 30 minutes.

Interpretation of the percent positivity

PP = <35%	Negative	*Salmonella*-specific antibodies are absent in the test sample.
PP = ≥35%	Positive	*Salmonella*-specific antibodies are present in the test sample.

In well-advanced *Salmonella* control programs the test can be used with adifferent cut-off. It remains the responsibility of the respective authorities/users to implement such cut-offs.

Customer and technical support

Technical support: visit **thermofisher.com/askaquestion**

Visit **thermofisher.com/support** for the latest in services and support,including:

- Worldwide contact telephone numbers
- Order and web support
- User guides, manuals, and protocols
- Certificates of Analysis
- Safety Data Sheets (SDSs; also known as MSDSs)

NOTE: For SDSs for reagents and chemicals from other manufacturers,contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their productsas set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com /us/en/home/global/terms-and-conditions.html**. If you have any questions,please contact Life Technologies at **thermofisher.com/support**.

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Lelystad | The NetherlandsThe information in this guide is subject to change without notice.

Revision history of Pub. No. MAN0013903 (English)

Rev.	Date	Description
A.0	10 September 2019	• New document. Converted the legacy document (PrioCHECK *Salmonella* Ab Bovine Dublin milk 7610640 v1.2_e.doc) to the current document template, with associated updates to the publication number, limited license information, warranty, trademarks, and logos. • The product name was changed from PrioCHECK® Salmonella Ab bovine Dublin to PrioCHECK™ Salmonella Dublin Ab Strip Kit.

Important Licensing Information: These products may be covered by one or more Limited Use Label Licenses. By use of these products, you accept the terms and conditions of all applicable Limited Use Label Licenses.

ThermoFisher SCIENTIFIC

Attachment 2 Literature Search

Most literature is found by reading through an article written by Waseem Shaukat, that started the research under Dr. Herman Barkema. Other literature is found when being referred to in other articles. Furthermore, Ruurd Jorritsma advised a book that was used for background and relevant articles were filtered out. When specific literature was needed, Pubmed was used as a first literature search machine, Scopus was also used if additional literature was needed. Results are limited by year (starting from 2010) and had to be open access. Results were, with this criteria, fifty or less. Based off the title and abstract it is determined which articles are useful and then the article is read carefully. Used terms are: 'dairy cows' 'prevalence' '*Salmonella* Dublin' 'BTM' in order to find articles that have done BTM testing for the prevalence of *Salmonella* Dublin in dairy cows. In order to find more about the effect of latent carriers infected with *Salmonella* Dublin, search terms were: 'latent carriers' and '*Salmonella* Dublin'. Since only eight results were left, all the abstracts were read to see if they contained information that was searched for. In order to find more literature on the herd prevalence of *Salmonella* Dublin in dairy cows, the following search terms were used: 'herd' 'prevalence' 'S*almonella* Dublin' 'dairy'. The abstract of the 24 remaining articles were read and if they seemed useful, the full article was read. Another search was done to get a better explanation of the chi-square test by searching for: 'chi-square' 'explained' and 'independence'. With the filters, the abstract of the eight results were being read and see if they explained the chi-square test. Other search terms were: 'fisher exact' 'chi square' 'correlation' 'difference'. The fifteen results were read on abstract to see if they could be used. In order to find out more about the zoonotic transmission of *Salmonella* Dublin, search terms '*Salmonella* Dublin' and 'zoonotic' were used in Pubmed. The abstract of the 36 results were read.

