



# Species Identification and Strain Typing of *Staphylococcus agnetis* and *Staphylococcus hyicus* Isolates from Bovine Milk by Use of a Novel Multiplex PCR Assay and Pulsed-Field Gel Electrophoresis

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**ABSTRACT** *Staphylococcus hyicus* and *Staphylococcus agnetis* are two coagulase-variable staphylococcal species that can be isolated from bovine milk and are difficult to differentiate. The objectives of this study were to characterize isolates of bovine milk origin from a collection that had previously been characterized as coagulase-positive *S. hyicus* based on phenotypic species identification methods and to develop a PCR-based method for differentiating *S. hyicus*, *S. agnetis*, and *Staphylococcus aureus*. Isolates ( $n = 62$ ) were selected from a previous study in which milk samples were collected from cows on 15 dairy herds. Isolates were coagulase tested and identified to the species level using housekeeping gene sequencing. A multiplex PCR to differentiate *S. hyicus*, *S. agnetis*, and *S. aureus* was developed. Pulsed-field gel electrophoresis was conducted to strain type the isolates. Based on gene sequencing, 44/62 of the isolates were determined to be either *S. agnetis* ( $n = 43$ ) or *S. hyicus* ( $n = 1$ ). Overall, 88% (37/42) of coagulase-positive *S. agnetis* isolates were found to be coagulase positive at 4 h. The herd-level prevalence of coagulase-positive *S. agnetis* ranged from 0 to 2.17%. Strain typing identified 23 different strains. Six strains were identified more than once and from multiple cows within the herd. Three strains were isolated from cows at more than one time point, with 41 to 264 days between samplings. These data suggest that *S. agnetis* is likely more prevalent on dairy farms than *S. hyicus*. Also, some *S. agnetis* isolates in this study appeared to be contagious and associated with persistent infections.

**KEYWORDS** *Staphylococcus*, cattle, milk

**S**taphylococci are the most common bacteria isolated from the cow's mammary gland and are frequently associated with subclinical or mild clinical mastitis (1–3). Staphylococci can be differentiated into groups using the coagulase test. Hemolytic coagulase-positive isolates are often presumptively identified as *Staphylococcus aureus*, a so-called “major” contagious mastitis pathogen, and coagulase-negative staphylococci are frequently grouped together as “minor” pathogens (4).

Currently, there are two coagulase-variable staphylococcal species that have been associated with bovine mastitis, *Staphylococcus hyicus* and *Staphylococcus agnetis*. *Staphylococcus hyicus* was the first staphylococcal species to be described as coagulase variable, with coagulase production being found in approximately 24 to 56% of strains (5). The coagulation reaction of *S. hyicus* was described as often having weak activity,

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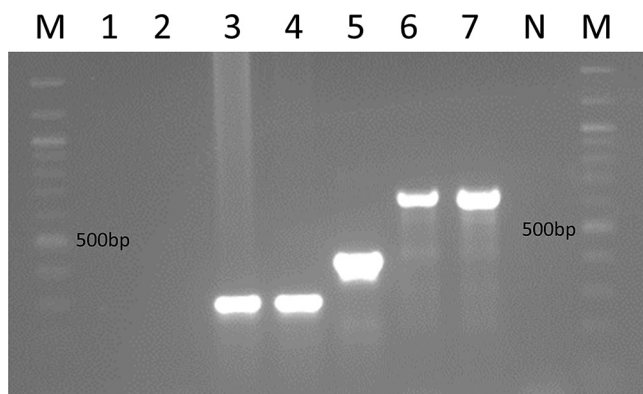
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requiring 18 to 24 h for detection (5). *Staphylococcus hyicus* has been associated with chronic intramammary infections (IMI) that cause subclinical or mild clinical mastitis in dairy cattle (6, 7). Historically, *S. hyicus* was found to be one of the most common organisms isolated from mammary gland secretions and teat canal keratin samples from primigravid heifers (8). When identified as a coagulase-negative staphylococcal (CNS) species, the reported prevalence of *S. hyicus* on dairy farms has been highly varied, with some researchers finding it to be one of the most prevalent species identified (7), while being rarely identified by others (3, 9). Coagulase-positive *S. hyicus* has been described as a low-prevalence mastitis pathogen and has been found to be a greater problem in herds with a low prevalence of *S. aureus* IMI (6). *Staphylococcus agnetis* is a newly described species that is genetically very similar to *S. hyicus*, making it difficult to differentiate from *S. hyicus* with routine phenotypic and genotypic tests. It was first reported in 2012 associated with subclinical and mild clinical bovine mastitis (10).

