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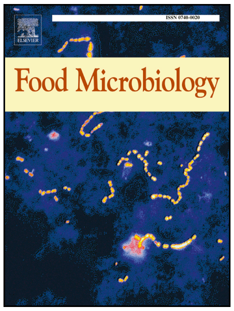

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Survival of *Mycobacterium avium* subspecies *paratuberculosis* in retail pasteurised milk

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Abstract

A survey of retail purchased semi-skimmed pasteurised milk (*n*=368) for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was conducted between May 2014 and June 2015 across the midlands of England using the Phage-PCR assay. Overall, 10.3 % of the total samples collected contained viable MAP cells, confirming that pasteurisation is not capable of fully eliminating human exposure to viable MAP through milk. Comparison of the results gained using the Phage-PCR assay with the results of surveys using either culture or direct PCR suggest that the phage-PCR assay is able to detect lower numbers of cells, resulting in an increase in the number of MAP-positive samples detected. Comparison of viable count and levels of MAP detected in bulk milk samples suggest that MAP is not primarily introduced into the milk by faecal contamination but rather are shed directly into the milk within the udder. In addition results detected an asymmetric distribution of MAP exists in the milk matrix prior to somatic cell lysis, indicating that the bacterial cells in naturally contaminated milk are clustered together and may primarily be located within somatic cells. These latter two results lead to the hypothesis that intracellular MAP within the somatic cells may be protected against heat inactivation during pasteurisation, accounting for the presence of low levels of MAP detected in retail milk.

Keywords

Mycobacterium avium subsp. *paratuberculosis; Johne's disease; human exposure; phage-PCR assay; pasteurised milk*

1.0 Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), a chronic wasting disease of cattle and other ruminants, characterised by a reduction in milk yield, severe diarrhoea, weight loss and intermandibular oedema (commonly referred to as bottle jaw). In addition to causing a significant health impact on the national herd, this disease has a significant economic impact on the dairy industry as a whole. MAP is an extremely slow growing bacterium, taking up to 8-12 weeks to grow in liquid culture media, which makes detection by traditional culture methods problematic (Sweeney et al., 2012). MAP is also implicated in the development of Crohn's disease (CD), an inflammatory bowel condition of humans with similar aetiology to JD (Bull et al., 2003; Feller et al., 2007; Rhodes et al., 2014), and although a causal relationship has not been fully established, it has been recommended that limiting human exposure would be sensible on a precautionary principle (NACMCF, 2010).

Cattle infected with MAP can shed substantial levels of the bacteria into their milk (up to 540 cfu ml^{-1}; Slana et al., 2008), faeces and semen (Antognoli et al., 2008), with milk being highlighted as a key transmission vehicle for human exposure to MAP, making the efficiency of pasteurisation an important factor in controlling transmission (Stabel and Lambertz, 2004). Although many studies have shown that the conditions used for pasteurisation are sufficient to inactivate MAP, some studies have shown that both low-temperature long-time (LTLT) and high-temperature short-time (HTST) pasteurisation did not totally inactivate MAP when present in milk at levels greater than 1 x 10^4 cfu ml^{-1} (Chiodini and Hermon-Taylor, 1993; Grant et al., 1996). This finding led to dairy processing centres to adjust the holding time of HTST pasteurisation from 15 s (the legal minimum in the UK), to 25 s to increase the likelihood of totally inactivating MAP. Despite this, MAP has been reported to survive the pasteurisation process and has been detected by culture in a number of surveys of retail pasteurised milk in different parts of the world with a prevalence ranging between

1.7 to 6.7 % of samples tested (Ayele et al., 2005; Grant et al., 2002a; Grant et al., 2002b; Paolicchi et al., 2012).

The detection of MAP has been historically difficult due to limitations in standard culture and PCR-based detection methods. Milk culture requires chemical decontamination to eliminate fast growing bacteria, but this also causes a 1-2 $\log_{10}$ drop in viable MAP cells, which in turn can decrease the chance of successful culture (Dundee et al., 2001). For pasteurised dairy products, PCR-based detection is less useful since it will detect any MAP DNA present in the sample, whether this is from viable bacteria or from cells that have been inactivated by the heat treatment. The bacteriophage amplification assay coupled with PCR (phage-PCR) has been developed to rapidly and sensitively detect viable MAP as an alternative to culture-based methods (Swift and Rees, 2013). This assay uses Mycobacteriophage D29 to infect any mycobacteria present in a milk sample, and any viable mycobacteria will be indicated by amplification of the bacteriophage within MAP cells and their subsequent lysis to release progeny phage and MAP DNA. PCR can then be used to confirm the identity of the mycobacteria detected (Stanley et al., 2007). The phage assay is faster than culture as results can be gained within 48 h, as well as being more sensitive than culture as there is no need for chemical decontamination. The phage assay has been successfully used to detect viable MAP in raw milk (Botsaris et al., 2010; Stanley et al., 2007), powdered infant formula (Botsaris et al., 2016), cheese (Botsaris et al., 2010) and blood of infected animals (Swift et al., 2013). In this study we used the phage-PCR assay to rapidly estimate the prevalence of MAP in retail pasteurised milk. In addition experiments were carried out to try and establish how MAP enters the milk and where the bacteria are located in naturally contaminated milk, and the pattern of results led to a hypotheses about how MAP may be able to survive pasteurisation.

