Rapid Purification of DNA with High PCR Efficiency from Mastitis Bacteria in Milk Using Silicon Carbide

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Abstract

Background:

Mastitis is the most damaging disease in the dairy cattle industry. The inflammation of the utter, mainly caused by various bacteria, leads to reduced milk quality and yield. Quick, sensitive and accurate detection of the causal bacteria is therefore needed to provide efficient control. DNA/PCR-based protocols for milk bacteria identification have been developed in recent years to replace the more laborious microbiological culture. However, the isolation of high quality bacterial DNA from milk was proved to be difficult. Inhibitors like proteins were commonly co-purified and hindered downstream applications like PCR. Moreover, while most studies focused on very mastitic samples, isolating DNA from small quantity of bacteria during the early stages of the disease was not examined extensively. The objectives of the research were to study the efficiency and sensitivity of a new silicon carbide-based method in isolating bacteria DNA from bovine milk and to evaluate the quality of the DNA isolated using various methods of PCR.

Methods:

20 raw bovine milk samples were collected. Bacterial DNA was isolated using a silicon carbide-based or a phenol:chloroform-based method. The quantity of DNA isolated was compared using gel electrophoresis. PCR with rRNA primers was used to compare the PCR success rate of all DNA samples. Quantitative PCR was used to assess the sensitivity of the DNA purification.

Results:

More bacterial DNA was isolated from raw bovine milk samples using the silicon carbide method than the organic solvent method. In addition, using E coli-spiked milk, it was demonstrated by qPCR that the current method could isolate DNA from as little as 10 cfu E coli in 1 mL of milk. Comparative end-point PCR studies showed that bovine milk bacterial DNA isolated using silicon carbide was of high quality. The PCR success rate using a general 16s rRNA specific primer was >90% for silicon carbide, ~50% for phenol:chloroform and <15% for using raw milk directly.

Conclusion:

A new method based on silicon carbide was demonstrated to isolate high quality, PCR-ready DNA and provide a sensitive protocol for bacterial detection in bovine mastitis.

Introduction

- Mastitis is the most damaging disease in the dairy cattle industry. The inflammation of the utter is mainly caused by bacteria including Staphylococcus aureus, Streptococcus agalactiae, and other coliform species such as Escherichia coli (1).

- Traditionally, the identification of the causal agent of mastitis involves microbiological culture; however this method is very time-consuming. In addition, an extensive amount of work may be required to identify a specific bacterial strain using this method. In light of such drawbacks for culturing techniques, molecular approaches have now become increasingly popular for bacterial identification of utter infections. In particular, the isolation of milk bacterial DNA followed by polymerase chain reaction (PCR) has become a standard diagnostic procedure. For example, the 16S rRNA gene and the 16S-23S rRNA spacer region of the bacterial genomic DNA have been commonly targeted for bacterial DNA PCR amplifications (1).

- While molecular approaches have contributed significantly to the identification of the causal agent of mastitis, a few drawbacks still exist. The aforementioned methods are sensitive to sample contamination, particularly during sampling handling. Many standard procedures require the use of organics such as phenol:chloroform for DNA extraction, which are considered hazardous (2). Moreover, inhibitors are commonly co-purified with the DNA, particularly in mastitis milk samples, and these inhibitors may affect the activity of the DNA polymerase used in PCR and qPCR. Finally, while most recent research has focused on improved quality of DNA isolation, the sensitivity of isolation methods has been overlooked.

- The goals of our research were to develop a sample preparation method for mastitis by:

    1. Isolating milk bacterial DNA with a minimal amount of handling and without the use of any organics

    2. Isolating milk bacterial DNA from a limited amount of input.

Methods

- A method to isolate bacterial DNA from milk samples based on the use of silicon-carbide was developed. Briefly, bacterial cells in the milk were pelleted and lysed using a Lysis Solution and Proteinase K. After incubation the lysate was loaded onto silicon carbide spin columns and the bacterial DNA was bound to the columns. The columns were then washed twice to remove any impurities and the bound DNA was eluted. For more details about the procedure please refer to the manual provided with Norgen's Milk Bacterial DNA Isolation Purification Kit (Cat # 21500).

