The effect of pasteurization on the amount of *Mycobacterium avium* subspecies *paratuberculosis* in colostrum

Research Project - Veterinary Medicine - University Utrecht

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Summary

Colostrum plays an important role in the transmission of Johne's disease to calves. Pasteurization of colostrum is an effective way to reduce this risk. Literature showed that a pasteurization at 60°C for 60 minutes gave the best results of reducing the concentration of *Mycobacterium avium* subspecies *paratuberculosis* and keeping IgG concentrations high. Most pasteurization studies use a culturing method for detecting differences in pre and post pasteurization samples. The disadvantage of this method is the duration of culture and the relatively high detection limit. Also PCR is used for detecting MAP in colostrum. The disadvantage of this method is the impossibility of making difference between viable and unviable MAP bacteria. In this research a fundament was established for creating a new diagnostic method, based on the differentiation between the MAP specific F57 gene and MbtA gene, by using a restriction enzyme to cut the MbtA gene. The difference between MbtA/F57 ratio of pre and post pasteurization samples will tell the difference of viable/unviable MAP bacteria. Electrophoresis and rtPCR were used in the experiments. Two restriction enzymes proofed to be useful for cutting the pure MbtA gene: Not-1 and Hinf-1. The best results for cutting the pure MbtA gene were given by the Hinf-1 restriction enzyme. Further experiments are required for the development of a new diagnostic method to make the difference between viable and unviable MAP bacteria after pasteurization.

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Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease in dairy cows. Johne's disease is a chronic wasting disease, caused by an infection of the small intestine with MAP, which leads to a chronic progressive enteritis. Cows with Johne's disease often show a loss of body condition, reduced fertility, reduced milk yield and reduced slaughter value. This will cause economic losses[1]. Cattle become infected with MAP as calves. After an incubation period of mostly two to five years, some infected cows will suffer from non-treatable diarrhea which leads to the death of the animal[2].

Infection occurs most of the time through fecal-oral transmission via ingestion of milk contaminated with MAP or through oral uptake of manure containing MAP present in the environment of the calf[1]. Also an intrauterine infection of MAP is possible[3]. Milk can be contaminated with MAP by the excretion of MAP through the mammary gland or through fecal contamination of teats[4]. After the oral intake of MAP, it will move to the ileum and will settle in the Peyer's patches in the gut wall of the ileum. MAP spreads via macrophages trough the body from Peyer's patches to the regional lymph nodes[1].

In Canada the cost of Johne's disease has been estimated to be 49 CAD per cow in an infected herd. The national costs of Johne's disease was approximately 15 million CAD, whereby the prevalence of Johne's disease was assumed at at least 7% per farm[5]. This amount is probably higher, because only ELISA positive cows were considered as MAP-infected. Because of this economic loss it is important to try to reduce the prevalence of Johne's disease. For this reason Alberta milk founded the voluntary Alberta Johne's Disease Initiative (AJDI). This control program is working together with dairy farmers and herd veterinarians to reduce the prevalence of Johne's disease. Because the main infection route of Johne's disease is by oral ingestion of MAP through calves, this risk factor has to be reduced.

There isn't much research done about the shedding of MAP in colostrum. Shedding of MAP seems to be linked to stage of infection and lactation stage[6]. According to Stabel et al. (2014) clinical cows shed about 250 cfu/ml in colostrum. Subclinical cows shed about 24 cfu/ml in colostrum[8]. These results were obtained by use of PCR, so it doesn't tell us if these MAP bacteria were viable or not, but it's likely that there are viable MAP bacteria in this tested colostrum as well. Calves fed colostrum from multiple cows have a higher chance of getting infected by MAP then cows only fed colostrum from their own dam[9]. Feeding raw colostrum from MAP positive dams has shown to be a significant predictor for transmission of MAP[9, 10].

Pasteurization projects with MAP done before

There are some pasteurization projects looking at MAP done before. Some projects are summarized below. The best way to pasteurize colostrum seems to be at 60 degrees Celsius for 60 minutes. With this method most of the MAP bacteria became unviable and there isn't a big decrease in IgG concentration. Using this procedure for pasteurization of colostrum seems to be the most appropriate option.

Godden et al. 2006[13]

In this research was looked what happened with MAP in colostrum when it was pasteurized at 60 degrees for several time periods. Before starting pasteurization, the colostrum was pasteurized using a commercial on-farm batch pasteurization system. After this the 30 liter batches were inoculated with MAP till a concentration of 10^3 cfu/ml was reached. Then the experimental pasteurization started. The pasteurized batches were cultured for MAP. The results of the research suggested that pasteurization for 60 minutes for 60 degrees should be sufficient to eliminate MAP. All the four batches didn't show any MAP growth after 60 minutes. Although, at one of those four batches growth was detected after 75 minutes and 90 minutes, but in a very low amount (<1 cfu/ml). According to the researchers this was probably a decontamination fault. The IgG concentration in colostrum wasn't changed after the pasteurization process.

Stabel, 2008[14]

In this study calves from naturally infected dams were used to determine if pasteurization of colostrum reduced the incidence of MAP in calves. Calves were divided in to two groups. One group received colostrum from their dam (DC; n=6). The other group was fed pooled pasteurized colostrum, from 3 cows tested negative for MAP shedding or ELISA (PC; n=5). The calves received colostrum for 3 weeks, followed by milk replacer. The colostrum fed to the PC group was pasteurized for 30 minutes at 65 degrees. After 6 weeks the calves were weaned and housed together. After 12 months calves were necropsied and 25 tissue sides were cultured for MAP. For DC calves 14 of the 25 tissue sides were positive versus 9 of the 25 tissue sides for PC calves. According to the researchers this points to a wider spread infection in DC calves.

The disadvantages of this study is the several other ways calves could have been infected. For example: The calves in the DC group were separated from their dam after 8 hours, in contrast to the PC group who were separated from their dam directly after birth. It is possible that calves are infected via contamination of their dams' teats[15].

It's doubtful that the lower infection rate in PC calves is only due to the pasteurized colostrum.

Stabel et al., 2004[16]

In 2004 Stabel et al. tested the effectiveness of High-Temperature, Short-Time pasteurization (HTST). In the experiment a commercial pasteurizer was used, which pasteurized at 71.7 degrees for 15 seconds. Spiked samples were used with inoculation of 10^2 cfu/ml and 10^6 cfu/ml for waste milk. Colostrum samples were spiked with 10^5 cfu/ml. A HEYM plate was used for culturing MAP. No viable MAP bacteria were recovered in waste milk and colostrum samples after pasteurization. The only disadvantage was the 25% loss of IgG in colostrum samples.