2.0 Material and Methods

2.1 Collection of pasteurised milk samples

Three-hundred and eighty-six semi-skimmed milk (1.7 % fat) samples were collected at four time points (May 2014, November 2014, January 2015, and June 2015) using volunteers from the University of Nottingham Sutton Bonington campus; this type of milk was chosen as it was the most popular purchased by the volunteers providing the samples. The milk had been purchased from either retail suppliers or doorstop providers primarily within three UK counties (Nottinghamshire, Derbyshire, and Leicestershire). Volunteers were provided with a sterile 50 ml tube and detailed instructions on how to take the sample, including specifying that it must be taken from a previously unopened carton (carton size not specified) that was shaken to uniformly mix the contents before samples were taken and that the 50 ml sample should be delivered to the laboratory within two days of purchase. Details of the date of pasteurisation and retail supplier were also recorded, but are not discussed here.

2.2 Total viable count

The total viable count (TVC) was performed using milk count agar according to Botsaris et al. (2013). Serial dilutions of the milk sample were prepared and samples (100 µl) plated on plate count agar before incubating aerobically at 30 °C for 3 d prior to enumeration of colonies and calculation of cfu ml^{-1}.

2.3 Phage-PCR assay

Briefly, 25 or 50 ml of milk was centrifuged at 2,500 *x g* for 15 min to separate the pellet, milk and cream layers. The upper two layers were removed, and the pellet resuspended in 3 ml Modified Middlebrook 7H9 media plus (MP; Middlebrook 7H9 broth supplemented with 10 % OADC (oleic acid, bovine albumin, dextrose and catalase; Becton Dickinson, UK), NOA (nystatin, oxacillin and aztreonam Mole et al., 2007), and 2 mM $CaCl_2$) and centrifuged at 2,500 *x g* for 10 min. The resulting pellet was finally resuspended in 1 ml of MP and mycobacteriophage D29 (100 µl of 10^9 pfu ml^{-1}

phage suspension) added to the sample. After incubation at 37 °C for 1 h a virucide (100 µl of 10 mM ferrous ammonium sulphate) was added and the sample incubated for 6 min at room temperature to destroy any exogenous phage. To inactivate the virucide 5 ml of MP was added, and the sample finally plated with *M. smegmatis* (1 ml of 10^8 cfu ml^{-1} culture grown in MP) and 6 ml of Middlebrook 7H10 agar. Plates were then incubated for 24 h and plaques enumerated. DNA was extracted from plaque agar using Zymoclean Gel DNA Recovery kit (Zymo Research) and the presence of MAP DNA detected by amplification of MAP-specific IS*900* region using a nested-PCR (Bull et al., 2003) with particular care being taken during sample preparation and handling to ensure that cross-contamination did not occur. As controls, plaques generated from the detection of *M. smegmatis* (non-MAP DNA control) and sterile water (no template control) were included for all sets of PCR reactions. Genomic MAP (K10) DNA was used as an additional positive control. Only samples that produced a positive IS*900* plaque-PCR result were classified as containing viable MAP cells.

2.4 Investigation of location of MAP cells in milk

One litre of raw milk was taken and split into twenty 50 ml samples and ten of these were tested using the phage assay (Section 2.3) for the presence of mycobacteria (termed 'whole' samples). Prior to conducting the phage assay pellet is washed with MP which lyses any somatic cell present and releases intracellular mycobacteria (Swift et al., 2013, Donnellan et al., 2017). The other ten samples were prepared for the phage assay in the same way, but after resuspending in MP each of these samples were further split into two 25 ml portions (termed 'paired' samples) and each was tested separately using the phage assay (Section 2.3).

2.5 Statistical Analysis

Pfu and cfu counts were compared using Pearson correlation and MAP status (positive or negative) with total viable count (TVC) by performing a t-test in IBM SPSS Statistics 22 (SPSS Inc., Chicago, USA). Further analysis of the correlation between the pfu counts in the paired split bulk tank milk samples was conducted in Microsoft Excel 2010. Details of the statistical methods used to determine the complete spatial randomness (CSR) of cells in bulk tank milk are provided in the text.

