Molecular Diagnostics Applied to Mastitis Problems on Dairy Farms

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KEYWORDS

• Mastitis • Molecular diagnostics • *Staphylococcus aureus* • *Mycoplasma bovis* • *Streptococcus uberis* • *Enterobacter*

KEY POINTS

- In the last decade, molecular diagnostics have been added to the toolkit of the mastitis researcher community.
- The choice of a molecular typing method depends on the needs, skill level, and resources of the laboratory.
- Many bacterial species have a large genetic variation and within a species many strains exist that have very different infection characteristics in the bovine mammary gland and epidemiologic characteristics within a herd.
- Accurate and cost-effective methods of identifying mastitis pathogens are important for the diagnosis, surveillance, and control of this economically important disease among dairy cows.

INTRODUCTION

Mastitis in dairy cows is among the most important diseases of dairy cattle worldwide. Mastitis is most often the response of the host to an intramammary infection (IMI) and is caused by a large number of bacterial species.[1] Accurate and cost-effective methods of identifying mastitis pathogens are important for the diagnosis, surveillance, and control of this economically important disease among dairy cows. Rapid identification methods, in particular nucleic acid–based tests, have the potential to be extremely specific and can also discriminate among closely related organisms.[2]

The authors have nothing to disclose.

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The development of diagnostic and monitoring tools is experiencing an unprecedented growth phase. In the last decade, molecular diagnostics have been added to the toolkit of the mastitis researcher community.[3] These new tools have resulted in a better understanding of epidemiology and pathobiology of IMI. The goal of molecular epidemiology, however, is not merely to classify organisms into taxonomic or phylogenetic groups but also to "identify the microparasites responsible for the infectious diseases and determine their physical sources, their biologic relationships, and their route of transmission and those of genes responsible for their virulence, vaccine-relevant antigens and drug resistance."[4]

STRAIN TYPING METHODS

These molecular methods include comparative typing methods, library typing methods, virulence gene arrays, and whole-genome sequencing.[5] This article describes the relationship among the epidemiology of some major bacterial infections of the mammary gland using molecular diagnostic techniques. The ideal diagnostic technique should be cost-effective and easy to perform. Its results should preserve a balance between increased discriminating power and applicability. Rapid identification methods, in particular nucleic acid–based tests, have the potential to be extremely specific and can also discriminate among closely related organisms.[2]

The process of subtyping is important epidemiologically for recognizing outbreaks of infection, detecting the transmission of nosocomial pathogens, determining the source of the infection, and recognizing particularly virulent strains of organisms.[6] Any subtyping method must have high differentiation power. It must be able to clearly differentiate unrelated strains, such as those that are geographically distinct from the source organism, and at the same time to demonstrate the genetic relationship of all organisms isolated from individuals infected through the same source.[7]

Many of the currently used molecular techniques for typing rely on electrophoretic separation of DNA fragments of different molecular lengths. The electrophoretic result is represented by a pattern of bands on a gel. Because these patterns may be extremely complex, the ease with which the patterns are interpreted and related is an important factor in evaluating the use of a particular typing method. Along with considerations related to a particular method's simplicity of interpretation, its convenience of use is also important. The technical difficulty, cost, and time to obtain a result must also be evaluated in assessing the use of a particular typing method. The choice of a molecular typing method depends on the needs, skill level, and resources of the laboratory.

Currently, sequencing of RNA or DNA is routinely used for molecular typing. Such methodologies allow for the sequence data to be available for whole genomes or selected areas, such as specific genes or repetitive elements. A major advantage of sequence data is that it is unambiguous, and can easily be stored and exchanged.[2]

In contrast, in the comparative typing methods, such as random amplification polymorphic DNA (RAPD) and pulse field gel electrophoresis, band sizes need to be expressed relative to each other. Many sequence-based typing methods and hybrids of banding pattern and sequences-based methods exist, and it is beyond the scope of this article to discuss all of them.