Gao et al., 2002[17]

In this research 18 pasteurization experiments were conducted. 7 batches were regular pasteurized with the method of 63 degrees for 30 minutes. 11 batches were pasteurized by HTST pasteurization, using 72 degrees for 15 seconds. The raw milk batches were spiked at levels of 10^3, 10^5 and 10^7 cfu/ml. Samples were cultured in Middlebrook 7H11 agar slants and Middlebrook 7H9 culture broth. Survival of MAP was confirmed using IS900 PCR on colonies from slants. 15 batches also underwent the BACTECT culture procedure. From the seven regular batches no MAP survivors were detected. In 2 of the 11 HTST batches, MAP was detected. One sample was spiked to 10^5 cfu/ml, the other was spiked tot 10^7 cfu/ml. This could indicate that MAP presented at more than 10^5 cfu/ml in milk is able to survive HTST pasteurization.

Diagnostic methods of MAP in milk

Most studies use the culturing method to diagnose MAP in colostrum or milk. The disadvantage of this method is the detection limit. The sensitivity of detection is 10^2 cfu/ml of liquid culture. The detection limit of HEY medium culture is 10^4 cfu/ml[8]. Using PCR the detection limit of MAP is 10 cfu/ml[18]. This difference is considerable. PCR will detect viable and unviable MAP bacteria, whereby culture will only detect viable MAP bacteria. There is no possible way to make the difference between viable and unviable MAP bacteria by using PCR up to now. The other benefit for PCR is the duration. For most culturing methods a minimum waiting period of 9 weeks is necessary.

Aim of the study

1. Determine if the use of a restriction enzyme could make the difference between viable and unviable *Mycobacterium avium* subsp. *paratuberculosis* bacteria in milk visible by using PCR
2. Determine the amount of *Mycobacterium avium* subsp. *paratuberculosis* in colostrum of cows from different lactation stages
3. Determine the effect of pasteurization on the amount of *Mycobacterium avium* subsp. *paratuberculosis* in the colostrum from dairy cows

Materials and methods

The research existed out of 2 parts. The first part is the development of a new diagnostic method. The other part is the set-up for getting on-farm pasteurization samples.

Development of diagnostic method

The new diagnostic method to measure the difference between viable and unviable MAP-bacteria, is based on the difference between the ratio of MbtA and F57 genes. The F57 gene is a very MAP specific gene[19]. A restriction enzyme will be used to cut the MbtA gene, so the MbtA gene isn't detectable by PCR anymore. A simplistic view of the principle is shown in figure 1.

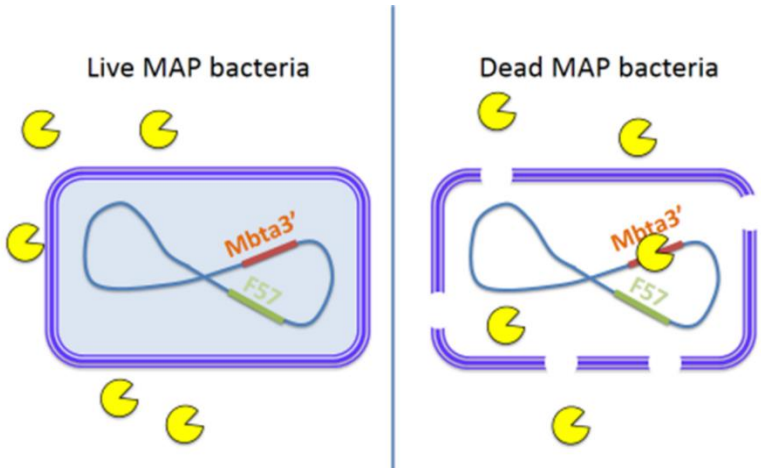


Figure 1; Simplistic view diagnostic method

Several experiments are necessary to test if a restriction enzyme is useful for a new diagnostic method. First it needs to be set that the restriction enzyme does cut the MbtA gene. If this is confirmed, it also needs to be able to cut the MbtA gene in milk. After this it needs to be tested if gDNA of MAP reacts with the restriction enzyme. Finally spiking experiments need to be done to see if the differentiation between MbtA and F57 tells us something about the viability of MAP bacteria in milk. The first experiments testing the efficiency of the restriction enzyme on the MbtA gene were performed in this research.

F57

The primers and probe used for the F57 rtPCR is described by Slana et al.(table 1). The primers that have been used are designed to amplify a 147–base-pair target sequence that can be detected with the PCR probe sequence.

Type	Name	Sequence
Probe	F57qPCRTM	CAATTCTCAGCTGCAACTCGAACACAC
Forward	F57qPCRF	GCCCATTTCATCGATACCC
Reverse	F57qPCRR	GTACCGAATGTTGTTGTCAC

Table 1; F57 primers and probe

MbtA

Table 2 shows the MbtA primers and probe used for MbtA rtPCR. The MbtA primers that have been used are designed to amplify a 203–base-pair target sequence that can be detected with the PCR probe sequence.

Type	Name	Sequence
Probe	MbtA3'NotFam	CGAAGATCTCCGAGGAACGTCAT
Forward	MbtA3'NotF	GCAAGTAGAATCGACTTC
Reverse	MbtA3'NotR	GTGTGAAAGTCGTATCTG

Table 2; MbtA primers and probe

Restriction enzymes

The restriction enzymes being used are the HINF-1, NOT-1 and EcoR-1 enzymes. It is important that the enzyme cuts the MbtA gene between the two primers and that it doesn't cut the F57 gene. Table 3 shows the binding sites of the restriction enzymes. Attachment 2, figure 2 shows the F57 gene, with the primers, probe and binding sites of the enzymes. Attachment 2, figure 3 shows those for the MbtA gene. Ecor-1 has no binding sites on the F57 and MbtA gene.

Enzyme	Binding site
HINF-1	G ANTC
NOT-1	GC GGCCGC
EcoR-1	G AATTC

Table 3; Binding site restriction enzymes

Experiments

Getting the MbtA gene in plasmid

To get the pure MbtA gene, the protocol "*PCR Using Taq DNA Polymerase and Q-Solution*" from Qiagen® was used[20]. The samples (See attachment 3, Experiment 1) were run on regular PCR followed by electrophoresis on a 1.5% agarose gel. To get the MbtA gene from the agarose gel, a "Qiaquick® Gel Extraction Kit" was used. The isolated MbtA gene was cloned into TOPO vector. This TOPO vector was inserted into TOP10 E. Coli cells and plate on LB/ampicillin plates. The plates were incubated at 37°C overnight. The 'GenElute Plasmid Miniprep Kit' was used to extract the MbtA gene from culture. The procedures were followed by manufacturer's protocol.

A confirmatory test was run to check if the MbtA gene was in the plasmid. Three samples were created. One containing plasmid, one MAP gDNA and one H2O (See attachment 3, Experiment 2). Samples were run on regular PCR followed by electrophoresis on a 1.5% agarose gel.