3.0 Results and Discussions

3.1 Detection of MAP in pasteurised milk

The phage-PCR assay detected viable MAP in 10.3 % (37/368) of the pasteurised milk samples collected as defined by the presence of plaques which gave a positive IS*900*-PCR result (see Figs. 1 & 2). Figure 1 shows the number of MAP-positive samples recorded at each of the four sampling time points (approximately 90 samples at each time point). The results show that although the overall average percentage of MAP-positive samples detected was 10.3 %, there was a large variation in the number of positive samples detected at each time point (range 1-27 %), but there was no seasonal pattern. This is consistent with the results of Grant et al. (2002) who detected variable levels of MAP in retail pasteurised milk using a direct PCR-method (average 10 %, range 0-27 %; Grant et al., 2002a).

Since each plaque arising from the phage-PCR assay represents detection of one viable MAP cell, counting the plaques allows enumeration of MAP as well as absolute detection. Figure 2 shows the distribution of plaque numbers detected in MAP-positive samples collected throughout the survey. Only 1.1 % of these contained more than 10 detectable MAP cells per 50 ml (range 10-32). It is known that chemical decontamination methods reduce the number of MAP cells detectable by culture by least 1 $\log_{10}$ giving a low limit of detection (Bradner et al., 2013). Therefore it is unlikely that samples that contained less than 10 MAP cells per ml would have given a culture-positive result in previous raw milk studies. On this basis, given that other published surveys of retail pasteurised milk generally report levels of detection in the 1-2 % range (Grant et al., 2002a), our results are in line with previous culture-based studies.

Direct PCR detection of MAP in milk is believed to be more sensitive than culture, but even then reported limits of detection are relatively high, ranging from 15-50 cfu per 50 ml (Gao et al., 2007) to 10^5 cfu ml^{-1} (Grant et al., 2000). However methods have been reported for direct PCR detection of MAP in faeces with a sensitivity as low as 2.4 cfu per g of sample (Logar et al., 2012) and therefore detection of very low numbers of cells is possible from complex samples. In this study, 3.5 % of MAP-positive samples (1.1 % >10 plus 2.4 % 3-9) contained more than 2 cells and therefore would theoretically be detectable by direct PCR. Previous published surveys of retail pasteurised milk samples by direct PCR have reported a prevalence of MAP detection of between 7 and 12 % (Grant et al., 2002b), thus our results are within the range predicted by the published literature. However it must be noted that direct PCR-methods used in these other surveys do not discriminate between viable cells and cells that have been inactivated by pasteurisation, and therefore the MAP-positive samples would also include samples where DNA from inactivated cells were detected, perhaps explaining the high prevalence reported in some of these direct PCR studies.

The largest percentage of samples in our dataset (6.8 %) contained 1-2 detectable MAP cells. Given that these samples contained fewer cells than the reported limit of detection of either culture or PCR-based detection, this result suggests that the level of MAP present would not be detected by either of these methods and that the phage-PCR assay is a more sensitive technique. This is consistent with our previous findings when low levels of MAP were detected in blood samples from infected animals that could not be detected by either culture or using a commercial direct PCR kit (Swift et al., 2013). Similarly in two recent surveys of the prevalence of viable MAP cells in powdered milk, the phage-based methods always detected more positive samples than either culture or direct PCR (Botsaris et al., 2016; Grant et al. 2017).

Commercial pasteurisation has been shown by many surveys of retail milk to not be sufficient to inactivate all MAP cells present in raw milk. Although determinations of D-values indicated MAP may survive HTST pasteurisation when the initial organism concentration is greater than 1 x 10^4 cells ml^{-1} (Grant et al., 1996), many other studies have shown that the cells are not intrinsically resistant to heat and pasteurisation should result in a seven-fold reduction in viable MAP (Rademaker et al., 2007). However Grant et al. (1996) has previously reported that after an initial rapid drop in viable cell number, a 'long tail' in inactivation curves is seen whereby low numbers of viable MAP cells in a sample survive, even after heating for an extended period of time. This type of kinetics indicates that there is a heat-resistant sub-population, and would be consistent with our observation that the largest number of samples contained only 1-2 detectable MAP cells. One possible explanation for this phenomenon could be if the MAP cells were internalised within somatic cells which provided some protection against heat inactivation.