PRIMARY DIAGNOSIS OF PATHOGENS

A wide range of DNA-based diagnostic assays have been developed and applied to the diagnosis of bovine IMI, and for the improved detection of pathogens in milk and animal feed.[8] These techniques have changed the molecular diagnostic scene

and must be considered as one of the more important developments during the last few years. Compared with the classical bacteriologic diagnosis the molecular technologies offer several important, well-known advantages, such as faster and higher throughput assays, lower costs per detected agent, if the equipment can be used for a large number of samples, and ability to differentiate between clonal outbreak and multiple strains. Particularly, real-time (RT) polymerase chain reaction (PCR) assays offer several important, well-known advantages:

1. Faster and higher throughput assays
2. Post-PCR handling of the amplicons/products is not required and hands-on time is greatly reduced compared with traditional detection using agarose gels followed by ethidium bromide staining
3. Probes can be labeled with several different fluorophores that function as individual reporter dyes for different primer sets; thus, it is suitable for the development of multiplex PCR systems
4. Lower costs per detected agent, if the equipment can be used for a large number of samples

Diagnostics of selected mastitis pathogens is likely an attractive application of the RT-PCR technology.

OBJECTIVES OF THIS ARTICLE

This article highlights the application in several real farm case studies of routinely used molecular techniques for primary diagnosis and epidemiologic investigation of IMI caused by major mastitis pathogens, such as *Staphylococcus aureus*, *Mycoplasma bovis*, *Streptococcus uberis*, *and Enterobacter* spp.

Staphylococcus aureus

Staphylococcus aureus is an important organism isolated in subclinical and chronic mastitis in bovines.[9] Bovine mastitis caused by *S aureus* inflicts enormous economic loss on dairy farms and is characterized by persistent and contagious in nature. Some strains of *S aureus* demonstrate antibiotic resistance and may persist for longer periods without overt symptoms. The diagnosis, surveillance, and control of this economically important mastitis pathogen are based on accurate, rapid, and cost-effective methods of identifying the pathogen. To formulate effective strategies for reducing the spread of *S aureus* infection it is very important to understand the distribution of the pathogen in dairy herds.[10] Various phenotyping techniques, such as biotyping, bacteriophage typing, and antibiotic sensitivity testing, have been routinely used in different epidemiologic studies of *S aureus* isolated from human and animal populations.[7,11] The use of phenotyping methods could be expensive, time consuming, and subject to considerable variation.[12] One of the genotyping method used extensively to fingerprint strains of various microorganisms is RAPD. The technique uses single, short, random sequence oligonucleotide primers resulting in discrete and characteristic patterns of DNA fragments.[12] The profiles obtained after electrophoretic separation of the amplified DNA fragments can be used to study the genetic diversity and structure of the natural population of a number of human and animal pathogens. The RAPD technique is rapid and discriminatory.[13]

Epidemiologic studies involving genotyping of strains from host and the environment are necessary to establish effective preventive measures in the spread of *S aureus* involved in IMI. The case study presented in this article describes the use of RAPD technique to determine the genetic relatedness of *S aureus* associated with

bovine IMIs in a dairy herd. The *S aureus* isolates came from an approximately 2000-cow dairy farm that had a low bulk milk somatic cell count at approximately 150,000 cells/mL. Data analysis of the farm indicated that based on individual cow somatic cell count data the average infection duration was approximately 2 months (cure risk was approximately 50%; average duration, in test day intervals, is estimated as the inverse of the cure risk); a low risk of new infections at approximately 5%; and clinical mastitis that was most prominent immediately after calving. This analysis of the herd data pointed more toward a noncontagious infection pattern than toward a classical contagious transmission pattern. The infection profile from clinical mastitis samples showed a large variety in the number of bacterial species causing IMIs without a predominance of a single bacterial species. Among the bacterial species was a relatively large number of *S aureus* isolates, and given the classical connection between *S aureus* and contagious transmission, the owner of the farm was concerned about a potential mastitis outbreak caused by *S aureus* and was determined to change milking procedures and overhaul his milking equipment. A farm risk assessment was performed and no major deficiency in milking procedures or functioning of the milking equipment was observed. Cow cleanliness was an issue and the use of sprinkler systems in the summer resulted in many teats dripping with water before entering the milking parlor. Therefore, risk assessment pointed more toward a high environmental infection risk rather than contagious transmission risk.

A total of 54 *S aureus* isolates were analyzed using RAPD. Briefly, amplification reactions were performed in 20-μL volumes containing 10 μL of GoTaq Green Mastermix (Promega WI, USA); 7 μL of water; 1 μL of 10-bp oligonucleotide primers AAG3 (5′-GGGACGGCCA-3′); and 2 μL of template DNA. The amplification consisted of initial denaturation at 95°C for 5 minutes, followed by 45 cycles of 30 seconds at 94°C, 1 minute at 35°C, and 2 minutes at 72°C. A final extension step of 72°C for 5 minutes was included in all amplifications.