- To test the developed method, 650 µL milk samples were spiked with 1.5×10^6 and 1.5×10^4 cfu of E. coli. The bacterial DNA was isolated using the silicon carbide-based method

and the DNA was run on a 1% agarose gel for visual inspection. Milk samples were also spiked with 1.5 x 10^6 E. coli cells and 1.5 x 10^6 B cereus cells to test the ability of the method to isolate DNA from Gram positive and Gram negative bacteria

- To test the sensitivity of the method milk samples were then spiked with decreasing amounts of E coli (10^7, 10^5, 10^3, 10^1 cells) and the bacterial DNA was isolated using the silicon-carbide based method. The isolated DNA was then used as the template in a qPCR reaction to detect the E. coli using a Bio-Rad iCycler Thermal Cycler. The DNA was also used as the template in an end-point PCR reaction, and the PCR products were run on a 1% agarose gel.

- To compare the silicon carbide-based method with traditional methods 18 raw bovine milk samples were collected from 18 different animals. Bacterial DNA was then isolated from 400 µL of each sample using two different methods:

    1. Silicon-Carbide Based Method (as described above).

    2. Tradtional Phenol:Chloroform Based Method. Bacterial cells in the urine were pelleted and treated with Proteinase K. After incubation the DNA was isolated using tradtional phenol:chloroform extraction followed by ethanol precipitation.

- The DNA isolated using the 2 different methods was run on 1% agarose gels (15 µL out of each 100 µL elution) for visual inspection and comparison.

- To test for the co-purification of inhibitors, the DNA isolated using the 2 different methods was then used to spike a non-related PCR reaction which amplifies the plasmid pCMVB. PCR reactions were set up which contained all the components including the template for the PCR reaction, and 1 µL of each elution was then added to the reactions to test for inhibitors. Each PCR was performed in duplicate. The PCR products were then run on a 1% agarose gel to look for inhibition.