Attachment 3 Statistical tests results PP%≥35

Geographic region and ELISA

Coefficients

	Odds ratio (OR)	OR 2.5%	(OR) 97.5%	P-value
Region North	Reference	Reference	Reference	Reference
Region Central	1.81	0.77	4.26	0.17
Region South	2.09	0.88	4.95	0.09

Herd size and ELISA

Coefficients

	Odds ratio (OR)	OR 2.5%	(OR) 97.5%	P-value
Small	Reference	Reference	Reference	Reference
Medium	1.26	0.64	2.48	0.51
Large	1.56	0.69	3.51	0.29

Herd Size, Geographic region and ELISA

Coefficients

	Odds ratio (OR)	OR 2.5%	(OR) 97.5%	P-value
Central Small	Reference	Reference	Reference	Reference
Central Medium	4.55	1.19	17.36	0.03
Central Large	4.48	1.09	18.33	0.04
North Small	1.15	0.22	5.88	0.87
North Medium	1.77	0.34	9.21	0.49
North Large	3.38	0.52	22.13	0.20
South Small	4.96	1.35	18.22	0.02
South Medium	2.15	0.49	9.39	0.31
South Large	1.58	0.15	16.23	0.70

Attachment 4 Statistical tests results PP%≥15

Geographic region and ELISA

	Odds ratio (OR)	OR 2.5%	(OR) 97.5%	P-value
Region North	Reference	Reference	Reference	Reference
Region Central	1.14	0.63	2.08	0.66
Region South	1.59	0.88	2.90	0.13

Herd size and ELISA

	Odds ratio (OR)	OR 2.5%	(OR) 97.5%	P-value
Small	Reference	Reference	Reference	Reference
Medium	1.20	0.71	2.01	0.50
Large	1.95	1.06	3.58	0.03