Out of the total 54 *S aureus* isolates 16 (30%) came from composite string milk samples, and the remaining 38 (70%) came from individual quarter milk samples. Amplification of the *S aureus* isolates by RAPD technique resulted in a polymorphic pattern composed of 8 to 12 clear bands in the range 200 to more than 2000 bp (**Fig. 1**). In the current case report, we found that identification of genetic diversity in *S aureus* strains from the farm (see **Fig. 1**) was primarily related to the problem of many different *S aureus* strains on the farm. In our study we identified eight common RAPD strain types and 46 distinct RAPD profiles. This fact suggests that for these eight clonal isolates, there may be a common source of *S aureus* in this herd. The results for the remaining isolates indicate a nonclonal transmission pattern of *S aureus* on this dairy farm. Communication back to the dairy producer emphasized the noncontagious nature of the *S aureus* IMI in this herd. It was advised to segregate the known infected cows, apply treatment to young animals with a recent infection, eventually cull older and long-term infected animals, and continue the excellent milking practices that have prevented infection transmission of the *S aureus* strains in the herd. In conclusion, molecular typing of the identified *S aureus* isolates showed a nonclonal infection transmission pattern. With this additional information on top of the species determination (*S aureus*), much more precise and accurate advice could be provided to the farm manager.

RAPD was a useful technique for distinguishing strains within species of *S aureus*. This technique provides useful information for understanding molecular epidemiology of *S aureus* within dairy herds and more specifically for investigating the source of *S aureus* mastitis outbreaks, thereby contributing to better management of *S aureus* mastitis in dairy herds.

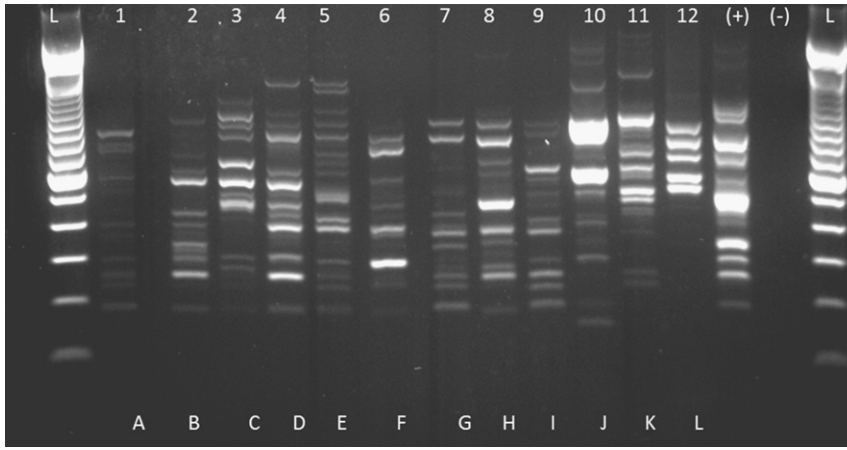

Fig. 1. RAPD gel showing a nonclonal outbreak of *Staphylococcus aureus* in 12 cows in a New York dairy farm. Mastitis isolates of *S aureus* from the farm are in lanes 1 to 12. Lanes coded with (+), (−), and L are positive and negative controls and DNA ladder, respectively.

Mycoplasma spp

Mycoplasma bovis, with increased cattle movement over recent years, is the most common disease implicated in pneumonia and mastitis.[14] Despite this, there is still poor appreciation of its impact on animal health and welfare, with underdiagnosis in many countries because of a lack of the specialized techniques required to detect the organism and incomplete understanding of the pathogenesis of infection. The standard laboratory diagnosis for mycoplasma mastitis is currently based on microbiologic procedures by bacterial isolation from bulk tank milk or samples from cows with clinical and subclinical mastitis. Over recent years enzyme-linked immunosorbent assay and PCR-based methods have gradually replaced culture as the method of choice for detecting *M bovis*, and the application of a novel RT-PCR method makes a valuable new contribution in this context.[15,16] Diagnosis for mycoplasma using cultures has intrinsic limitations in terms of sensitivity and test turnaround time and it is not expected that these drawbacks will be overcome by significant improvements in culture-based methods in the near future. The isolation and subsequent subculturing for mycoplasma identification is a time-consuming process that may take up to 15 days before a sample is considered negative or positive for mycoplasma.[17] In addition to a significant time delay in detection, the traditional culture-based methods often fail to isolate mycoplasmas because the viability of the organism declines rapidly during transport and storage.[18]