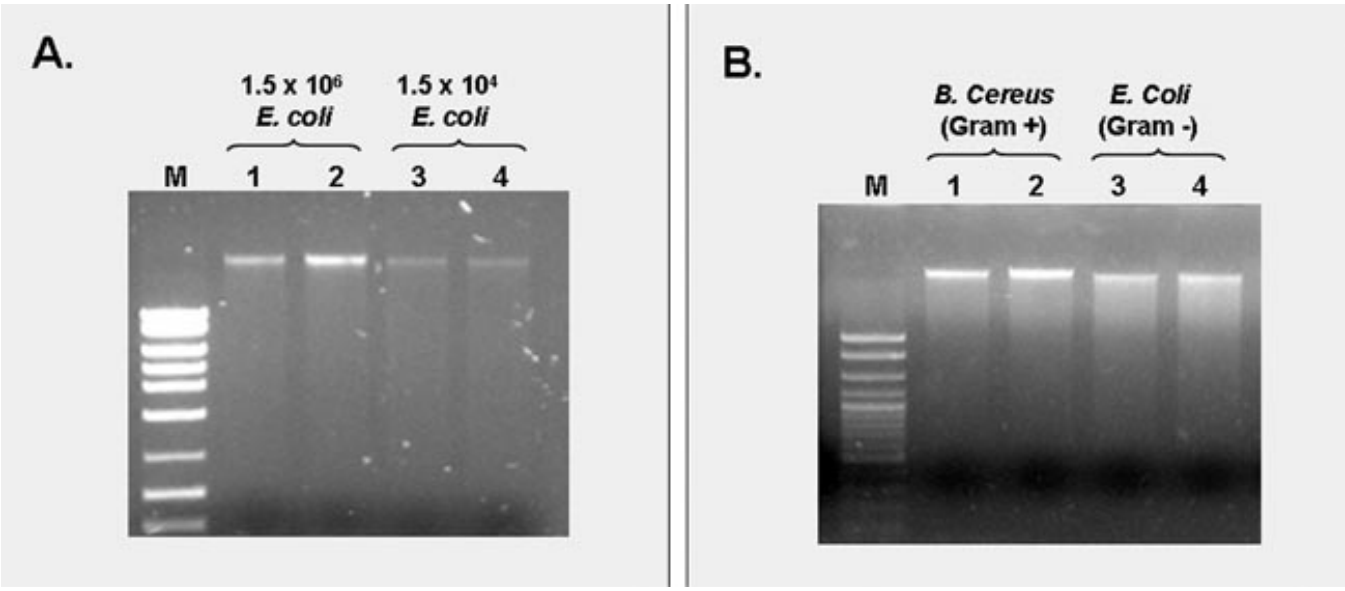

Figure 1: Isolation of Bacterial DNA from Both Gram Positive and Gram Negative Bacteria Found in Milk Using a Silicon-Carbide Based Method. 650 µL milk samples were spiked with the either 1.5 x 10^6 or 1.5 x 10^4 E. coli cells (cfu/mL). The silicon-carbide based method described in the Methods section was then used to isolate the bacterial DNA. As can be seen in Panel A, the method successfully isolated the bacterial DNA from these samples, and it can be detected on a gel. In order to further test the method, milk samples were spiked with 1.5 x 10^6 cfu's of E. coli or B. cereus, and again the method was used to isolate the bacterial DNA. From observing Panel B, it can be seen that the silicon-carbide based method can successfully isolate the bacterial DNA found in both Gram negative and Gram positive bacteria found in milk.

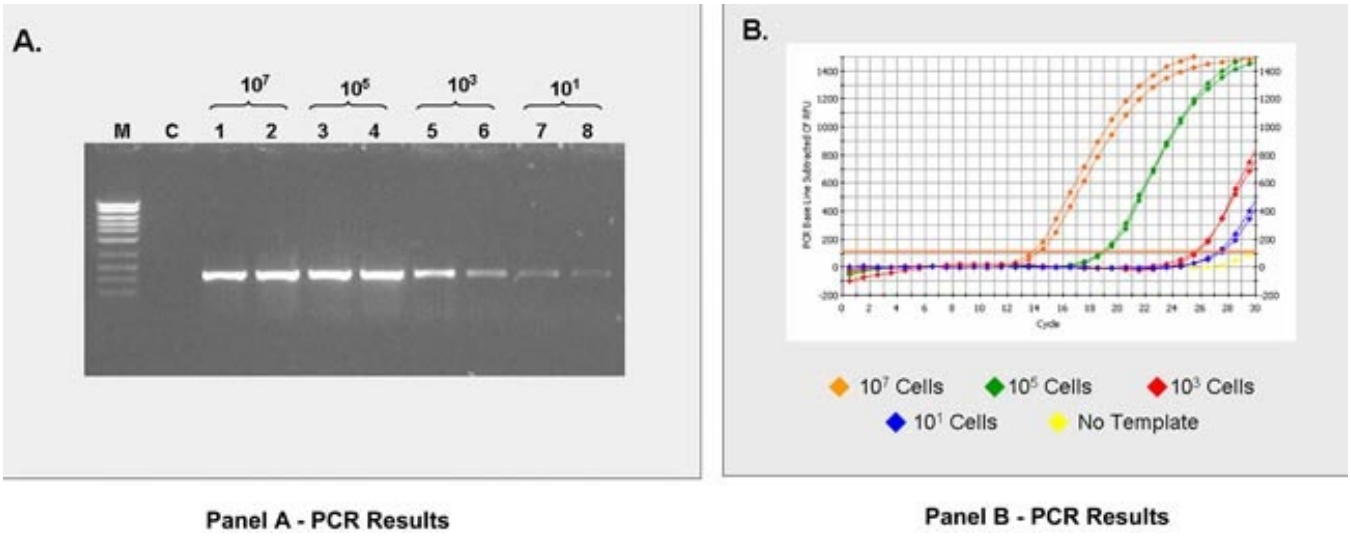

Figure 2: High Sensitivity of Bacterial Genomic DNA Isolation from 1 mL Milk Samples. Decreasing amounts of E.coli were spiked into 1 mL samples of pasteurized milk (10^7, 10^5, 10^3, 10^1) and the bacterial DNA was isolated using the silicon carbide-based method as outlined in

