The quest for the perfect test: Phenotypic versus genotypic identification of coagulase-negative staphylococci associated with bovine mastitis

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ABSTRACT

Coagulase-negative staphylococci (CNS) are a frequent cause of bovine intramammary infection and the objective of this paper is to discuss the utility of phenotypic identification systems used for species identification of CNS relative to use of genotypic identification. Mastitis control programs have been developed for major mastitis pathogens but few are specifically targeted for control of CNS. Few documented differences in treatment outcomes of mastitis caused by different species of CNS have been published, and at least one study has reported no differences in bacterial cures of mastitis caused by CNS based on genotypic identification. A number of commercial identification kits for species identification of CNS have been evaluated by mastitis researchers. Most phenotypic systems are considered to accurately identify >80% of staphylococci but have not been designed to detect all taxa that have been associated with bovine mastitis. Typical results were observed in the evaluation of agreement between 2 systems used for identification of staphylococci ($n = 54$) isolated from cases of mastitis. Satisfactory agreement (Kappa > 0.87) was achieved for API Staph but low agreement at the species level was seen for the BBL Crystal Gram-Positive system (Kappa = 0.25). Results of this small study are typical of similar studies and confirm that differences occur among phenotypic identification systems. In spite of the limited precision of some phenotypic identification systems, their consistent use with an adequate number of isolates in the diagnostic algorithm is probably sufficient for most mastitis control programs, which are currently not based on species level identification. However, genotypic identification will be useful for advancing knowledge of the role of CNS in bovine mastitis.

1. Introduction

In many modern dairy herds, coagulase-negative staphylococci (CNS) are a frequent cause of bovine mastitis and their importance as mastitis pathogens has been recently reviewed (Taponen and Pyörälä, 2007). On farms that have successfully controlled mastitis caused by *Staphylococcus aureus* and *Streptococcus agalactiae*, opportunistic bacteria such as CNS are frequently associated with bovine mastitis. In Wisconsin, it is quite common for CNS to be recovered from about 15 to 20% of bovine milk samples obtained from cows experiencing subclinical and clinical mastitis (Table 1) (Hoe and Ruegg, 2005; Makovec and Ruegg, 2003; Pol and Ruegg, 2007). Likewise, in prevalence studies conducted in other regions CNS are commonly recovered from milk samples obtained from cows affected by mastitis (Myllys et al., 1998; Wilson et al., 1997; Bradley et al., 2007). CNS are the most common pathogens recovered from heifers and a variety of CNS species have been recovered from teat skin, the streak canal and pre-calving udder secretion obtained from heifers (Nickerson et al., 1995; Borm et al., 2006).

The genus *Staphylococcus* contains at least 40 species and 17 subspecies (http://www.bacterio.cict.fr/s/staphylococcus.html; Bannerman, 2003) and it is conceivable that under the right circumstances most of these organisms

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Table 1
Proportion of coagulase-negative staphylococci recovered from bovine milk samples in selected studies in Wisconsin, USA.

Study	Origin of isolate	Type of sample	Number of samples	Number of farms	Recovery of CNS
Makovec and Ruegg (2003)	State diagnostic laboratory submissions	Quarter and composite	83,650 Milk samples	Not specified	11,062 (13.2%)
Hoe and Ruegg (2005)	Cases of mild or moderate clinical mastitis	Duplicate quarter samples	133 Cases	4 Farms	26 (19.5%)
Pol and Ruegg (2007)	Subclinical mastitis	Single quarter milk samples	5,672 Milk samples	40 Farms	850 (15.0%)

Table 2
Quarter prevalence of *Staphylococcus* species by parity and lactation period (adapted from Matthews, 1992).

Species	Prepartum teat orifice swabs		Parturition (duplicate 1/4 milk)		Week 5 postpartum (duplicate 1/4 milk)	
	Lact. 1 (%)	Lact. 2+ (%)	Lact. 1 (%)	Lact. 2+ (%)	Lact. 1 (%)	Lact. 2+ (%)
S. aureus	6.9	2.3	7.6	0.6	3.5	0.3
S. hominus	2.8	7.1	0.7	0.6	1.4	1.0
S. chromogenes	28.5	28.9	12.5	10.4	9.7	6.2
S. warneri	0.7	3.9			0.7	0.7
S. xylosus	0.7	2.6	0.7			
S. epidermidis	2.1	3.2	2.8	1.0		0.6
S. simulans	3.5	1.0	4.2		2.8	2.8
Staph spp. no ID	0.7	3.6	0.7	0.3		

could be capable of causing bovine mastitis. *S. chromogenes, S. hyicus* and *S. simulans* are generally considered to be predominant species associated with bovine mastitis but many species of CNS have been recovered from milk samples (Sears and McCarthy, 2003). One study that characterized CNS recovered from bovine mastitis occurring on 16 commercial farms in Vermont, [0] identified more than 10 *Staphylococcus* species, i.e. *epidermidis, hyicus, hominis, xylosus, simulans, warneri, haemolyticus, sciuri, capitis, saprophyticus* and *lentus* (Hogan et al., 1987). Many species of CNS have been associated with bovine mastitis, but epidemiological differences among various species have not been well defined. While recovery of *S. aureus* has been associated with stage of lactation and parity, similar trends for individual species of CNS have not been consistently documented (Table 2). In spite of the high incidence of intramammary infections associated with CNS, species specific control programs are rare and most diagnostic laboratories do not even utilize phenotypic identification for clinical specimens. The aim of this paper is to discuss the utility of phenotypic identification systems for CNS in light of evolving systems of genotypic identification, which are described in a companion paper (Zadoks and Watts, 2009).