Early detection of mycoplasma infections is important in preventing disease and reducing the spread to other animals; in this regard it is essential to develop laboratory methods faster than the traditional bacteriologic approach. The routine mycoplasma diagnosis may also be based on serologic and biochemical assays, but it is well documented that these microorganisms share common surface components responsible for cross-reactivity.[19] In addition to this it has been observed that mycoplasma have the ability to vary their size, shape, and surface antigens further complicating identification by these techniques.[20] The molecular approaches represent a valid and promising option to overcome these limits. However, reliability and sensitivity of these methods are highly dependent on the extraction of adequate amounts of pure DNA using appropriate methods (Gioia, personal communication, 2012).

The molecular epidemiology of mastitis-associated *Mycoplasma* spp has been investigated by such approaches as Amplified Fragment Length Polymorphism (AFLP), pulse field gel electrophoresis, and restriction enzyme typing analysis.[3] The PCR assays for the detection of mycoplasma are mostly based on in vitro amplification of the highly conserved 16S rRNA gene. However, PCR tests developed on the 16S target without further sequences analysis fail to differentiate among different *Mycoplasma* species.[21]

The low degree of variation in the 16S rRNA gene sequences suggests a potential misdiagnosis using only 16S rRNA-based PCR assays. Subramaniam and colleagues[22] developed a PCR based on the DNA repair *uvrC* gene, which was shown to clearly differentiate between *M bovis* and other *Mycoplasma* species. The authors recently developed a single-step duplex PCR assay using a combination of two primer pairs, one universal for *Mycoplasma* genus designed on the 16S target and one specific for *M bovis*, targeting the *uvrC* gene, thus allowing the simultaneous and unequivocal detection and differentiation *M bovis* from other *Mycoplasma* spp. This assay performed on culture-positive *Mycoplasma* samples was able to generate amplicons with different size respectively for *Mycoplasma* spp and *M bovis*. The single-step multiplex PCR ensured high sensitivity and specificity with quick turnaround time for test results. The multiplex PCR assay represents an additional tool for epidemiologic studies and routine disease assessment in areas endemic for the multiple *Mycoplasma* species.

The represented case study is from a dairy herd of 600 Holstein milking cows with a mean daily milk production of 32 kg per cow per day. This dairy farm experienced a sudden increase in the incidence of respiratory problem in lactating heifers and after approximately 50 days the same animals showed signs of clinical mastitis. Forty-three cases of clinical mastitis were recorded over a 15-day period. During the outbreak, bacteriologic examination was performed on samples from clinically affected cows (including bulk tank milk and lungs). Identification of *Mycoplasma* infection and determination of the species (*M bovis)* was performed in one diagnostic procedure with the developed duplex PCR (**Fig. 2**). Early detection of the *M bovis* from the submitted samples assisted in quick diagnosis and management of *Mycoplasma* outbreak in this herd. Cows were segregated immediately and culled where possible. Appropriate

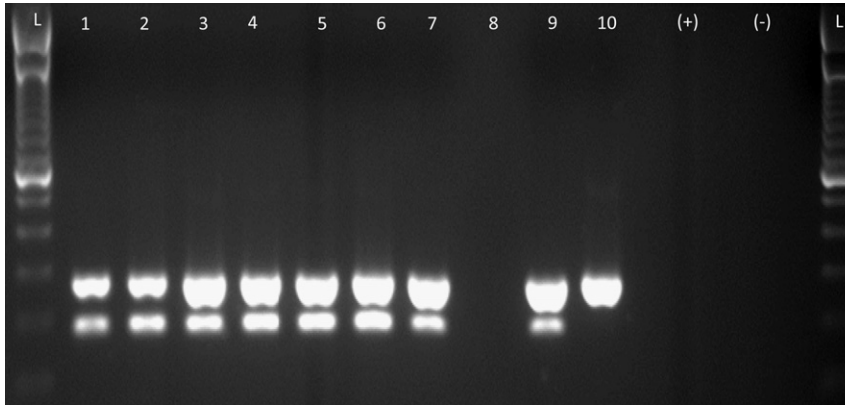

Fig. 2. Duplex PCR for detection of *Mycoplasma* spp and *Mycoplasma bovis*. Lane 1, lung sample positive for *M bovis*; lanes 2 to 7, milk samples positive for *M bovis*; lane 8, milk sample negative for *Mycoplasma*; lane 9, positive control to *M bovis* (ATCC 25523); lane 10, positive control to *Mycoplasma* spp (ATCC 29103); lanes coded with (+), (−), and L are positive and negative controls and DNA ladder, respectively.