The phenotypic tests used to differentiate staphylococcal species have likely led to classification errors. Prior to 1986, *Staphylococcus chromogenes* and *S. hyicus* were not recognized as different staphylococcal species (11), and phenotypic classification methods, such as API 20 (API test; bioMérieux, Hazelwood, MO, USA), have been shown to have low specificity for detecting *S. hyicus* (12). Only since 2012 has *S. agnetis* been recognized as a species and mastitis agent (10); thus, prior to this date, most coagulase-positive non-*aureus* *Staphylococcus* spp. were likely classified as *S. hyicus*. Further, from a practical standpoint, when a staphylococcal species is identified as coagulase positive, it is often presumptively identified as *S. aureus*, and thus *S. hyicus* and *S. agnetis* may be misclassified by practitioners or dairy farmers performing their own milk microbiology (6, 13), particularly since approximately one-fifth to one-fourth of *S. aureus* isolates from bovine mastitis do not present any detectable beta-hemolytic activity in primary culture (14).

Recently, genotypic methods to identify staphylococcal species have been described as being superior to phenotypic methods (12). Analysis of the 16S rRNA gene sequence is the most commonly used method for the identification and classification of bacteria (15). However, the usefulness of 16S rRNA gene sequencing is limited when applied to certain staphylococcal species because of the high degree of similarity between closely related species (15, 16). For example, 16S rRNA gene sequences of *S. agnetis* isolates were found to exhibit 99.7% identity to *S. hyicus* ATCC 11249<sup>T</sup> and 99.1% identity to *S. chromogenes* ATCC 43764 (10). Using the recommended cutoff value of 98.7% to differentiate a species (17), these results would be inconclusive. Several other genes have been used as targets for sequenced-based species identification of staphylococcal isolates, including *rpoB* (18), *tuf* (19), and *cpn60* (20). When using *rpoB* gene sequencing with a cutoff of  $\geq 97\%$  for identification of these species, *S. agnetis* can be differentiated from the swine-origin type strain of *S. hyicus* (ATCC 11249), as these isolates exhibit only 93.5% identity. However, the partial sequences of the *S. agnetis* *rpoB* genes are 99% similar to those of some bovine-origin *S. hyicus* strains, e.g., strain KSOS1-02, an *S. hyicus* isolate from bovine milk, thus leading to further confusion in making a definitive diagnosis. It is unknown if the *rpoB* gene of *S. hyicus* of bovine origin is different from that of swine origin or if there is an error in the database, which has been previously reported (21). The *tuf* gene can be used to differentiate *S. agnetis* and *S. hyicus* when following the recommended cutoff values of  $\geq 98.0\%$  identity with  $>0.8\%$  separation between species (22). However, if this gene is not the primary gene used to identify staphylococcal isolates to the species level, several gene sequencing reactions may need to be performed before arriving at a definitive identification. In addition, access to gene sequencing equipment is needed. Hence, a rapid, accurate, and inexpensive method to differentiate coagulase-positive staphylococci isolated from bovine milk would improve diagnostic accuracy and allow a true understanding of the epidemiology of *S. hyicus*, *S. agnetis*, and *S. aureus* on dairy farms.



**FIG 1** Multiplex PCR amplification profiles. The M lanes contain a 100-bp molecular weight ladder marker. Lane 1, *S. chromogenes* ATCC 43764<sup>T</sup>; lane 2, *S. chromogenes* MU 970; lane 3, coagulase-negative *S. agnetis* DSM 23656<sup>T</sup>; lane 4, coagulase-positive *S. agnetis* 430E; lane 5, *S. hyicus* ATCC 11249<sup>T</sup>; lane 6, *S. aureus* ATCC 12600<sup>T</sup>; lane 7, *S. aureus* ATCC 29740. The N lane contains a negative control.