3.2 Evidence that MAP in milk is primarily intracellular

3.2.1 Comparison of TVC and Phage-PCR

It has been well established that low level faecal contamination of milk can occur during routine milking practises (Vissers et al., 2007), therefore providing a route of entry for MAP into raw milk, and it would be expected that these cells would be released into the milk along with other enteric bacteria. Since we have previously shown that MAP present in the circulating blood is located within white blood cells (Swift et al., 2013), MAP cells entering the milk from the udder may be expected to be intracellular, either within infected somatic cells or macrophages. Total viable count is a common method used to determine the level of faecal contamination and therefore hygienic status in milk. To provide evidence that the MAP cells being detected in the somatic cell pellet represented cells being shed directly into the milk rather than being introduced by faecal contamination, a comparison was made of the TVC of bulk tank milk samples and levels of MAP detected using the phage-PCR assay. The samples were collected from 225 separate farms as previously described by Botsaris et al. (2013). Since the phage D29 has a very broad host range the phage assay results (plaque number) report on the total number of viable mycobacteria present in a sample. Figure 3a shows a comparison of the TVC results for all samples that were found to contain mycobacteria due to the formation of plaques (n = 218). These samples were then further stratified in to MAP-positive and MAP-negative as determined by the detection or non-detection of the *IS*900 genetic element by PCR in the DNA extracted from plaques, respectively. The number of plaques detected for the samples in these two groups were then compared with the TVC results for each of the samples (Fig. 3b). As there was no significant difference in the mean TVC value of the MAP-positive samples and those that contained mycobacteria but with no MAP detected (approx. 1×10^4 cfu ml^{-1}; $p = 0.276$), this suggests that there is no relationship between the likelihood that MAP will be detected and the level of faecal contamination. Therefore the presence of MAP does not appear to correlate with the hygienic status of the milk, suggesting that faecal contamination is not the main route of entry of the MAP into the bulk milk. Similarly when TVC and the plaque number results of the individual MAP-positive samples are compared, there is no correlation ($p = 0.270$) between the number of plaques detected in a MAP-positive sample and the TVC (Fig. 3b) again indicating that a high plaque count, which is associated with a high probability that MAP will be present in a sample (Botsaris et al., 2013), is not due to faecal contamination. These results are in agreement with a previous study that showed that there was no correlation between the total bacteriological count, somatic cell count and the detection of IS*900* sequences by PCR in a survey of bulk tank milk (Corti and Stephan, 2002) and therefore faecal contamination alone cannot account for the presence of MAP in raw milk and rather suggests that these cells are being directly shed into the milk.

3.2.2 Evidence for asymmetric distribution of MAP in raw milk due to intracellular location

If MAP is not present in the milk due to faecal contamination, it must have entered the milk by a different route. As an intracellular pathogen, one such route is via infected somatic cells which are shed into the milk, and in this case it is predicted that the cells would not be free in the milk matrix, but would be clustered together inside the infected cells, since intracellular MAP have been shown to located together inside vacuoles (Bannantine and Stabel, 2002).

To provide evidence that the MAP being detected in raw milk were primarily intracellular, raw bulk tank milk was obtained which was likely to contain viable MAP cells from a farm with a known Johne's disease problem. To investigate the spatial distribution of MAP, the variation in number of mycobacteria present in individual samples taken from bulk tank milk was investigated. To do this ten 50 ml samples were taken from one litre of raw milk and were tested using the standard phage assay method for the presence of mycobacteria (termed 'whole' samples). To determine if variability in the number of Mycobacteria detected was due an asymmetric distribution of cells in the milk rather than the sampling method, another ten samples were prepared for the phage assay, but after the pellet had been washed with MP which lyses any somatic cells and releases the mycobacteria into the media, each of these samples was then further split into two portions (termed 'paired' samples) and each of these was tested separately using the phage assay (see Fig. 4A for experimental plan). The results in Figure 4B shows that for each of the two groups of samples ('whole' or 'paired'), the median number of mycobacteria detected per 50 ml was the same (50 vs 49) and the range of the plaque numbers detected within each group of samples was also very similar (range for 'whole' samples = 16-145 per 50 ml; range for paired samples = 13-131 per 50 ml). These results showed that processing the samples in two different ways did not affect the overall number of mycobacteria detected per 500 ml but that there was quite a large range in the number of mycobacteria detected per 50 ml sample, typical of the pattern seen when low numbers of cells are asymmetrically distributed in a sample.

When the numbers of mycobacteria detected in the paired samples is examined, it can be seen that although there is still variation in the number of mycobacterial detected in individual 50 ml samples, there is a good correlation between the number of mycobacteria detected for each of the pairs when individual 50 ml samples were further split into two samples (r^2 = 0.79; Fig. 4C), showing that the method reproducibly detects mycobacteria in samples predicted to contain the same number of free Mycobacteria cells.

