PCR, PFGE, ABCD…Understanding and Using Molecular Tests to Diagnose and Control Mastitis

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Introduction

The technical definition of mastitis is "inflammation of the mammary gland" but on a practical basis, almost all bovine mastitis is caused by bacteria [1]. Appropriate mastitis control is based on knowledge of the etiology, thus identification of pathogens is a fundamental aspect of mastitis control programs. Mastitis occurs after an infective dose of a pathogenic organism passes through the streak canal and progresses to a subclinical or clinical state but many intramammary infections are spontaneously cured as a result of the cows immune response [2]. However, detection of mastitis is based on observation of inflammation that occurs as a result of that response. Thus, recognition of mastitis occurs after the immune response and in many instances, culture of milk samples obtained from inflamed quarters may not result in bacterial growth. The use of molecular techniques to identify bacterial DNA from milk samples is increasingly used in an attempt to improve diagnosis. The purpose of this paper is to discuss practical aspects of using these tests in mastitis control programs.

Definitions

Subclinical Mastitis. By definition, milk obtained from mammary gland quarters of cows experiencing *subclinical mastitis* appears visually normal (even when millions of somatic cells are present) but the milk contains an excessive number of somatic cells, (with or without the detectable presence of pathogenic organisms)[3]. The SCC of healthy quarters is usually well below 100,000 cells/mL and a threshold of < 200,000 cells/mL is usually considered to be the most practical value to use to define a mammary quarter as healthy[3-6]. The occurrence of SCC >200,000 cells/mL is an extremely specific indicator of IMI, but the failure to recover bacteria from a high SCC gland does not indicate that the gland is healthy. An increased SCC in a microbiologically negative milk sample is a common occurrence that can occur because the immune response has reduced the number of bacteria to below normal laboratory detection limits (usually 100 cfu/mL). The increased SCC is part of an immune response that has the purpose of elimination of pathogens. This response is often effective and at least 10-25% of quarters that have SCC > 200,000 cells/mL will be apparently bacteriologically negative [3, 4, 6].

Clinical mastitis. Inflammation that results in visible abnormalities of milk is defined as clinical mastitis, regardless of SCC level. Most symptoms of clinical mastitis are quite mild and cannot be detected unless foremilk is observed before attaching the milking cluster. In a study that enrolled almost 800 cases of clinical mastitis occurring on 51 Wisconsin dairy farms, only 15% of clinical cases presented with systemic symptoms, while 50% and 35% of cases presented with solely abnormal milk or abnormal milk and swelling of the affected quarter, respectfully[7] . About 25-40% of milk samples obtained from cases of clinical mastitis are typically bacteriologically negative when submitted for culture [7-9].

Bacterial Terminology Used in Molecular Testing. Terminology used to describe mastitis pathogens can be very confusing but a few key terms are used in relatively standard ways[10]. When referring to bacteria, ***genus*** is the broadest level of discrimination and an ***isolate*** is

considered to be a pure culture of microbes identified to the species level. A **strain** is basically a subset of isolates that share some specific characteristics. For example, mastitis pathogens may be broadly identified as Staphylococci (genus level), more narrowly identified as *Staphylococcus aureus* (an isolate at the species level) or considered to be a specific strain based on phenotypic (such as penicillin resistance) or genotypic characteristics. Genotypic characteristics are identified through the use of laboratory techniques that examine characteristics of the bacterial DNA. Identification of strains using genotypic testing is considered to be more reproducible and discriminatory as compared to the use of phenotypic methods [10].

Selected Molecular Tests Currently Used in Mastitis Laboratories

Several molecular methods are used for diagnosis of mastitis pathogens and to investigate mastitis outbreaks and milk quality problems [10, 11]. Some methods (such as polymerase chain reaction (PCR) are used to improve diagnosis of pathogens for which biochemical methods for identification of species are known to be inaccurate [12, 13] or to more rapidly arrive at a diagnosis [14]. Other methods (such as the use of pulsed field gel electrophoresis (PFGE) are used to identify specific strains of bacteria to confirm failure of bacteriological cure, determine the source of exposure or identify the mode of transmission of a pathogen [15].

*PCR Testing for Determination of Bacterial Etiology. G*enotypic methods of identifying bacteria are based on identification of unique sections of DNA that are compared to characteristics of known library strains. The use of PCR is very basic to many molecular procedures. A PCR is based on the concept that the nucleus of bacterial cells contains DNA composed of nucleotides with unique sequences. When PCR is used for identification of mastitis pathogens, bacterial DNA is extracted from milk samples and then mixed with “primers” which are basically templates of known nucleotide sequences from particular bacterial species (not strains). The primers duplicate matching sequences until enough copies are made so that they can be matched to a bacterial species in the library of known bacterial sequences. A key concept for interpretation of results of these tests is to understand that PCR tests can only identify organisms for which a specific primer is included in the “test mix.” It is also important to understand that the PCR copies the sequences of both living and dead bacteria. Identification of bacterial DNA does not ensure that an active bacterial infection is currently present in the mammary gland. Thus, the use of molecular testing for making individual cow decisions is not yet well defined.