Authors have recently published whole-genome sequences for *S. hyicus* (23), *S. agnetis* (24), and *S. chromogenes* (25). Examination of these whole-genome sequences identified unique gene sequences that were postulated to be good diagnostic targets for differentiating *S. hyicus* from *S. agnetis* isolated from bovine milk using a multiplex PCR platform. Hence, the objectives of this study were to (i) develop a simple multiplex PCR-based assay to differentiate *S. hyicus*, *S. agnetis*, and *S. aureus*, and (ii) use this assay along with pulsed-field gel electrophoresis strain typing to characterize a collection of coagulase-positive non-*aureus* *Staphylococcus* spp. isolated from bovine milk samples from 15 dairy herds in Washington and Idaho. A further objective was to compare the prevalences of *S. aureus*, *S. agnetis*, and *S. hyicus* isolated from milk samples from each herd.

## RESULTS

A total of 62 isolates that had been previously identified as coagulase-positive *S. hyicus* based on coagulase testing and API Staph (26) were studied. Among the 62 isolates received from Washington State University, 15 isolates were found to be coagulase negative and 47 isolates were coagulase positive. Based on *rpoB* gene sequencing, 13 of the coagulase-negative isolates were identified as species other than *S. agnetis* or *S. hyicus*, including *S. chromogenes* ( $n = 8$ ), *Staphylococcus hominis* ( $n = 2$ ), *Staphylococcus epidermidis* ( $n = 1$ ), *Staphylococcus simulans* ( $n = 1$ ), and *Staphylococcus xylosus* ( $n = 1$ ). One of the coagulase-negative isolates was identified by *rpoB* (99.8% sequence homology to *S. hyicus* and 93.5% sequence homology to *S. agnetis*) and *tuf* gene sequences as *S. hyicus*, and the remaining coagulase-negative isolate was definitively identified as *S. agnetis* by the *tuf* gene sequence. Among the 47 coagulase-positive isolates, 5 isolates were identified as *S. aureus* by *rpoB* gene sequence, and the remaining 42 isolates could not be definitively identified using the *rpoB* gene sequence, as all sequences were 99% homologous with both *S. agnetis* and *S. hyicus*. Using *tuf* gene sequencing, all of the remaining 42 coagulase-positive staphylococcal (CPS) isolates were definitively identified as *S. agnetis*. A total of 88% (37/42) of the coagulase-positive *S. agnetis* isolates were found to be coagulase positive at 4 h. The remaining coagulase-positive *S. agnetis* isolates (12% [5/42]) exhibited delayed coagulation and were found to be coagulase negative at 4 h and coagulase positive at 24 h.

The *tuf* gene sequencing results were used as the definitive identification method against which the newly designed multiplex PCR was tested. All 43 *S. agnetis* isolates and the four *S. agnetis* control isolates each produced a single 293-bp band on gel electrophoresis following multiplex PCR (Fig. 1), whereas the single isolate that was identified as *S. hyicus*, based on *rpoB* and *tuf* gene sequencing, and the *S. hyicus* type strain (ATCC 11249) each produced a single 425-bp band. Sanger sequencing and DNA



**FIG 2** Comparison and alignment of *aroD* gene sequences from all *S. agnetis* isolates. The dendrogram shows that all isolates were at least 99.25% homologous. The dendrogram includes the *S. agnetis* type strain (DSM 23656 [6VT]), the reference strain DSM 23658 (55 OE), and two strains (100 VT and 43 OE) originally isolated from cows in Finland (10). A consensus sequence is at the top of the figure, with all other isolates below. All nucleotide bases in agreement with the consensus sequence are noted by a vertical bar (|), and any single-nucleotide changes are noted by the presence of the variant nucleotide base present within that column.

alignment of the *aroD* gene fragments found 99 to 100% homology of all isolates identified as *S. agnetis* (Fig. 2). Sanger sequencing of the single *S. hyicus* test isolate *aroD* gene showed 99% homology with the *S. hyicus* type strain (ATCC 11249) *aroD* gene sequence. Alignment of the *S. agnetis* and *S. hyicus* gene fragments that were produced using the multiplex PCR showed that the fragments were only about 58% similar. No electrophoretic band was detectable when the two *S. chromogenes* control isolates were tested using the multiplex PCR.