management techniques were emphasized in the milk parlor and for the hospital pen. Because of quick diagnosis and immediate response, further expansion of the outbreak was halted.

This case study signifies the importance of application of a novel diagnostic tool, such as duplex PCR, for early detection and screening of *Mycoplasma* in a dairy herd thereby aiding in control and management of the outbreak.

Streptococcus uberis

Streptococcus uberis is a major mastitis-causing pathogen that is generally classified as an organism of environmental origin. *S uberis* has been associated with subclinical and clinical IMIs in lactating and nonlactating cows.[23] Typically, the authors classify *S uberis* as an environmental organism, meaning that infection occurs because of organisms in the environment of cows.[24] In the case of *S uberis*, one would therefore predict that most strains causing infections in a herd should be genotypically different because an enormous number of different genotypes of *S uberis* exist in the environment of the cow.[25] In some cases it has been observed that a single strain of *S uberis* caused mastitis in multiple cows.[26] This observation of a single strain in multiple IMIs would potentially indicate that transmission between animals occurs, and that the bacterium behaves more like a contagious organism.

RAPD fingerprinting has also been used for confirmation of *S uberis* after intramammary challenge with *S uberis* and identified new *S uberis* infections in challenged quarters. Subtyping of *S uberis* and *Streptococcus dysgalactiae* by RAPD fingerprinting demonstrated isolates from New Zealand were distinct from isolates from the United States.[12]

A 1700-cow dairy farm was concerned about an increase in *S uberis* clinical mastitis observed during a 3-month period. Clinical mastitis was observed in approximately 5% of cows on a monthly basis. The herd manager focused on improving stalls and cow hygiene given the classical environmental organisms observed in clinical cases (*S uberis* and coliforms). Subsequent data analysis and risk assessment on the farm pointed toward a high risk of transmission, particularly during milking. The authors observed that known infected cows were not segregated and postmilking teat disinfection was done with a spray system and showed very poor teat coverage with the disinfectant. Dynamic measurements of the milking equipment showed that there was a high fluctuation of vacuum under the teat-end. This high fluctuation of vacuum was caused by a low effective reserve relative to the size of the milking parlor.

Molecular methods were used for strain typing and to confirm the species identity of isolates that had been classified as *Streptococcus* spp based on phenotypic characteristics. Strain typing was performed using RAPD PCR. Briefly, crude DNA extracts from *S uberis* isolates were obtained by 10-minute boil preparation and used as templates for RAPD PCR with primer set OPE-04 (5k-GTGACATGCC-3k; Operon Technologies, Alameda, CA) and cycling conditions described previously.[25] Electrophoresis of amplified products was performed using 1.5% agarose gels, with 20 5-mm-wide wells, run in 0.5× Tris-borate-EDTA buffer for 1.5 hours in a horizontal electrophoresis system at approximately 95 V. Gels were stained with ethidium bromide and visualized through ultraviolet transillumination with the Molecular Imager Gel Doc XR system and Quantity One software, version 4.4.1 (Bio-Rad, Hercules, CA).

Fig. 3 clearly shows a clonal outbreak of *S uberis* IMIs in this dairy farm with all isolates from the clinical cases belonging to the same *S uberis* clone. Based on the result of RAPD typing, it was concluded that this farm had a clonal outbreak of *S uberis* isolated from the clinical cases.