Test MbtA plasmid with restriction enzymes on electrophoresis

The MbtA plasmid is first tested with the restriction enzymes on electrophoresis. In the first attempt, three samples were run on a 1.5% agarose gel on electrophoresis. Containing Not-1, Ecor-1 and H2O (See attachment 3, Experiment 3). The buffers added to the restriction enzymes are conform the manufacturer's recommending's.

The second attempt was also a confirmatory test. Samples contained pure MbtA insert (what was put into the TOPO vector) in combination with the Not-1 enzyme. Other samples contained MbtA plasmid with Not-1/Ecor-1 and a control sample (See attachment 3, Experiment 4). The samples were run on a 1.5% agarose gel electrophoresis.

In the fifth and sixth experiment the restriction enzymes were tested in milk. Commercial milk was used. One sample contained plasmid, Not-1 and milk, another contained plasmid, Not-1 and water. Three control samples were run on a 1.5% agarose gel electrophoresis as well (See attachment 3, experiment 5 and 6). In experiment 6 one sample contained milk only, to see the smear of milk on electrophoresis.

Test MbtA plasmid with restriction enzymes in milk on rtPCR

Not-1 and the plasmid were added to milk in experiment 7 (see attachment 3, Experiment 7). Samples were incubated at 37°C for 2 hours and heat restricted for 20 minutes in a water bath, to inactivate the restriction enzyme. Samples then underwent the milk extraction procedure. The first procedure followed is the procedure created by Gao et al.[21]. The DNA extraction is performed with a QIAmp Blood Mini kit, with a procedure created by Slana et al.[22]. After these procedures the samples were run on a Bio-Rad CFX96™ Real Time PCR System.

In experiment 8 Hinf-1 and a combination of Not-1+Hinf-1 were added, together with the plasmid, to milk (See attachment 3, Experiment 8). Plasmid was diluted 10x and 100x before adding. Samples were then incubated at 37°C for 2 hours and heat restricted at 80°C for 20 minutes in a water bath. After that samples underwent the earlier mentioned milk extraction procedure. In experiment 9 Hinf-1, plasmid and milk were added together (See attachment 3, Experiment 9). The plasmid was diluted 10x, 100x and 1000x. Samples were treated the same as in experiment 9.

Pasteurization Project set-up

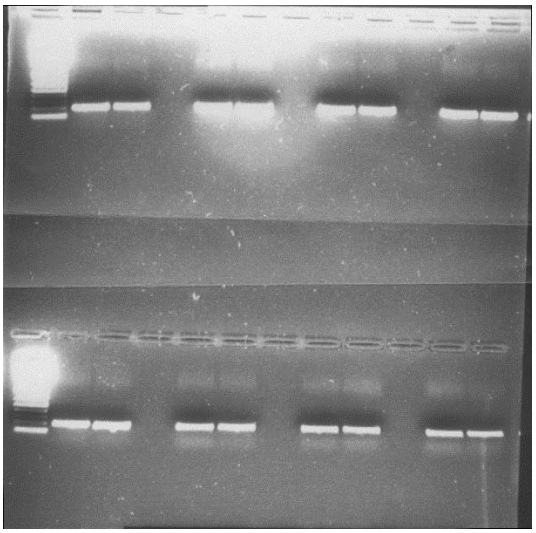
The research took place on a high MAP prevalence farm, at which more than 30% of the cows were infected, which is identified in the Alberta Johne's Disease Initiative. From all dry cows serum and manure samples were taken. Dry cows were considered as MAP-infected if antibodies were present in serum and/or when manure samples contained MAP bacteria. The presence of the F57 gene was used for the detection of shedding MAP in manure. Colostrum samples were taken from cows which are considered as MAP-infected. Expected calving dates of these cows were recorded. As soon as one of these cows calved, the colostrum was collected in sterile buckets. The pasteurization took place on the farm using the 'Coloquick' pasteurizer. The pasteurization process was carried out according to the manufacturer's recommendations. The manufacture recommends to pasteurize for 60 minutes at 60 degrees Celsius. The farmer filled in a question farm, to get insight if there could be any contamination of the colostrum (see attachment 1). During the pasteurization process the temperature was recorded using a HOBO temperature recorder. The recorder will be put in a bag with colostrum.

First three pre-pasteurization samples were taken, to estimate the amount of MAP bacteria. After this, the pasteurization process began. During the process, the temperature was recorded using an automatic temperature recording system. Because the pasteurization process couldn't bed stopped, no samples were taken during the process. Samples will be analyzed by using rtPCR to quantify the presence of MAP bacteria. A restriction enzyme will be added to make the MbtA gene of unviable MAP bacteria undetectable by rtPCR, so only the MbtA gene of viable MAP bacteria would be detectable. The MbtA/F57 ratio from samples pre and post pasteurization will tell the difference between viable and unviable MAP bacteria.

Results

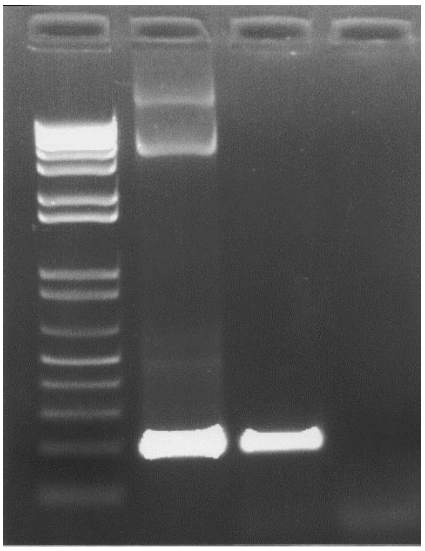
Getting the MbtA gene in plasmid

- *Experiment 1 – Getting pure MbtA gene*



The MbtA genes are visible as clear bands. The DNA fragments were excised from the agarose gel with a clear and sharp scalpel.

- *Experiment 2 – Confirmatory test MbtA plasmid*



Lane 1 – Ladder

Lane 2 – Plasmid

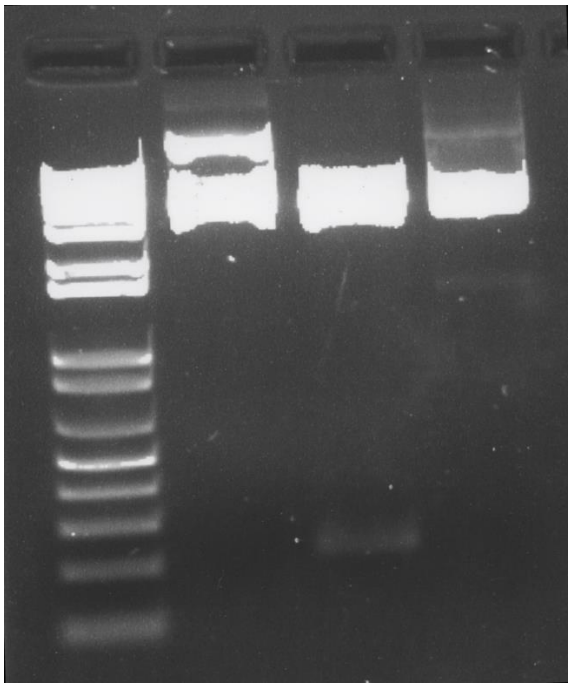
Lane 3 – MAP gDNA

Lane 4 – Control

There is a clear band in lane 2 and 3 at the same place. This confirms that the MbtA gene is in the created plasmid.