2. Role of species identification in control programs for mastitis caused by CNS

The principles of mastitis control have been understood for decades and are based on reducing new infections and limiting the duration of existing infections (Bramley and Dodd, 1984). Coagulase-negative staphylococci are generally considered to be opportunistic pathogens that result in mildly elevated somatic cell count and occasional bouts of clinical mastitis. Researchers that ribotyped CNS recovered from subclinical cases of mastitis reported that a variety of types were isolated from different quarters of the same cow and suggested that infections were acquired independently, thus reinforcing their opportunistic nature (Aarestrup et al., 1999).

High rates of spontaneous cure and acceptable responses to antimicrobial treatment are generally reported for mastitis caused by CNS and few differences in these outcomes have been identified among the various species (Wilson et al., 1999; Taponen and Pyörälä, 2007). While some early research has suggested that virulence varies among CNS species (Myllys, 1995; Aarestrup and Jensen, 1997), more recent research has reported only minimal or no difference in virulence based on species (Taponen et al., 2006). A recent study that utilized both species identification and amplified fragment length polymorphisms (AFLP) for identification of CNS, found that there was no association between species and production of β-lactamase, severity of the case or response to antimicrobial treatment (Taponen et al., 2006). Likewise, there was no statistical difference in bacterial cure based on AFLP cluster (Taponen et al., 2006).

Some initial research suggested that duration of infection may vary among species of CNS with particular attention directed toward enhanced persistence of *S. simulans* as compared to other species (Aarestrup and Jensen, 1997). However, more recent research did not confirm that hypothesis, as the same CNS species and isolates with similar AFLP patterns were found in persistent and transient cases of mastitis (Taponen et al., 2007). It is likely that CNS are acquired from both the cow's environment and via contagious transmission from infected or colonized cattle and general mastitis control principles are considered effective (Sears and McCarthy, 2003).

There has been some suggestion that prevalence and distribution of various species of CNS could be influenced by the choice of teat dips but these differences did not appear to be clinically relevant because there was no

association between CNS species and the somatic cell count of affected quarters (Hogan et al., 1987). Thus, the clinical relevance of species identification of CNS has not yet been demonstrated. The inability to confirm that the behavior (pathogenicity, persistence or response to treatment) of CNS varies by species is probably why providers of clinical diagnostic services do not feel compelled to utilize existing phenotypic identification systems or the even more precise genotypic methods. A compelling argument can be made for the use of genotypic methods to enhance precision in research about CNS as consistency in identification of CNS would result in improved ability to integrate research results from different laboratories and in different regions.

3. Microbiological examination of milk samples

Veterinarians and mastitis researchers are generally in agreement that microbiological examination of milk samples is vital for implementation of effective mastitis control. However, the necessity to identify traditional contagious pathogens (S. aureus and S. agalactiae) has lessened as the prevalence of these pathogens has diminished (Makovec and Ruegg, 2003) and emphasis has turned to control of environmental pathogens. Perhaps as a consequence, North American dairy farmers do not seem to feel compelled to arrive at pathogen specific mastitis diagnoses. In a recent statewide survey, only 13% of Wisconsin dairy producers reported that they submit milk samples from all clinical cases and 51% indicated that they rarely submitted any milk samples for microbiological analysis (Hoe and Ruegg, 2006). Producers with larger farms (>200 lactating cows) were more likely to culture clinical cases but few farms (<17% of all farm sizes) were utilizing microbiological examination of milk samples obtained from suspected cases of subclinical mastitis or from fresh cows (Hoe and Ruegg, 2006). Farmers with larger herds reported greater use of microbiological assessment of mastitis pathogens but this use is probably associated with adoption of rudimentary on-farm culture systems.

On-farm culture systems are based on the use of selective media to differentiate Gram-positive from Gram-negative organisms. The use of these systems is advocated as a mechanism to reduce antibiotic usage by targeting antibiotic therapy toward infections caused by Gram-positive infections (Hess et al., 2003). Usage of these systems was reported by 15% of Wisconsin dairy farms (*n* = 154) that participated in a milk quality improvement program in 2006 (Ruegg, unpublished). However, only 41% of farmers indicated that they used results to direct treatment programs (Ruegg 2006, unpublished).