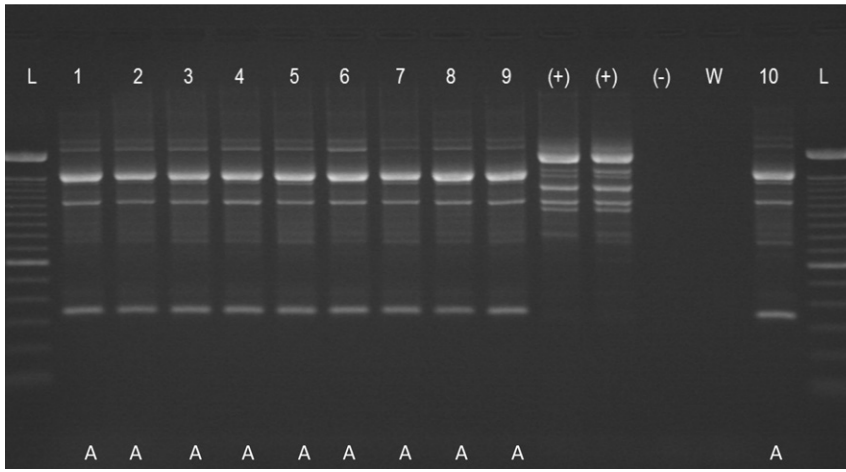

Fig. 3. RAPD gel showing a clonal outbreak of *Streptococcus uberis* in 10 cows in a New York dairy farm. Mastitis isolates of *S uberis* from the farm are in lanes 1 to 10. Lanes coded with (+), (−), and L are positive and negative controls and DNA ladder, respectively. W is a negative control lane with only water.

In this farm, data analysis, risk assessment, and infection profile all pointed toward a herd diagnosis of contagious transmission. The result of the data analysis, risk assessment, and molecular strain typing were reported back to the herd manager. Advice to resolve the issue focused on preventing infection transmission in the herd. Segregation of known infected cows was implemented, postmilking teat disinfection was changed to dipping rather than spraying, and milking equipment was upgraded to match the size of the milking parlor. Implementation of the advice was swift and clinical mastitis in the farm dropped dramatically over a period of 6 months to approximately 1.5% of cows per month.

Again, RAPD strain typing turned out to have a large value on top of classical culture-based diagnostics. Farm advice was based on the combination of data analysis, risk assessment, and advanced diagnostic methods.

Enterobacter spp

Enterobacter spp has been reported among the causes of clinical mastitis on dairy farms.[27] In general, no further speciation of the *Enterabacter* spp is performed. However, in several recent studies rpoB sequencing was performed and the dominant *Enterobacter* spp was reported to be *E cloacae*.[27,28] Clinical signs associated with *E cloacae* were generally mild compared with other coliform mastitis cases and spontaneous cure of infection is generally high. The authors report here on well-managed dairy farms of approximately 550 cows with a bulk milk somatic cell count of approximately 200,000 cells per milliliter. All cases of clinical mastitis were sampled by the owner and on-farm culture was performed. The owner noted a large number of gram-negative mastitis cases that were generally mild. Culture on the farm was inconclusive and further diagnostics were requested by the owner. Because the treatment routing on the farm was not to treat gram-negative mastitis cases, the identified cows with gram-negative mastitis remained in the lactating cow pen and affected cows were not segregated. The abnormal milk was discarded in a separate milking can, but no disinfection or elaborate cleaning of the milking unit was performed after

milking the clinical cases. Data analysis on the farm revealed a relative high risk of new infections (~10%), with approximately 8% chronic high cell count cows. Risk assessment on the farm showed few weaknesses with excellent milking procedures and milking equipment that passed the ISO equipment test. The gram-negative isolates from the farm were tentatively identified as *Enterobacter* spp by classical microbiology. All *Enterobacter* isolates were subjected to *rpo*B sequencing and were identified as *E cloacae* (99% species identity based on 100% coverage of 900-bp fragment). All identified *Enterobacter* isolates were used for strain typing. Strain typing was performed by means of RAPD PCR.[6] Briefly, crude DNA extracts from *Enterobacter* isolates were obtained by 10-minute boil preparation and used as templates for RAPD PCR with primer set ERIC-2/ERIC-1026 (5'-AAGTAAGTGACTGGGGTGAGCG-3' and 5′-TACATTCGAGGACCCCTAAGTG-3′, respectively). Electrophoresis of amplified products was performed using 1.5% agarose gels, with 20 5-mm-wide wells, run in 0.5× Tris-borate-EDTA buffer for 1.5 hours in a horizontal electrophoresis system at approximately 95 V. Gels were stained with ethidium bromide and visualized through ultraviolet transillumination with the Molecular Imager Gel Doc XR system and Quantity One software, version 4.4.1 (Bio-Rad).