Test MbtA plasmid with restriction enzymes on electrophoresis

- *Experiment 3 – Restriction attempt 1*



Lane 1 – Ladder

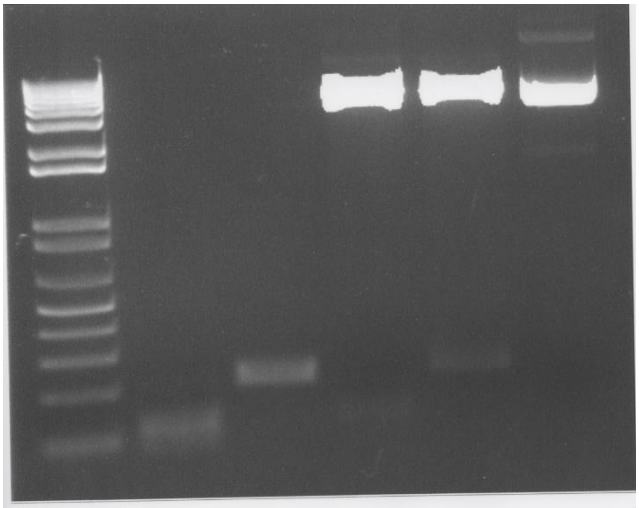
Lane 2 – Not-1 with plasmid

Lane 3 – Ecor-1 with plasmid

Lane 4 – Control

Only a band is visible in lane 3 at the bottom of the gel, this could be the MbtA gene. In lane 2 no bands are visible on the agarose gel.

- *Experiment 4 – Restriction attempt 2/Confirmatory digest*



Lane 1 – Ladder

Lane 2 – Not-1 with MbtA insert

Lane 3 – Control MbtA insert

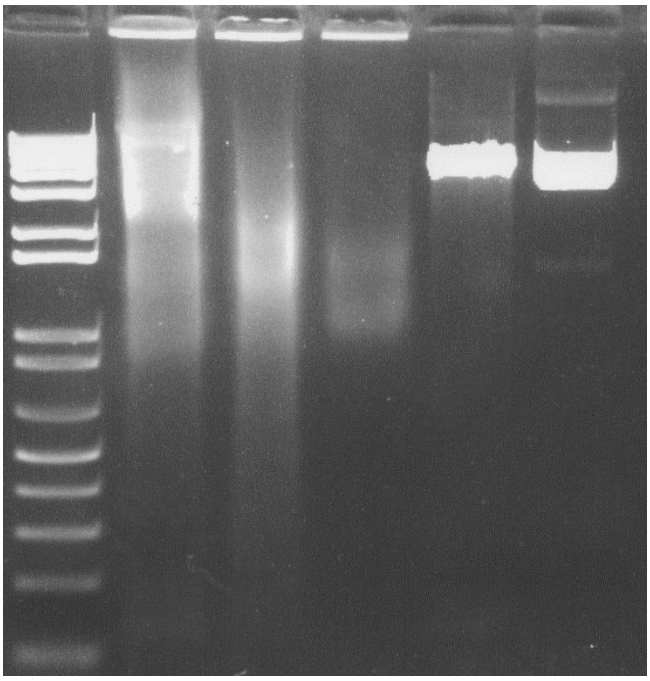
Lane 4 – Not-1 with plasmid

Lane 5 – Ecor-1 with plasmid

Lane 6 – Control plasmid

Lane 2 and lane 4 have the same band visible at the bottom of the gel. This is the same for lane 3 and lane 5. This confirms that the Not-1 restriction enzyme cuts the MbtA gene and the Ecor-1 doesn't do this.

- *Experiment 5 – Milk digest 1*



Lane 1 – Ladder

Lane 2 – Not-1 with plasmid in milk

Lane 3 – Control plasmid in milk

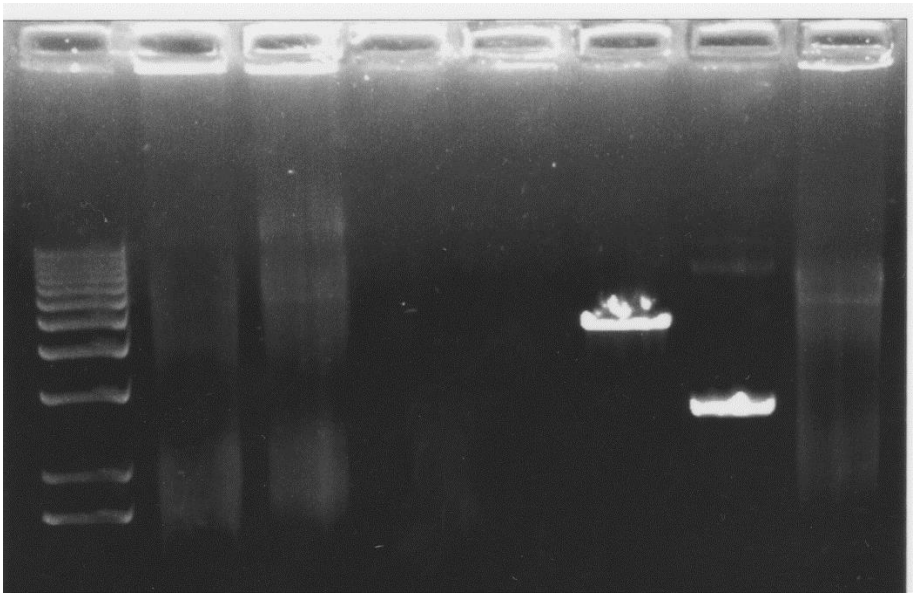
Lane 4 – Negative control

Lane 5 – Not-1 with plasmid in water

Lane 6 – Control plasmid in water

There are some differences between the lanes, but no clear bands are visible.

- *Experiment 6 – Milk digest 2*



Lane 1 – Ladder

Lane 2 – Not-1 with plasmid in milk

Lane 3 – Control plasmid in milk

Lane 5 – Negative control

Lane 6 – Not-1 with plasmid in water

Lane 7 – Control plasmid in water

Lane 8 – Milk

There aren't any clear bands visible that shows the cutting of the MbtA-gene by Not-1. Lane 8 shows similarities with lane 2 and 3. Lane 6 and 7 have differences, but this wouldn't suggest the cutting of the gene neither, because the band of the control plasmid is more at the bottom of the gel.