Attachment 5 R-code

```
#installing needed packages
library(ggplot2)
library(openxlsx)
install.packages("openxlsx")
install.packages("ggplot2")
install.packages("dplyr")

# open/activate data-object of PP%>35
attach(Dublin_List_NBw)

library(magrittr)
library(dplyr)
# see which variables are in the file
names(Dublin_List_NBw)
# summary per variable
summary(Dublin_List_NBw)
head(Dublin_List_NBw)
summary(Plate.place)
summary(PP.)

#summarize information
table(Region)
table(Size)
table(POS.NEG)
prop.table(table(Region))
prop.table(table(POS.NEG, Region))
prop.table(table(POS.NEG, Size))
table(POS.NEG, Region)
table(POS.NEG, Size)

#make a table where Region and Size are combined
combined_df <- data.frame(Value1 = Region, Value2 = Size)
print(combined_df)
table(combined_df)

str(Dublin_List_NBw)

#R-studio code on Region and Elisa result (pos/neg)
#convert label of results to numeric values (NEG = 0, POS = 1)
Dublin_List_NBw$POS.NEG <- ifelse(Dublin_List_NBw$POS.NEG == "POS", 1,
                                  ifelse(Dublin_List_NBw$POS.NEG == "NEG",
0, NA))
print(Dublin_List_NBw)

#convert label of region to numeric values (North = 1, Central = 2, South =
3)
Dublin_List_NBw$Region <- factor(Dublin_List_NBw$Region, levels =
c("North", "Central", "South"))

#check levels of Region, to ensure good coding
levels(Dublin_List_NBw$Region)

#Convert region to numeric values (North = 1, Central = 2, South = 3)
Dublin_List_NBw$region_numeric <- as.numeric(Dublin_List_NBw$Region)
```

```
#Verify updated dataset
print(Dublin_List_NBw)

#Obtain p-value to see if further testing is needed for Region.
# Full model
full_model <- glm(POS.NEG ~ Region, data = Dublin_List_NBw, family =
binomial)
print(full_model)

# Reduced Model
reduced_model <- glm(POS.NEG ~ 1, data = Dublin_List_NBw, family =
binomial)

# Only the intercept
# Perform the likelihood ratio test
lr_test <- anova(reduced_model, full_model, test = "Chisq")

# Extract the p-value
p_value_lr <- lr_test["2", "Pr(>Chi)"]

print(p_value_lr)

#Obtain p-value to see if further testing is needed for Herd size
# Full model
full_model <- glm(POS.NEG ~ Size, data = Dublin_List_NBw, family =
binomial)
print(full_model)

# Reduced Model
reduced_model <- glm(POS.NEG ~ 1, data = Dublin_List_NBw, family =
binomial)

# Only the intercept
# Perform the likelihood ratio test
lr_test <- anova(reduced_model, full_model, test = "Chisq")

# Extract the p-value
p_value_lr <- lr_test["2", "Pr(>Chi)"]
print(p_value_lr)

#with the linear regression model the baseline is one of the values
(region) when they are categoric
#therefore make 3 different linear regression models to compare all of the
regions as baselines

#linear regression model, baseline North
#set North as the basline category
Dublin_List_NBw$Region <- relevel(Dublin_List_NBw$Region, ref = "North")
#fit logistic model
log_model <- glm(POS.NEG ~ Region, data = Dublin_List_NBw, family =
binomial)
summary(log_model)
#output is on logistic scale, therefore get the exponent to get the odds
ratio:
exp(coefficients(log_model))
#calculate the 95% confidence interval
confint.default(log_model)
```

```
#confidence interval of the odds ratio
exp(confint.default(log_model))

#R-studio code on Herd size and Elisa result (pos/neg)
#convert label of results to numeric values (NEG = 0, POS = 1)
Dublin_List_NBw$POS.NEG <- ifelse(Dublin_List_NBw$POS.NEG == "POS", 1,
                                  ifelse(Dublin_List_NBw$POS.NEG == "NEG",
0, NA))
print(Dublin_List_NBw)

#make sure size is seen as a factor
#specify the levels within size
Dublin_List_NBw$Size <- factor(Dublin_List_NBw$Size, levels = c(1, 2, 3))

#with the linear regression model there is a baseline needed, therefore
three models
#herd size 1 as baseline
Dublin_List_NBw$Size <- relevel(Dublin_List_NBw$Size, ref = "1")
#fit logistic model
log_model <- glm(POS.NEG ~ Size, data = Dublin_List_NBw, family = binomial)
summary(log_model)
#output is on logistic scale, therefore get the exponent to get the odds
ratio:
exp(coefficients(log_model))
#calculate the 95% confidence interval
confint.default(log_model)
#confidence interval of the odds ratio
exp(confint.default(log_model))

#since no significant result, combine both herd size and region with ELISA
test result
#combine region and size to a new variable in order to compare later
Dublin_List_NBw$Region_Size <- factor(paste(Dublin_List_NBw$Region,
Dublin_List_NBw$Size))
# Check unique levels in Region_Size
levels(Dublin_List_NBw$Region_Size)

#make a table of the new variable
table_region_size <- table(Dublin_List_NBw$Region_Size,
Dublin_List_NBw$POS.NEG)
print(table_region_size)
prop_table <- prop.table(table_region_size, margin = 1)
print(prop_table)

#table not 1 in total, so need to fix
total_sum <- sum(prop_table)