the Materials section. The DNA was then subjected to PCR using E.coli-specific primers which recognize the 16S rRNA gene. The resulting PCR products were run on a 1% agarose gel for visualization, and can be seen in Panel A above. The number of E. coli cells used to spike the milk is indicated above the gel. Lane M contains the marker, and Lane C contains the non-template control. It can be seen that the PCR reaction resulted in the expected 544 bp PCR product in all cases, even when as little as 10 E. coli cells were used for spiking. Since the milk used was pasteurized, no bacterial cells were initially present (as verified by a lack of bacterial growth on LB agar plates - data not shown). Therefore, this method is sufficiently sensitive to isolate and detect as little as 10 bacterial cells in 1 mL of milk. Small aliquots of the milk bacterial DNA were also amplified in a real-time PCR reaction, and the Ct graph generated can be seen in Panel B. Again, it can be seen that DNA can be isolated and detected from as little as 10 E. coli cells in 1 mL of milk. Thus, the silicon carbide-based method for the isolation of bacterial DNA from milk is extremely sensitive and can be used.

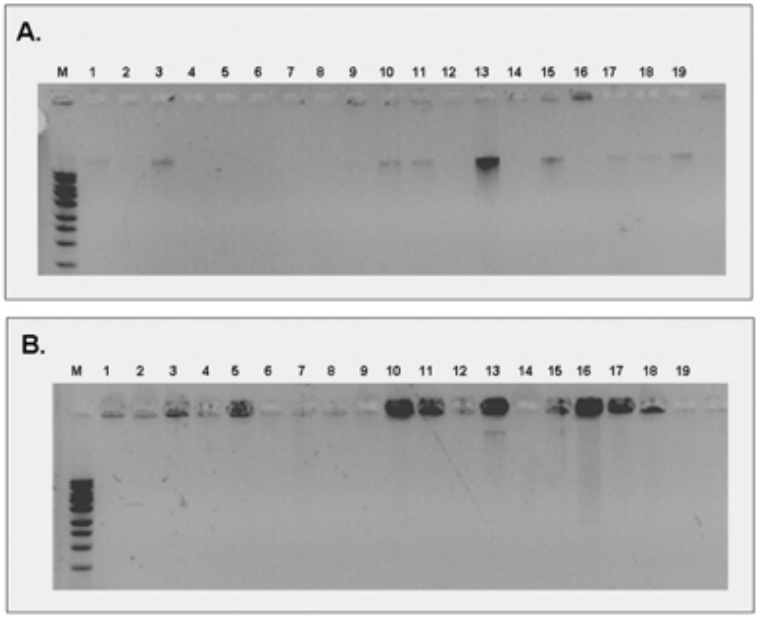

Figure 3: Comparison of the Silicon Carbide-Based Method and Phenol:Chlorofom Method for Bacterial DNA Isolation from Milk. Bacterial DNA was isolated from 400 µL of 20 different raw mastitis milk samples using the method based on silicon carbide (Panel A) and the traditional method of using phenol:chloroform extractions (Panel B). Aliquots of 19 of the different samples were run on 1% agarose gels for visual inspection. From observing the agarose gels above, it can be seen that the silicon carbide-based method resulted in higher yields of genomic DNA with minimal amounts of RNA or protein contamination (Panel A). The phenol:chloroform method resulted in very low yields of genomic DNA (Panel B), with high amounts of contaminating proteins appearing in the wells. Furthermore, the phenol:chloroform method is a much longer procedure, and relies on the use of harmful chemicals. Thus the silicon carbide-based method for bacterial genomic DNA isolation is superior to phenol:chloroform extraction in terms of yeild, ease of use and speed.