Test MbtA plasmid with restriction enzymes in milk on rtPCR

- *Experiment 7 – Not-1 restriction digest in milk*

	FAM (630 ng)
Uncut control	13.9
Not I digest 1	14.64
Not I digest 2	14.25
Average value	14.45
Average difference	0.55

The difference in ct-value between the uncut control and the Not-1 digest is 0.55(14.45-13.9).

- *Experiment 8 – Not-1+Hinf-1 restriction digest in milk*

	10x dilution (63 ng)	100x dilution (6.3 ng)
Uncut control	15,15	19,31
Not I + Hinf I Digest	16,85	19,26
Hinf I Digest	18,97	24,22
Not I + Hinf I difference	1,7	-0,05
Hinf I difference	3,82	4,92

A combination with Not-1 and Hinf-1 gives a difference of 1.7(16.85-15.15) with a 10x dilution and no difference with a 100x dilution. The Hinf-1 enzyme gives an average difference of 3.82(18.97-15.15) in the 10x dilution and a difference of 4.92(24.22-19.31) in the 100x dilution.

- *Experiment 9 – Hinf-1 restriction digest in milk*

	10x dilution (63 ng)	100x dilution (6.3 ng)	1000x dilution (0.63 ng)
Uncut control	16.25	36.22/19.31	25.25
Hinf I digest 1	21.23	24.29	27.37
Hinf I digest 2	20.86	24.22	27.38
Hinf I digest 3	22.35	24.61	27.93
Average value	21.48	24.37	27.56
Average difference	5.23	5.06	2.31

With a 10x dilution Hinf-1 gives a difference of 5.23(21.48-16.25). With a 100x dilution a difference of 5.06(24.37-19.31) is given and a difference of 2.31(27.56-25.25) with a 1000x dilution.

Pasteurization Project

Sampling dry cows

Eight dry cows were considered as MAP-positive. Two cows were positive for MAP in manure and ELISA positive. Three cows were shedding MAP in manure, but weren't ELISA positive. Three cows were only ELISA positive. See table 4.

Cow ID	F57 ct value	ELISA result
2774	38.16	+
Neck 120	39.59	+
2746	36.65	-
2741	37.86	-
2675	37.95	None
2736	0	+
2665	0	+
2589	0	+

Table 4; Positive dry cows

Temperature recording pasteurization process 'Coloquick'

The whole pasteurization process took 120 minutes. The temperature was 60 degrees or above for 44 minutes. For 59 minutes the temperature was above 59 degrees. See figure 2. Attachment 4 shows the temperature recordings per minute.

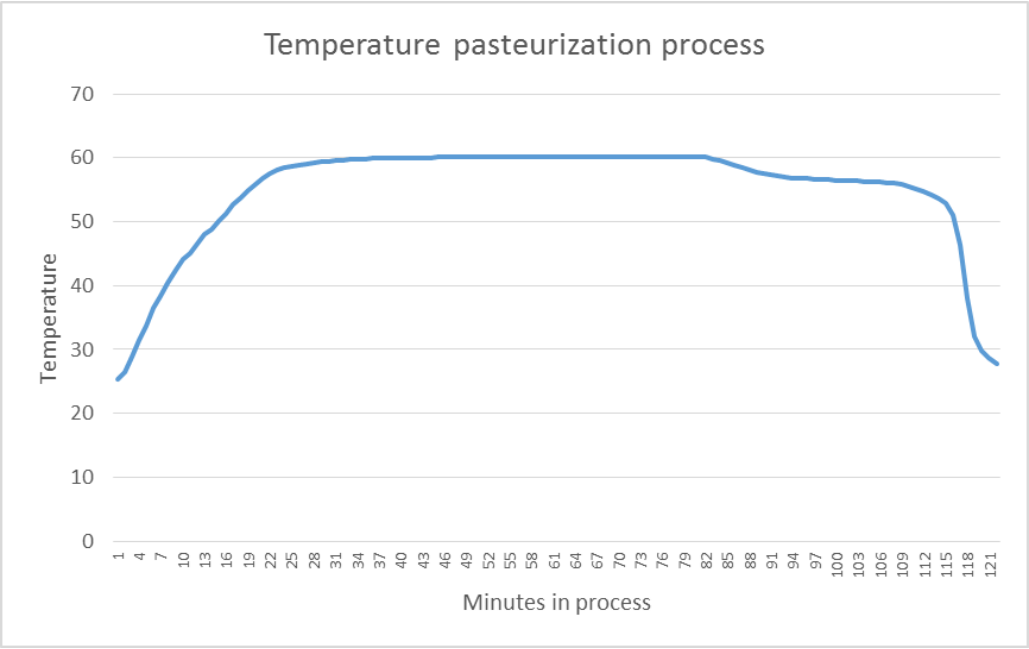


Figure 2; Temperature recording

Discussion

This study proved the working of a restriction enzyme cutting the MbtA gene. Several experiments proofed this. There wasn't any research done before about this new possible method. Experiment 4 shows a clear digest of the MbtA gene by the use of the Not-1 enzyme. Experiment 5 and 6 weren't very successful, probably because of the reaction of milk on electrophoresis. The smear of milk gives unclear bands on the agarose gel. In experiment 7 the rtPCR is used to identify the working of the restriction enzyme. Not a very big difference was noticed between the control sample and the Not-1 sample. Because of this reason Hinf-1 was introduced as a new restriction enzyme. A combination of Not-1 and Hinf-1 was more successful than Not-1 only, but still not sufficient. The digestion with Hinf-1 restriction enzyme was very successful and did cut almost all the MbtA genes. The reason why the Hinf-1 enzyme works better than Not-1 is not clear. It could have something to do with its binding sites. Hinf-1 cuts the MbtA gene at the same place as the binding place of the forward primer (see attachment 2). The use of the Ecor-1 enzyme wasn't necessary, because this enzyme didn't have matching binding sites with the MbtA gene. A critical point of the process could be the inactivation of the restriction enzyme. It could possibly be affective to viable MAP bacteria[23].

If the restriction enzyme is inactivated pretty quickly it shouldn't be a problem, but if it takes some minutes MAP bacteria could be damaged. In combination with pasteurized or unpasteurized samples this is a crucial step in the process.

The cutting of the MbtA gene due to the restriction enzyme in milk is not enough. Next steps are necessary to see if the enzyme is able to cut the MbtA gene in gDNA and in MAP itself. First it has to be tested with gDNA in milk. If this works an experiment can be created whereby 2 milk samples spiked with MAP are used. One sample needs to be boiled, so all MAP bacteria will be dead. The other sample is the control sample. If it works, there will be no difference between the quantity of MbtA gene and F57 gene in the unboiled sample. The boiled sample should theoretically contain no detectable MbtA genes, but should still contain all F57 genes. What could be a problem with pasteurization is the cell wall of MAP. It could be possible that the MAP bacteria become unviable after pasteurization, but the restriction enzyme isn't able to reach the DNA of MAP, because the cell wall is still intact. This theory isn't evidenced based, but is hypothetical.