#normalize proportion table
prop_table_normalized <- prop_table / total_sum
print(prop_table_normalized)

#with the new factor variable fit the logistic regression model
#baseline is Central 1
log_model <- glm(POS.NEG ~ Region_Size, data = Dublin_List_NBw, family =
binomial)
summary(log_model)
#output is on logistic scale, therefore get the exponent to get the odds
ratio:
```

```
exp(coefficients(log_model))
#calculate the 95% confidence interval
confint.default(log_model)
#confidence interval of the odds ratio
exp(confint.default(log_model))

#close dataset PP>35%
detach("Dublin_List_NBw")

# open/activate data-object PP%>15
attach(Dublin_List_PPg)

#summarize information
table(Region)
table(Size)
table(POS.NEG)
prop.table(table(Region))
prop.table(table(POS.NEG, Region))
prop.table(table(POS.NEG, Size))
table(POS.NEG, Region)
table(POS.NEG, Size)

#make a table where Region and Size are combined
combined_df <- data.frame(Value1 = Region, Value2 = Size)
print(combined_df)
table(combined_df)
str(Dublin_List_15PP.xlsx)

#R-studio code on Region and Elisa result (pos/neg)
#convert label of results to numeric values (NEG = 0, POS = 1)
Dublin_List_PPg$POS.NEG <- ifelse(Dublin_List_PPg$POS.NEG == "POS", 1,
                                  ifelse(Dublin_List_PPg$POS.NEG == "NEG",
0, NA))
print(Dublin_List_PPg)

#convert label of region to numeric values (North = 1, Central = 2, South =
3)
Dublin_List_PPg$Region <- factor(Dublin_List_PPg$Region, levels =
c("North", "Central", "South"))

#check levels of Region, to ensure good coding
levels(Dublin_List_PPg$Region)
#Convert region to numeric values (North = 1, Central = 2, South = 3)
Dublin_List_15PP.xlsx$region_numeric <-
as.numeric(Dublin_List_15PP.xlsx$Region)
#Verify updated dataset
print(Dublin_List_PPg)
#Obtain p-value to see if further testing is needed for Geographic region
# Full model
full_model <- glm(POS.NEG ~ Region, data = Dublin_List_PPg, family =
binomial)
print(full_model)

# Reduced Model
reduced_model <- glm(POS.NEG ~ 1, data = Dublin_List_PPg, family =
binomial)
# Only the intercept
```

```
# Perform the likelihood ratio test
lr_test <- anova(reduced_model, full_model, test = "Chisq")
# Extract the p-value
p_value_lr <- lr_test["2", "Pr(>Chi)"]
print(p_value_lr)

#Obtain p-value to see if further testing is needed for Herd size
# Full model
full_model <- glm(POS.NEG ~ Size, data = Dublin_List_PPg, family =
binomial)
print(full_model)

# Reduced Model
reduced_model <- glm(POS.NEG ~ 1, data = Dublin_List_PPg, family =
binomial)
# Only the intercept
# Perform the likelihood ratio test
lr_test <- anova(reduced_model, full_model, test = "Chisq")
# Extract the p-value
p_value_lr <- lr_test["2", "Pr(>Chi)"]
print(p_value_lr)

#with the linear regression model the baseline is one of the values
(region) when they are categoric
#therefore make 3 different linear regression models to compare all of the
regions as baselines
#linear regression model, baseline North
#set North as the basline category
Dublin_List_PPg$Region <- relevel(Dublin_List_PPg$Region, ref = "North")
#fit logistic model
log_model <- glm(POS.NEG ~ Region, data = Dublin_List_PPg, family =
binomial)
summary(log_model)
#output is on logistic scale, therefore get the exponent to get the odds
ratio:
exp(coefficients(log_model))
#calculate the 95% confidence interval
confint.default(log_model)
#confidence interval of the odds ratio
exp(confint.default(log_model))

#R-studio code on Herd size and Elisa result (pos/neg)
#convert label of results to numeric values (NEG = 0, POS = 1)
Dublin_List_NBw$POS.NEG <- ifelse(Dublin_List_NBw$POS.NEG == "POS", 1,
                                  ifelse(Dublin_List_NBw$POS.NEG == "NEG",
0, NA))
print(Dublin_List_NBw)

#R-studio code on Herd size and Elisa result (pos/neg)
#convert label of results to numeric values (NEG = 0, POS = 1)
Dublin_List_PPg$POS.NEG <- ifelse(Dublin_List_PPg$POS.NEG == "POS", 1,
                                  ifelse(Dublin_List_PPg$POS.NEG == "NEG",
0, NA))
print(Dublin_List_PPg)
#make sure size is seen as a factor
#specify the levels within size
Dublin_List_PPg$Size <- factor(Dublin_List_PPg$Size, levels = c(1, 2, 3))
```

```
#with the linear regression model there is a baseline needed, therefore
three models
#herd size 1 as baseline
Dublin_List_PPg$Size <- relevel(Dublin_List_PPg$Size, ref = "1")
#fit logistic model
log_model <- glm(POS.NEG ~ Size, data = Dublin_List_PPg, family = binomial)
summary(log_model)
#output is on logistic scale, therefore get the exponent to get the odds
ratio:
exp(coefficients(log_model))
#calculate the 95% confidence interval
confint.default(log_model)
#confidence interval of the odds ratio
exp(confint.default(log_model))

#detach file
detach("Dublin_List_PPg")
```