At least one real-time PCR method for diagnosis of bovine mastitis pathogens is widely commercially available (Pathoproof®; Thermo Fisher Scientific). The Pathoproof™ kit contain of primers that can bind with and multiply DNA from a number of organisms. Some kits include primers that can identify bacterial DNA from just a few organisms (M. bovis; Staph aureus and Strep agalactiae) and other kits include primers for DNA of up to 16 organisms. Intramammary infection is not the only source of bacterial DNA found in milk samples, as teat skin, the streak canal and sampling methods can contaminate milk with bacterial DNA. To ensure a useful result, milk samples used for PCR testing must be collected using aseptic technique. Even when aseptically collected milk samples are used, false positive results of PCR testing occur [16, 17]. In one study, the use of this test resulted in identification of bacterial DNA of potential mastitis pathogens in 43% of culture negative milk samples [16]. However, 31% of the culture negative milk samples contained DNA from $\geq$2 types of

organisms and major mastitis pathogens were grown from some of the PCR negative samples [16]. Bacteria vary among farms and evolve, thus it is possible that some mastitis pathogens may not contain the nucleotide sequences that are used in the primers.

The use of the commercially available PCR test to evaluate milk quality of bulk tank milk has also been investigated [18]. As expected, PCR testing is useful to detect the presence of obligate udder pathogens (such as *S. agalactiae*) in bulk milk, especially when there is a low prevalence of infected cows within the herd [18]. However interpretation of PCR test results for other bacteria found in bulk milk is difficult. PCR test results are interpreted relative to the cycling threshold values (Ct) which indicate the number of PCR cycles that are required to make enough copies to reach the signaling threshold. In general, the lower the Ct value the greater the amount of the specified DNA in the sample. When using PCR on bulk milk samples, there is limited understanding of how to interpret Ct values for bacteria that can originate from either the environment or from IMI and validated guidelines for interpretation at the herd level are not available.

DNA Fingerprinting. When determination of individual strains of a bacterial species is desired, then a process of DNA fingerprinting is used. There are several different methods for comparing the DNA of a bacterial species, but they are all based on extraction of bacterial DNA followed by separation of the bacterial DNA into columns based on size of specific fragments. Some of the methods include Pulsed Field Gel Electrophoresis (PFGE) or Random Fragment Length Polymorphism (RFLP)[10]. These methods are performed by adding restriction enzymes that cut the bacterial DNA in specific areas. In PFGE, those cut pieces are then put in a gel column and separated by use of an electrical field to resemble a bar code. The resulting bands of DNA fragments are then compared to each other and form the basis for deciding if strains are identical, similar or different. Bands that are identical or very similar are considered to be the same strain or a slightly different substrain.

There are several instances where the identification of a strain may be useful in mastitis control programs. The identification of failure to achieve bacteriological cure after treatment versus development of a new infection is an example of when DNA fingerprinting can be useful. Historically, bacteriological cure has been defined based on comparing results of cultures taken before and after treatment. In general, if the same bacterial species is found in the post-treatment samples, a treatment failure was assumed. However, comparison of the DNA fingerprints can be used to determine if that species is the same strain as the strain found in the original IMI (and then presumed to be a persistent infection) or if the bacteria is a different strain (and presumed to be a new infection). Comparison of strains of bacterial species can also be used to determine if diverse strains of opportunistic organisms from the environment are causing mastitis versus identical strains which may indicate cow-to-cow transmission. The recovery of identical strains should not be considered to be absolute proof of cow-to-cow transmission because identical strains can also be recovered if the dominant pathogenic strain in the environment is a consistent strain. Thus, even DNA fingerprints do not necessarily provide conclusive evidence that indicates contagious transmission of mastitis pathogens.

Conclusions & Recommendations

Numerous molecular methods have been developed and the use of these methods is commonplace in diagnostic laboratories. However, decision making for management of milk quality based on some of these tests is still evolving. The use of PCR to decide on therapy of mastitis is complicated because DNA from bacteria that have been successfully eliminated by the cow's immune response will potentially be detected. The application of these diagnostic methods to daily decision making on farms is also unknown. Diagnostic microbiology for mastitis pathogens is based on combining knowledge of pathogen behavior with a firm understanding of how the pathogens behave in culture media. For example, the occurrence of a few colonies of environmental pathogens on a blood agar plate are not typically sufficient evidence to indicate that a treatment for an IMI is indicated [1]. Likewise, identification of bacterial DNA from milk is not always sufficient evidence upon which to base a treatment protocol. When molecular methods are used, producers must understand that there are multiple sources of bacterial DNA and the utility of the samples will be vastly improved when aseptic methods are used to collect the milk samples[17]. To facilitate decision making, the medical history and SCC of the cow should be combined with the results of the molecular test. This is especially important before making critical decisions about culling or segregation. For some pathogens, such as *Mycoplasma* spp, *Streptococcus agalactiae* or *Staphylococcus aureus* the cow history will generally support the presumed diagnosis; for other pathogens the relationship may be less apparent. Unless a herd surveillance program is focused on detection of *Streptococcus agalactiae*, interpretation of results of molecular tests to screen bulk tank milk is currently not well defined. It is clear that the cost of molecular methods will decrease and that use of these methods will increase. However, continued research is needed to help define practical ways of best using these methodologies in a cost effective manner.

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