Another risk could be the reaction of the restriction enzyme with other components in colostrum than MAP. Such as other bacteria, confounding microorganisms, fungi and molds[8]. This problem could be solved by adding enough restriction enzyme.

The results of the first colostrum samples taken are not included in this research. The temperature recording noticed some abnormalities. The Coloquick manufacturer claims that the 'Coloquick pasteurizer' pasteurizes at 60 degrees for 60 minutes. The Hobo temperature recorder showed that colostrum in the bag, reaches a temperature of 60 degrees or above for only 44 minutes. This could be due to an insignificancy in the temperature recorder or a settings fault in the pasteurizer.

Due to the limited time and the duration of the MbtA experiments, the aims of the study could not be fulfilled.

Conclusion

Experiments with electrophoresis showed the working of the Not-1 enzyme on the MbtA-gene. RtPCR showed that Hinf-1 was the best working restriction enzyme to cut the MbtA-gene in milk. This research is a good start for the development of a new diagnostic method. Many other experiments should be performed to see if the restriction enzyme could make a clear difference between MbtA and F57 ratios, to make the difference between viable and unviable MAP bacteria. The potential new diagnostic method would make the visibility of the effect of pasteurization more accurate and quicker.

Attachments

Attachment 1: On-farm questions pasteurization project

Questions colostrum pasteurization project

1. *Cow information*
    a. Cow number ……..
    b. Lactation stage ……..
    c. Medication no/yes ……..
2. *Dates*
    a. Date of calving ……..
    b. Time of calving ……..
    c. Expected calving date ……..
3. *Parturition information*
    a. Observed parturition no/yes
    b. Milk spill no/yes
4. *Calf information*
    a. Time of removal ……..
    b. Removed before standing yes/no
    c. Did you see the calf suckle no/yes
5. *Colostrum information*
    a. Time of milking ……..
    b. Contamination during milking yes/no
    c. Liters of colostrum ……..
    d. Storage condition refrigerated/not refrigerated
6. *Pasteurization information*
    a. Time of pasteurization ……..
    b. Quantity of colostrum pasteurized ……..

Comments

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Attachment 2: Primers, probe and restriction enzymes

Ggatctcggccccgatagctttcctctccttcgtcaccaactggcgcgggtccaggaacgcttggcactcgtcaatcaccacgaacaccagcggaacctgcggcgtcgggccgcgatcccaaaagttggacgatccgaatatgttgttgccctgtctaattcg
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ccaacgggagcgactggtagacgcccatttcatcgatacccaaactcagagaccacgagtgggcggctggcaca
gacgaccattcaagcggcgaactaagcggatcgacaattctcagctgcaactcgaacacacctgggaccggctc
ggtgacaacaacattcggtacac caatagctgacgccagcttcggagccgccacctgccactgtctgagatcc

Forward Primer Reverser Primer Probe **HINF-1**

Figure 4; F57 gene with primers, probe and restriction enzymes

CGACGGGTCCCCGGATGAATCGGCGGGCGGGGCCGCCGAATCGGTGTCCTGGCGGCCGGCGGTGACCAGTTGTGCCCAGGCCTCGATGGTGGGCGTCTCGGCCAGCGTGGCGAAGTCGACGGCGATGCCCCGGCGCCGCCAGCGGCCGGCCAGGGTCATGATC
CGGATCGAGTCCAAGCCCTGGCCGATGAGGTTGCTACCCGGCTGAACTGCATCGACGTCGACGCCGAGCAGTTCAGCCACCTCTTCCCGGATGTCCTCCGAGCGTGCCGGGGCGTGCACCACAGACCTCCCTGAATTAGCACAGGCTGCCCTTAAATGATTTG
GTTACCCTATCCTTACTCCCTGCGTCCCCGCTACTAGCCCCGTTCGAAGCCCGCTTAGAGGAGCCATGAGCCCCAACACCACGCCGCCGGCCGGAGTCCTCGACGGGTTCGTGCCGTTTCCCGCCGAGCGCGCCGCCGCCTACCGGGCGGCCGGTTTGTGGAC
GGGGCGAGCCCTGGACACGATCCTGACCGACGCGGCCCGGCGGTGGCCCGACCGAACCGCCGTCCTCGACGCCTCCGGCGGCACCGGTTTCAGCTACGCGGGCCTCGACGAGCAGGCTAACCGCGCCGCCGCAGGTTTGGCGGACGCGGGTATCGCCCCCGGC
GACCGGGTGCTGCTGCAGCTGCCGAACGGCTGCCAGTTCGCGGTGGCGCTGTTCGCGCTGTTACGGGCGGGGGCGATCCCGGTGATGTGCCTGCCCGGCCACCGCGCCGCCGAGCTTGGGCATTTCGCCGCGCTCAGCCAGGCCACCGCACTGCTGATCGCCG
ACACCGCAGCGGGTTTCGATTATCGAACGATGGCCGCGGGGCTCATCGAGGAGCATGAAGCGCTCGCCCACGTGATCGTCGACGGCGACCCGGGGCCGTTTCTGTCCTGGGCGCAGCTGTGCGAGCGTGCGCCCGCGGGGCGGCCGGCGACGCCGGTCGATCC
GGGATCGCCTGCGCTGCTTCTGGTTTCCGGTGGCACCACCGGCACACCAAAGCTCATCCCGCGCACCCACAACGATTACGTCTTCAACGCCACCGCCAGCGCCGAACTGTGCGGGCTCACCCGCGACGACGTCTATCTGGCGGTGCTGTCGGCCGGCCACAAT
TTCCCGCTCGCCTGCCCCGGCCTGCTCGGCGCGATGACGGTGGGCGCGACCACGGTGTTCGGCACCGACCCCAGCCCCGAGGCGGCCTTTGCCACCATCGCGCGCCACGGCGTCACGGTGACCGCCCTGGTCCCGGCCCTGGCCAAACTATGGGCGCAGGCCT
GCGAATGGGAGGACAATCCACCGAAGTCCTTGCGGCTCTTGCAAGTCGGCGGCGCCAAGTTGGAGGCCGACGACGCCCGTGTGATCCGCAGCGCGTTGAGGGTGCGGCCGGCGAAGACGGCGTCCAGCTCGGCCACGCAGGCCGCGCAGTTGATGCGCCGGGC
CGAAACCCCCGAACCGCAAGGCACCTCCCGCAACTCGGTCACGGCCATGCCGCAAGCATTCCAGAACCGCGCAGGGATGTCAGCAACTTTCCCGCACCTTCTATCGAA
AAAAGCGTCGGCAAGTA**GAATC**GACTTCTCGAACCGTAAGAAACGAAGATCTCCGAGGAACGTCATGGGTTCTG
CGCGACGGCTCGCCCGGAAGACCTCGACGCTCGCCGGCGCTCTGGCTTTC***GCGGCCGC***GGCATGCCCGCCAATC
GCGCAGGCCGCCATGCCTTTCGGCAACTACGACCTCCACGTCGAGGGCAGATACGACTTTCACACCTGGCTGTG
GGCGATCACCGCATGCCCGGGCGGCGGCTGCGTGCATGTGAATGCCATCGCCAGGCCGGTGGCCAAGGCATTCCCGTACGTCGGGGACGCGCGGTCGGTCGACGGCCAATACGTCTTGACGGTCGACGTTCCCGACG
GCCTGAGGTGTGACGACATCTATTACGGCCCAACCGTTCCCACCCACGACGTCTACACGTGGGATCCGACCACGCTGGCCGGCTCGCTGCAATCCTCCTTCGACGCGGGTTGCCATGGAGCGCCCGCCGGTTCCTACACGTATCCGTTCACGTTGTCACGCTT
GTAACTCTCGCTCCCGCTGAGCGCGTTCGGCCTTCTGCCCAGCCCTTTGCATCGCATCCATGCACTGCTGCAACGCGAACGCCGGGTTCAGCTCGAGCGCCTTGGTTCGTTCCCGATAGATCAGGATGTGCCCGGCCGGAAAAAATACACCTCGCCGGCCGTC
GCCGTCTCGTCCGGCCAGTCGGTGTTCGGATAGGTCGCGCGGATCGAG