It is not possible to carry out the same simple correlation analysis for the number of mycobacteria detected in the replicate 'whole' samples as there is no non-arbitrary method that can be used to pair them. Hence two statistical analyses were performed to examine a null hypothesis of complete spatial randomness (CSR) of cell distribution within the bulk milk when samples are split into sub-samples by these two different methods. The first analysis examined the number of bacteria present in each of the 50 ml samples, and the data set used consisted of ten 'whole' samples, and (by summing the counts in each of the two paired samples) ten 'paired samples', giving 20 samples in total. To analyse the initial allocation into 50 ml sub-samples, a Pearson Chi-squared test was used, in which there are 19 d.f. and the predicted number of cells in each sample would be 59.4 (i.e. the total number of cells detected divided by 20). This test essentially assumes that the counts in each subsample are drawn from a Poisson distribution with mean equal to 59.4. The result of this analysis was that this null hypothesis of CSR across the subsamples was strongly rejected ($p<0.001$) and suggests that the distribution of mycobacteria present in the bulk tank milk is uneven so that when an individual 50 ml sample is taken there is likely to be a significant variation in the numbers of mycobacteria detected.

The second analysis used a permutation test to compare the total number of mycobacteria detected in the 'paired' samples with that expected from a random (binomial) allocation. Expressing each of the 10 allocations as a proportion $p_i=N_{1i}/N_{Ti}$ of the total $N_{Ti}=N_{1i}+N_{2i}$ of the two halves for that sample, 10,000 sets of equivalent proportions were generated from a binomial distribution. Each of these sets takes the form $p_i^*=r_i/N_{Ti}$ where r_i~$B(N_{Ti},0.5)$ for $i=1,\ldots 10$, thus being a random allocation of the same total count as the corresponding sample. For each of these sets, a summary statistic - in this case the variance of these proportions - is calculated. The position (order statistic) of the variance of the proportions from the data within this list of 10,000 variances from the binomial allocations, gives the p-value. As a two-tailed test is to be used, the smaller of the two order statistics are taken and doubled to obtain the final p-value, which was p=0.625. Thus for the 'paired' samples the null hypothesis of randomness cannot be rejected, since the evidence of randomness seen in the initial analysis is no longer present after this lysis step has been carried out to release the mycobacteria into the media.

The difference in the treatment of the 'whole' and 'paired' samples was rather than resuspending the pellet in 1 ml and independently testing each 50 ml 'whole' sample, the pellet from the 'paired' samples was resuspended into 2 ml of media which lyses the somatic cells releasing the Mycobacteria into the media before further splitting into two samples (see Fig. 4A). The overall plaque number detected per 50 ml sample was not affected by the change in sample preparation method (Fig. 4B), this pattern of results could be best explained if the mycobacteria in the original milk were not free in the milk matrix but were located inside somatic cells, explaining the heterogeneous distribution of MAP cells in the 50 ml milk samples. This hypothesis is consistent with the fact that Patel et al. (2006) have shown that bovine mammary epithelial cells can contain up to 10^4 MAP cells after infection, and MAP are known to be able to replicate within macrophage (Arsenault et al., 2014), so it is also likely that MAP-infected somatic cells will not contain only a single bacterium.

The results of the survey carried out in this study, and the results of other published surveys of retail pasteurised milk, clearly show that MAP in retail milk is able to survive pasteurisation. Laboratory studies of heat inactivation of MAP milk have consistently shown that HTST pasteurisation conditions are effective at reducing the levels of viable MAP by 3-6 $\log_{10}$ (McDonald et al., 2005). Since levels of MAP previously reported in milk from infected cows are generally not reported to be more than 10^2 cfu ml^{-1} MAP (Slana et al., 2008; Sweeney et al., 1992), pasteurisation should achieve complete inactivation of MAP. However these heat inactivation studies used laboratory grown bacteria that were artificially added to milk. Our results suggest that MAP in raw milk is primarily located inside somatic cells which are made up of polymorphonuclear leukocytes, macrophages, lymphocytes and a small number of mammary epithelial cells (Boutinaud and Jammes, 2002). Despite the fact that it has never been demonstrated that MAP cells found intracellularly are resistance to heat, it has previously been shown that MAP surviving inside amoeba are approximately 2-fold more resistant to chorine disinfection (Whan et al., 2006) and this in part is due to the physical protection provided by the large mass of the host cell surrounding the bacteria (amoeba = 15-35 µm). Although further experiments are required to confirm this hypothesis, it is therefore possible that the survival of low levels of MAP during pasteurisation of naturally infected milk could be as a result of their intracellular location, with the somatic cell providing sufficient protection to prevent complete inactivation of the bacteria by heat treatment. If some of these infected cells do contain high levels of MAP cells (Patel et al., 2006), this would increase the likelihood that the low numbers of survivors detected after pasteurisation represent a residual population of cells that have been physically protected from the heat treatment, especially as it has been previously reported that high numbers of MAP cells can infer heat resistance during pasteurisation (Klijn et al., 2001).