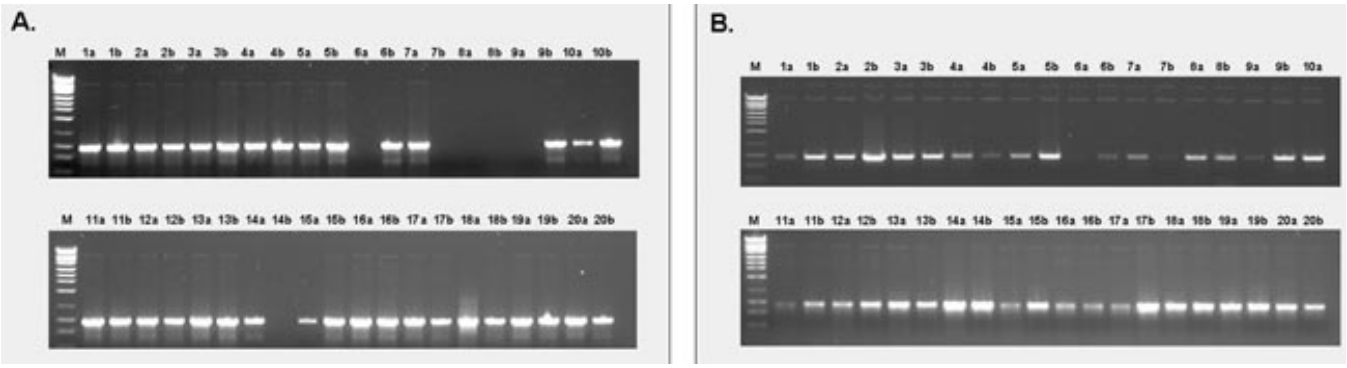

Figure 4: Comparison of PCR Compatibility of Milk Bacterial DNA Isolated using Silicon Carbide-Based Method and Phenol:Chloroform Method. The milk bacterial DNA isolated from the 20 different raw mastitis milk samples was used to spike non-related pCMVB PCR reactions in order to detect the presence of inhibitors. A PCR reaction was considered inhibited if either one of the duplicate samples showed a band intensity lower than the control on an agarose gel. Panel A shows the results from samples 1 - 20 isolated using the silicon carbide method, and Panel B shows the results from samples 1 - 20 isolated using the phenol:chloroform method. For the DNA isolated using the silicon carbide method, only 2 samples showed inhibition (8 and 14), and thus the success rate of the PCR was > 90%. For the DNA isolated using the phenol:chloroform method, 7 of the samples showed inhibition (4,6,7,8,11,12,16), corresponding to a success rate of only 65%. Therefore, the silicon carbide method allows for the isolation of bacterial DNA with less contaminants and PCR inhibitors than traditional methods, further indicating that it is a successful method to use for bacterial detection in milk.

Conclusions

1. A procedure based on the use of silicon carbide columns was developed that allows for the isolation of bacterial DNA from milk samples without the use of harmful chemicals such as phenol or chloroform (Figure 1).

2. The silicon carbide method can isolate milk bacterial DNA from both Gram negative and Gram positive bacteria (Figure 1).

3. The method is extremely sensitive and can isolate and detect as little as 10 bacterial cells in 1 mL of milk (Figure 2). Since the legal limit for the amount of bacteria present in milk samples is 15,000 cfu/mL, this method is clearly sensitive enough to isolate and detect bacterial DNA from this amount of bacteria.

4. The isolated DNA can be successfully used in PCR and RT-PCR reactions to detect bacterial species found in 1 mL milk samples (Figure 2).

5. The silicon carbide method results in higher yields of DNA than traditional phenol:chloroform based methods (Figure 3). The silicon carbide method is also much more rapid than the phenol:chloroform method.

6. Bacterial DNA isolated using the silicon carbide method has less PCR inhibitors present than DNA isolated using phenol:chloroform based methods. When the isolated DNA was used to spike

a non-related PCR, >90% of the samples spiked with silicon carbide isolated DNA could be successfully amplified, while only 65% of the samples spiked with the phenol:chloroform isolated DNA could be successfully amplified (Figure 4).

References

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