Forward Primer Reverser Primer Probe **HINF-1** ***NOT-1***

Figure 4; MbtA gene with primers, probe and restriction enzymes

Attachment 3: Experiments samples

Experiment 1 – Getting pure MbtA gene

Sample

10x buffer = 5 µL
Q buffer = 10 µL
MgCl2 = 2 µL
dNTPs = 2 µL
Forward Primer = 2 µL
Reverse Primer = 2 µL
Top taq = 0.2 µL
H2O = 21.8 µL
MAP gDNA = 5 µL
Total volume = 50 µL

Experiment 2 – Confirmatory test MbtA plasmid

Sample 1 – 5 µL plasmid
Sample 2 – 5 µL MAP gDNA
Sample 3 – 5 µL H2O

Added to each sample:

10x buffer = 5 µL
Q buffer = 10 µL
MgCl2 = 2 µL
dNTPs = 2 µL
Forward Primer = 2 µL
Reverse Primer = 2 µL
Top taq = 0.2 µL
H2O = 21.8 µL
Total volume = 50 µL

Experiment 3 – Restriction attempt 1

Sample 1	Sample 2	Sample 3
Not-1 = 1 µL	Ecor-1 = 1,5 µL	H2O = 17 µL
BSA = 2 µL	H-buffer = 3 µL	plasmid = 3 µL
Triton = 2 µL	H2O = 12,5 µL	
H-buffer = 2 µL	plasmid = 3 µL	
H2O = 10 µL		
plasmid = 3 µL		

Experiment 4 – Attempt 2/Confirmatory test

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
MbtA insert = 3 µL	MbtA insert = 3 µL	Plasmid = 3 µL	Plasmid = 3 µL	Plasmid = 3 µL
Not-1 = 1 µL		Not-1 = 1 µL	Ecor-1 = 1.5 µL	H2O = 17 µL
BSA = 2 µL	BSA = 2 µL	BSA = 2 µL		
Triton = 2 µL	Triton = 2 µL	Triton = 2 µL		
H-buffer = 2 µL	H-buffer = 2 µL	H-buffer = 2 µL	H-buffer = 3 µL	
H2O = 10 µL	H2O = 11 µL	H2O = 10 µL	H2O = 10 µL	H2O = 17 µL

Experiment 5 – Milk digest 1

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Not-1 = 1 µL			Not-1 = 1 µL	
BSA = 2 µL	BSA = 2 µL	BSA = 2 µL	BSA = 2 µL	BSA = 2 µL
Triton = 2 µL	Triton = 2 µL	Triton = 2 µL	Triton = 2 µL	Triton = 2 µL
H-buffer = 2 µL	H-buffer = 2 µL	H-buffer = 2 µL	H-buffer = 2 µL	H-buffer = 2 µL
Milk = 10 µL	Milk = 10 µL	Milk = 10 µL		
	H2O = 1 µL	H2O = 4 µL	H2O = 10 µL	H2O = 11 µL
plasmid = 3 µL	plasmid = 3 µL		plasmid = 3 µL	plasmid = 3 µL

Experiment 6 – Milk digest 2

Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Not-1 = 1 µL			Not-1 = 1 µL		
BSA = 2 µL	BSA = 2 µL	BSA = 2 µL	BSA = 2 µL	BSA = 2 µL	
Triton = 2 µL	Triton = 2 µL	Triton = 2 µL	Triton = 2 µL	Triton = 2 µL	
H-buffer = 2 µL	H-buffer = 2 µL	H-buffer = 2 µL	H-buffer = 2 µL	H-buffer = 2 µL	
Milk = 10 µL	Milk = 10 µL	Milk = 10 µL			Milk = 10 µL
	H2O = 1 µL	H2O = 4 µL	H2O = 10 µL	H2O = 11 µL	
plasmid = 3 µL	plasmid = 3 µL		plasmid = 3 µL	plasmid = 3 µL	

Experiment 7 – Not-1 restriction digest in milk rtPCR

Sample 1	Sample 2
Not-1 = 5 µL	
BSA = 30 µL	BSA = 30 µL
Triton = 30 µL	Triton = 30 µL
H-buffer = 30 µL	H-buffer = 30 µL
Milk = 198 µL	Milk = 198 µL
H2O = 5 µL	H2O = 10 µL
plasmid = 2 µL	plasmid = 2 µL

Experiment 8 – Not-1+Hinf-1 restriction digest in milk rtPCR

Sample 1	Sample 2	Sample 3
Not-1 = 5 µL		
Hinf-1 = 5 µL		Hinf-1 = 5 µL
BSA = 30 µL	BSA = 30 µL	
Triton = 30 µL	Triton = 30 µL	
H-buffer = 30 µL	H-buffer = 30 µL	React2-buffer = 30 µL
Milk = 198 µL	Milk = 198 µL	Milk = 198 µL
	H2O = 10 µL	H2O = 65 µL
plasmid = 2 µL	plasmid = 2 µL	plasmid = 2 µL

All samples were made in duplicate with plasmid dilutions of 10x and 100x

Experiment 9 – Hinf-1 restriction digest in milk rtPCR

Sample 1	Sample 2
Hinf-1 = 5 µL	
React2-buffer = 30 µL	React2-buffer = 30 µL
Milk = 198 µL	Milk = 198 µL
H2O = 65 µL	H2O = 70 µL
plasmid = 2 µL	plasmid = 2 µL

All samples were made in triplicate with plasmid dilutions of 10x, 100x and 1000x