Results of strain typing are shown in **Fig. 4**. RAPD patterns for *E cloacae* isolates all showed a band of approximately 290 bp as shown in **Fig. 4**. Three different clones of *E cloacae* were identified: clone A was present in lanes 1, 7, 11, 13, and 16; clone B in lanes 2, 3, 4, 5, 6, 9, 10, 14, and 15; and clone C only in lane 8. Clearly, clonal transmission seems to play an important role in *E cloacae* infections in this herd, with two dominant strains present.

The results were discussed with the owner and it was advised to segregate all cows with clinical mastitis in a sick cow pen. After each milking of a clinically affected cow the unit should be disinfected before being used on the next cow. Cows will only be

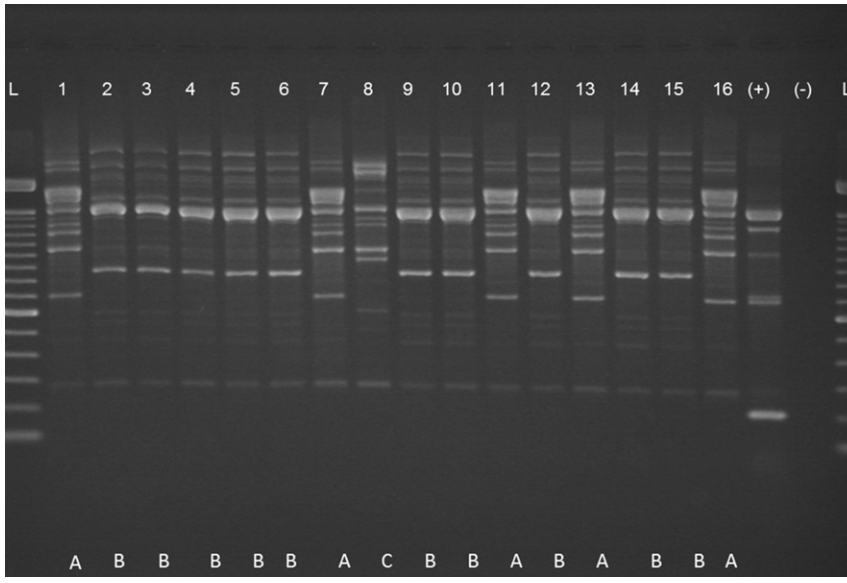

Fig. 4. RAPD gel showing a nonclonal outbreak of *Enterobacter* spp in cows in a New York dairy farm. Mastitis isolates of *Enterobacter* from the farm are in lanes 1 to 16. Lanes coded with (+), (−), and L are positive and negative controls and DNA ladder, respectively.

allowed to return to the milking pens with visibly normal milk and the absence of gram-negative bacteria from the milk.

Although *E cloacae* are generally not considered to be contagious organisms, the specific management practices on this farm with no treatment of affected cows and milking the affected cows within the lactating cow pens resulted in a high exposure of other cows in the same pen to this gram-negative organism. By eliminating this transmission route, it was possible to prevent further expansion of the clonal outbreaks.

SUMMARY

The use of molecular diagnostic tools has not changed the true pathobiology of mastitis or mastitis pathogens, but has definitely assisted in a much more accurate diagnosis and management of mastitis problems at the herd level. Molecular diagnostic techniques have contributed to the understanding of infection sources, transmission, and prognosis of major mastitis pathogens on a dairy farm. Using the molecular epidemiologic techniques, it has become possible to monitor spread of pathogens, to identify virulent strains, and to differentiate between environmental and contagious nature of infectious agents in the dairy farm environment.

In the coming years, with the advent of rapid DNA-based diagnostic technologies, the use of molecular diagnostics in mastitis diagnosis will become inevitable to improve the quality and precision of herd health management. The added costs must be considered, in connection with all of the technical benefits provided by the assay, when making decisions on implementation of the molecular diagnostic assays in routine mastitis testing programs. The authors have showed that genotypic methods are considered to be faster (in the case of RT-PCR) and more discriminatory (in the case of RADP typing) than phenotypic methods and therefore should be increasingly used in diagnostic laboratories. It has been shown that the combination of herd data analysis, herd risk assessment, and molecular diagnostic methods allows an accurate and precise herd diagnosis.

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