4. Conclusion

The possible association between MAP and Crohn’s disease has been discussed for many years (Feller et al., 2007; Rhodes et al., 2014), with pasteurised milk thought to be a key vehicle of transmission to humans. One of the advantages of using phage to detect bacteria is that the bacterial cell must be viable before detection can occur and therefore, unlike PCR-based methods, the phage-based method differentiates between live and dead cells (Stanley et al., 2007; Swift et al., 2014). This makes the method very useful for studying pasteurised products which could contain both viable and inactivated MAP cells. In this study the phage-PCR assay was shown to be able to detect low levels of viable MAP in pasteurised milk with 10 % of the samples found to contain viable

MAP. The prevalence of MAP was higher than that reported in a number of other published studies that used culture to detect MAP (1.7 to 6.7%) but this does not suggest that our samples had a higher prevalence rate, just that the phage-PCR assay is more sensitive and therefore increases the frequency with which samples containing very low numbers of MAP cells give a positive result. The specificity of the assay is demonstrated by the end-point PCR, which has been previously demonstrated as being specific for MAP (Bull et al., 2003). Given the rapidity of this method, there is now potential to perform a larger trial to ascertain ability of this method to monitor the efficacy of milk pasteurisation processes on a larger scale. There is a clear need for further research to be carried out to fully understand this hypothesised mechanism of survival and the fact that mycobacteria that reside inside eukaryotic cells can be protected from external sources of stress. Here we have demonstrated the ability to rapidly detect and enumerate MAP using the phage assay providing a useful tool to allow such studies to be completed without the need for prolonged incubation of cultures required when using traditional culture methods.

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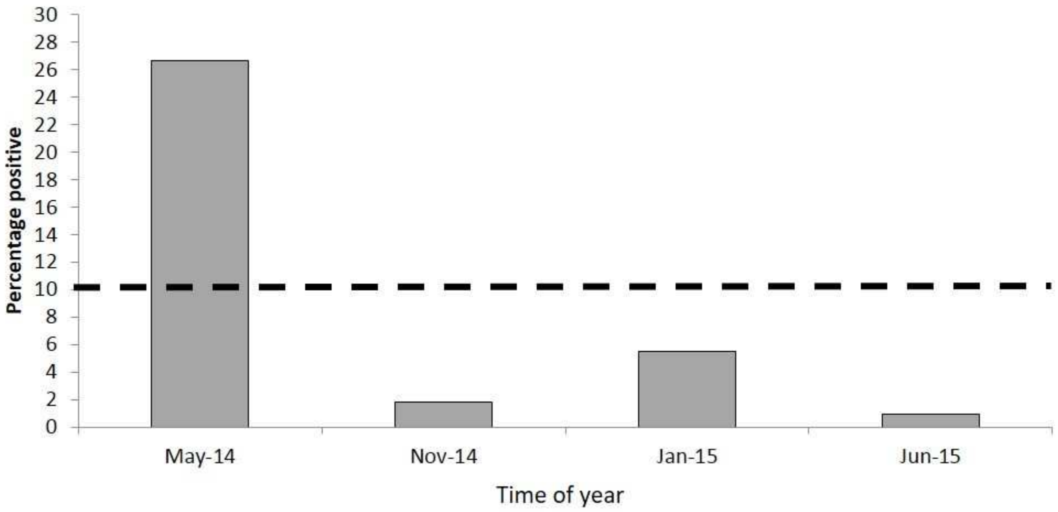
Figure 1. Seasonality of MAP positive samples isolated from pasteurised milk
Dashed line represents the average percentage prevalence of viable MAP detected in pasteurised milk over the four sampling periods. The graph shows the seasonal variation in the frequency of MAP-positive samples detected using the phage-PCR assay.