Attachment 4: Temperature recording pasteurization process

Minute	Temp		Minute	Temp		Minute	Temp		Minute	Temp
1	25,355		31	59,534		61	60,134		91	57,299
2	26,5		32	59,662		62	60,134		92	57,138
3	28,941		33	59,748		63	60,134		93	56,978
4	31,561		34	59,79		64	60,134		94	56,898
5	33,704		35	59,833		65	60,134		95	56,818
6	36,444		36	59,919		66	60,134		96	56,738
7	38,365		37	59,919		67	60,134		97	56,659
8	40,343		38	59,962		68	60,134		98	56,579
9	42,475		39	60,005		69	60,177		99	56,54
10	44,073		40	60,005		70	60,134		100	56,5
11	45,123		41	60,048		71	60,134		101	56,46
12	46,577		42	60,048		72	60,134		102	56,381
13	48,007		43	60,048		73	60,134		103	56,342
14	48,871		44	60,048		74	60,134		104	56,303
15	50,025		45	60,091		75	60,134		105	56,263
16	51,313		46	60,091		76	60,134		106	56,184
17	52,637		47	60,091		77	60,134		107	56,145
18	53,813		48	60,091		78	60,134		108	56,067
19	54,906		49	60,091		79	60,177		109	55,871
20	55,91		50	60,091		80	60,177		110	55,56
21	56,818		51	60,134		81	60,134		111	55,136
22	57,501		52	60,134		82	60,091		112	54,678
23	58,031		53	60,134		83	59,876		113	54,187
24	58,402		54	60,134		84	59,62		114	53,627
25	58,651		55	60,134		85	59,28		115	52,855
26	58,86		56	60,134		86	58,86		116	50,996
27	59,069		57	60,134		87	58,485		117	46,449
28	59,196		58	60,134		88	58,072		118	37,976
29	59,365		59	60,134		89	57,786		119	32,047
30	59,45		60	60,134		90	57,501		120	29,765

References

1. Anderson DE, Michael Rings D. Anderson DE, Rings DM, eds. *Food Animal Practice (Fifth Edition).* Saint Louis: W.B. Saunders; 2009:xv.

2. Stabel JR. Johne's Disease: A Hidden Threat. *J Dairy Sci*. 1998;81:283-288.

3. Sweeney RW. Transmission of paratuberculosis *Vet. Clin. North Am. Food Anim. Pract.* 1996;12:305-312.

4. Streeter RN. Isolation of Mycobacterium paratuberculosis from colostrum and milk of subclinically infected cows. *Am J Vet Res*. 1995;56:1322-1324.

5. McKenna SLB. Johne's disease in Canada Part II: Disease impacts, risk factors, and control programs for dairy producers. *Canadian veterinary journal*. 2006;47:1089-1099.

6. Sweeney RW. Mycobacterium paratuberculosis cultured from milk and supramammary lymph nodes of infected asymptomatic cows. *J Clin Microbiol*. 1992;30:166-171.

7. Stabel, J.R. and Goff, J.P. Efficacy of immunologic assays for the detection of Johne's disease in dairy cows fed additional energy during the periparturient period. *J Vet Diagn Invest*. 2004;16:412-420.

8. Stabel JR, Bradner L, Robbe-Austerman S, Beitz DC. Clinical disease and stage of lactation influence shedding of Mycobacterium avium subspecies paratuberculosis into milk and colostrum of naturally infected dairy cows. *J Dairy Sci*. 2014;97:6296-6304.

9. Nielsen SS, Bjerre H, Toft N. Colostrum and milk as risk factors for infection with Mycobacterium avium subspecies paratuberculosis in dairy cattle. *J Dairy Sci*. 2008;91:4610-4615.

10. Pithua P. Efficacy of feeding plasma-derived commercial colostrum replacer for the prevention of transmission of Mycobacterium avium subsp paratuberculosis in Holstein calves. *J Am Vet Med Assoc*. 2009;234:1167-1176.

11. Doré E, Paré J, Côté G, et al. Risk factors associated with transmission of Mycobacterium avium subsp. paratuberculosis to calves within dairy herd: A systematic review. *Journal of Veterinary Internal Medicine*. 2012;26:32-45.

12. Nielsen SS, Toft N. Effect of management practices on paratuberculosis prevalence in Danish dairy herds. *J Dairy Sci*. 2011;94:1849-1857.

13. Godden S, McMartin S, Feirtag J, et al. Heat-treatment of bovine colostrum. II: Effects of heating duration on pathogen viability and immunoglobulin G. *J Dairy Sci*. 2006;89:3476-3483.

14. Stabel JR. Pasteurization of colostrum reduces the incidence of paratuberculosis in neonatal dairy calves. *J Dairy Sci*. 2008;91:3600-3606.

15. Pithua P. Evaluation of the association between fecal excretion of Mycobacterium avium subsp paratuberculosis and detection in colostrum and on teat skin surfaces of dairy cows. *J Am Vet Med Assoc*. 2011;238:94-100.

16. Stabel JR, Hurd S, Calvente L, Rosenbusch RF. Destruction of Mycobacterium paratuberculosis, Salmonella spp., and Mycoplasma spp. in raw milk by a commercial on-farm high-temperature, short-time pasteurizer. *J Dairy Sci*. 2004;87:2177-2183.

17. Gao A, Mutharia L, Chen S, Rahn K, Odumeru J. Effect of Pasteurization on Survival of Mycobacterium paratuberculosis in Milk. *J Dairy Sci*. 2002;85:3198-3205.

18. Bradner L, Robbe-Austerman S, Beitz DC, Stabel JR. Chemical decontamination with N-acetyl-L-cysteine-sodium hydroxide improves recovery of viable Mycobacterium avium subsp. Paratuberculosis organisms from cultured milk. *J Clin Microbiol*. 2013;51:2139-2146.

19. Möbius P, Hotzel H, Raßbach A, Köhler H. Comparison of 13 single-round and nested PCR assays targeting IS900, ISMav2, f57 and locus 255 for detection of Mycobacterium avium subsp. paratuberculosis. *Vet Microbiol*. 2008;126:324-333.

20. Qiagen. *Taq PCR Handbook.* 3rd ed. ; 10/2010.

21. Gao A, Mutharia L, Raymond M, Odumeru J. Improved template DNA preparation procedure for detection of Mycobacterium avium subsp. paratuberculosis in milk by PCR. *J Microbiol Methods*. 2007;69:417-420.

22. Slana I, Kralik P, Kralova A, Pavlik I. On-farm spread of Mycobacterium avium subsp. paratuberculosis in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. *Int J Food Microbiol*. 2008;128:250-257.

23. Stabel, J.R., Lambertz, A. Efficacy of Pasteurization Conditions for the Inactivation of Mycobacterium avium subsp. paratuberculosis in Milk . *Journal of Food Protection*. 2004;67:2719-2726.