A number of genotypic identification methods such as polymerase chain reaction (PCR), sequencing of housekeeping genes or genotyping using amplified fragment length polymorphism have been used for identification of CNS (Bes et al., 2000; Stepanović et al., 2005; Taponen et al., 2007). The use of genotypic methods adds precision in identification of CNS but the utility of enhanced precision may not be apparent for many users of the resulting information. Farmers that have controlled mastitis caused

by S. aureus and Mycoplasma bovis using either somatic cell count records or simplistic microbiological identification systems may have little motivation to pay for more expensive diagnostic methods that involve molecular methods. Because few management decisions are dependent on the outcome of the test, mastitis control programs have been developed for major contagious and environmental pathogens, but virtually no specific recommendations exist for control of CNS. Farmers may be interested in reducing costs and enhancing efficacy of mastitis treatments but it is unlikely that the use of genotypic identification for identification of CNS would achieve those objectives. The efficacy of treatment of CNS infection is generally high although between farm variation has been observed (Borm et al., 2006). If differences in treatment outcomes of CNS could be demonstrated to be associated with variation in species, farmers may be more inclined toward the use of precise diagnostic methods.

4. Accuracy of methods used to identify CNS

Historically, identification of mastitis pathogens has been based on conventional microbiological procedures which include growth on various media, observation of colony morphology and hemolysis patterns, Gram staining characteristics, agglutination tests and use of biochemical profiles (Bannerman, 2003). Very few clinical or research laboratories routinely utilize classical identification methods because of time constraints and cost. A number of rapid and simple commercial identification kits and diagnostic schemes have been developed to speciate staphylococci. Mastitis researchers have performed a plethora of studies to evaluate the accuracy of many of these tests. When a confidence limit of >90% is used for interpretation, most phenotypic systems can accurately identify >80% of staphylococci, although accuracy is higher for human than for bovine CNS isolates (Table 3). In general, most identification systems have been developed for human healthcare and validated using clinical isolates obtained from human infections. When used on isolates of bovine origin, the identification systems tend to be consistent within laboratories but variation among laboratories can occur based on differences in methodologies (Oliveira et al., 2006).

A typical outcome was observed in the evaluation of two commercial identification systems (API Staph32 and BBL Crystal Gram-Positive) used for identification of Staphylococci (Oliveira et al., 2006). Clinical isolates previously identified as staphylococci were obtained from subclinical (*n* = 26) or clinical (*n* = 28) cases of mastitis or originated from commercially purchased American Type Culture Collection (ATCC) strains (*n* = 8). All ATCC isolates were properly identified by both testing systems but considerable divergence in identification of clinical isolates was seen among tests. Satisfactory agreement (Kappa > 0.87; Martin et al., 1987) was achieved at the genus and species levels for API Staph but low agreement was seen for the BBL Crystal Gram-Positive system (Kappa of 0.25 for species identification). These results were not unexpected because the BBL Crystal Gram-Positive database does not include the commonly reported CNS species

Table 3
Summary of selected previous studies comparing identification systems for staphylococci.

Study	Sample type	No. of isolates	Reference method	Agreement with reference method				
				API32	StaphZym	API StaphIdent or StaphTrac	Vitek	Staphase
Ieven et al. (1995)	Human fluids	444	Classical microbiology	93	98			
Renneberg et al. (1995)	Human blood	89	Classical microbiology	82	82			
Watts et al. (1984)	Bovine milk	179	Classical microbiology				92	89
Matthews et al. (1990)	Bovine milk	130	Classical microbiology			81	45	
Thorberg and Brändström (2000)	Bovine milk and feline	77 bovine; 5 feline	Classical microbiology	77	94			
Taponen et al. (2007)	Bovine milk	120	AFLP	72				

of *chromogenes* or *hyicus*. While this type of study confirms that differences occur among phenotypic identification systems, virtually no changes in treatment or control programs would have been recommended based on different bacterial species identification.

Interpretation and integration of research results could be improved if a consistent method for phenotypic identification of staphylococci was adopted. Variation in responses to control programs for CNS mastitis have been reported (Borm et al., 2006) and the utility of these type of studies could be enhanced if the isolates had been speciated. The consistent use of a phenotypic identification system containing an adequate set of species of veterinary importance in the diagnostic algorithm should be sufficiently precise for mastitis control programs and many research needs. While an argument could be made that precise species identification of CNS is necessary for research about antimicrobial resistance, few mastitis specific breakpoints are currently available, breakpoints for staphylococci do not currently extend below the genus level and in many instances, identification of actual resistance genes would be preferable. The use of genotypic methods for identification of CNS should be encouraged for study designs that require precise identification of pathogens.

5. Conclusions

A compelling argument for the necessity of enhanced precision in identification of CNS clinical isolates has not been identified yet. Control programs for CNS are not based upon species level identification and current research has not identified differences in treatment outcomes based on genotypic identification. CNS are widely distributed in nature and their ability to cause mastitis is undisputed. The first step to enhance knowledge about the role of CNS in bovine mastitis is to encourage their identification at the species level using a consistent phenotypic system that is widely available for veterinary clinical laboratories that are directly serving farmers. Moreover, the use of genotypic identification should be encouraged in appropriately designed research studies.

Conflict of interest statement

None.

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