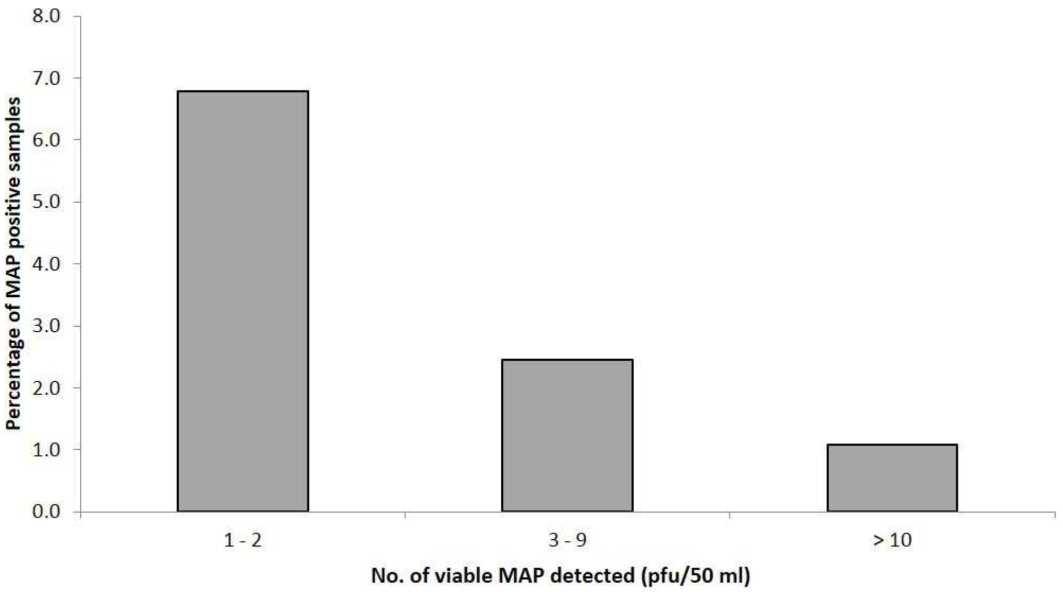
Figure 2. Distribution of the number of viable MAP samples across the whole survey
A total of 38 MAP-positive samples were detected, defined as those that produced plaques that were IS900-PCR positive. Data were grouped by plaque number per 50 ml sample and the results for each group reported as percentage of the total sample set (n = 368 semi skimmed (1.7 % fat) milk). The groupings were based on the reported sensitivities of other methods *i.e.* 1-2 cells, no other method reports being able to detect this low level, 3-9 cells and >10 cells, potentially detectable by direct PCR; >10 cells, potentially detectable by culture.

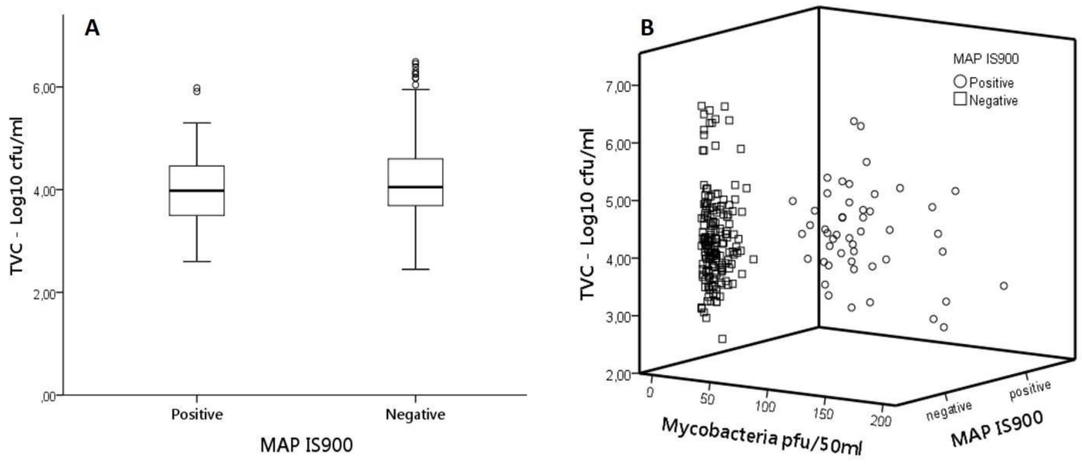
Figure 3. Comparison of MAP status and TVC of bulk milk samples

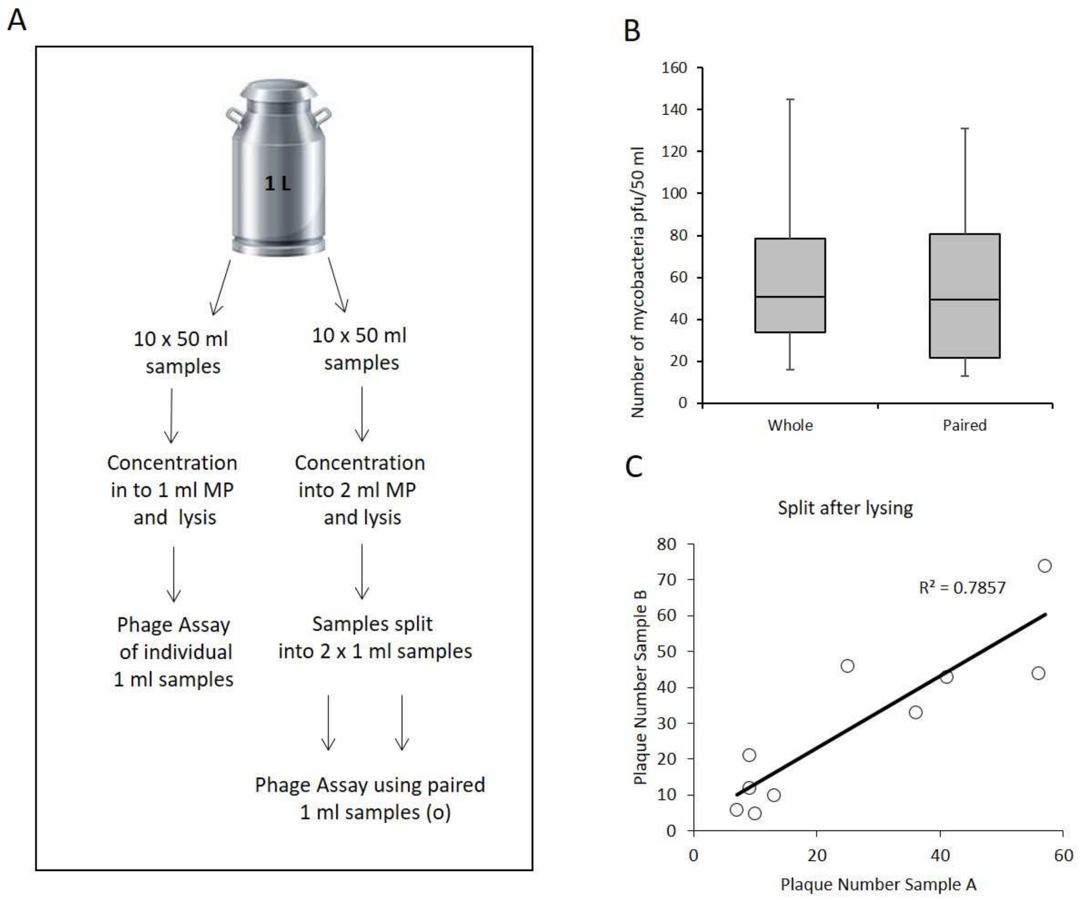
Samples of raw bulk tank milk (n = 225) were tested using the phage-PCR assay and the TVC of each sample was separately determined. Samples were classified as MAP-positive or MAP-negative according to the results of the Phage-IS*900* PCR assay. Panel A. For each data set, the distribution of TVC values is represented by a Box and Whisker plot. An independent samples t-test revealed no significant difference between the TVC results of the MAP-positive samples and the MAP-negative samples (p=0.276). Panel B. MAP-positive samples are shown as open circles and MAP-negative samples are shown as open squares. For each group, the distribution of TVC values is compared with the plaque count for individual samples. For the MAP-positive samples, a Pearson correlation indicated no significant relationship existed between these two variables (p=0.270).

Figure 4. Distribution of viable MAP in whole milk samples with and without somatic cell lysis
Panel A. One litre of raw milk split into twenty 50 ml samples. Ten of these were tested using the phage assay for the presence of mycobacteria ('whole' samples). The other ten samples were centrifuged, and the pellet resuspended in 2 ml MP to lyse the somatic cells. This sample was further split into two 1 ml samples ('paired' samples) before each one was tested separately using the phage assay. Panel B. Box and Whisker plot showing the median number and range of mycobacteria detected for 'whole' (mean 50, range 16 to 145) and 'paired' (mean 49, range 13 to 131) samples for the two different treatments. To normalise the data the results for the two different treatments for the 'paired' samples were multiplied by 2 as the equivalent of 25 ml and not 50 ml had been tested, before determining the median and range of data. Panel C. Plot to determine the correlation between plaque numbers detected in individual pairs of samples. Circles show the pfu results for the paired samples per 25 ml ($r^2 = 0.79$).


30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Percentage positive
May-14
Nov-14
Jan-15
Jun-15
Time of year


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Percentage of MAP positive samples
1 - 2
3 - 9
> 10
No. of viable MAP detected (pfu/50 ml)


A
TVC - Log10 cfu/ml
6,00
4,00
2,00
,00
Positive
Negative
MAP IS900
B
MAP IS900
Positive
Negative
TVC - Log10 cfu/ml
7,00
6,00
5,00
4,00
3,00
2,00
0
50
100
150
200
Mycobacteria pfu/50ml
negative
positive
MAP IS900


A
1 L
10 x 50 ml samples
10 x 50 ml samples
Concentration in to 1 ml MP and lysis
Concentration into 2 ml MP and lysis
Phage Assay of individual 1 ml samples
Samples split into 2 x 1 ml samples
Phage Assay using paired 1 ml samples (o)
B
Number of mycobacteria pfu/50 ml
0
20
40
60
80
100
120
140
160
Whole
Paired
C
Split after lysing
R² = 0.7857
Plaque Number Sample B
0
10
20
30
40
50
60
70
80
Plaque Number Sample A
0
20
40
60

Highlights

- Survey of UK retail pasteurised milk for presence of viable MAP
- Use of phage-PCR to rapidly and sensitively detect viable MAP in milk
- Presence of viable *M. paratuberculosis* observed in 10 % of samples
- Intracellular MAP may aid their survival during pasteurisation