Overall, 40/42 coagulase-positive *S. agnetis* isolates could be categorized according to one of the sampling periods, including the three herd cultures or cattle at parturition (Table 1). Data on isolate collection time point were missing for 2 isolates; therefore, they were not included in the prevalence data. Overall, all herds had at least one cow with a CPS isolated from the milk (Table 1). Of the 15 herds, 10 herds had at least one *S. agnetis* strain isolated from milk samples. In 5 herds, no *S. agnetis* was isolated, and on 1 herd (herd 4), the only recorded CPS isolated from milk samples was an *S. agnetis* isolate. Overall, the prevalence of *S. agnetis* was low, ranging from 0.0% to 2.2%. However, the prevalence of *S. agnetis* was equal to or higher than that of *S. aureus* at least once among the different sample collection periods on 5/15 (33%) herds (Table 1). The only *S. hyicus* isolate was from herd 2.

Pulsed-field gel electrophoresis (PFGE) was completed on all 44 non-*aureus* *Staphylococcus* isolates (42 coagulase-positive *S. agnetis*, 1 coagulase-negative *S. agnetis*, and 1 coagulase-negative *S. hyicus*). Two of the coagulase-positive *S. agnetis* isolates were not typeable by PFGE and produced consistent smearing of the electrophoretic bands. Among the 42 typeable isolates, 23 different PFGE banding patterns were identified. Overall, 17 strains were only identified once, and 6 strains were identified more than one time (Table 2). One of the strains found only once was the *S. hyicus* isolate, which had a different banding pattern from all of the *S. agnetis* isolates. Additionally, one of the strains only identified once was the coagulase-negative *S. agnetis* isolate. Further analysis was completed on 39 of the isolates, which included all the *S. agnetis* isolates that originated from a herd ( $n = 8$  herds) with more than one *S. agnetis* strain isolated (Fig. 3). Of the 6 strain types that were identified more than once, all 6 types were identified from more than one cow within the herd. Overall, 3/6 (50%) strains that were

**TABLE 1** Total number and percentage of cows with CPS isolated within each herd from each of the three whole-herd milk samplings and milk samples collected from heifers and cows at calving at 15 commercial dairy herds<sup>a</sup>

Herd no.	Prevalence of CPS at herd cultures									Prevalence of CPS at parturition					
	Herd culture 1			Herd culture 2			Herd culture 3			Heifers			Cows		
	Herd size <sup>b</sup>	<i>S. aureus</i> (no. [%])	<i>S. agnetis</i> (no. [%])	Herd size <sup>b</sup>	<i>S. aureus</i> (no. [%])	<i>S. agnetis</i> (no. [%])	Herd size <sup>b</sup>	<i>S. aureus</i> (no. [%])	<i>S. agnetis</i> (no. [%])	<i>n</i> <sup>c</sup>	<i>S. aureus</i> (no. [%])	<i>S. agnetis</i> (no. [%])	<i>n</i> <sup>c</sup>	<i>S. aureus</i> (no. [%])	<i>S. agnetis</i> (no. [%])
1	233	5 (2.2)	1 (0.4)	262	4 (1.5)	0	279	4 (1.4)	2 (0.7)	105	1 (1.0)	<b>5 (4.8)</b>	155	0	0
2	274	8 (2.9)	0	261	3 (1.2)	1 (0.4)	288	4 (1.4)	3 (1.0)	15	0	<b>1 (6.7)</b>	185	2 (1.1)	<b>2 (1.1)</b>
4	166	0	0	173	0	<b>1 (0.6)</b>	178	0	0	34	0	0	50	0	0
5	198	2 (1.0)	0	215	0	0	213	1 (0.5)	<b>1 (0.5)</b>	76	0	0	165	1 (0.6)	<b>2 (1.2)</b>
6	48	1 (2.1)	0	54	0	0	42	0	0	10	0	0	8	0	0
7	168	3 (1.8)	0	154	5 (3.3)	0	148	2 (1.4)	0	16	0	0	61	3 (4.9)	0
8	82	3 (3.7)	1 (1.2)	101	7 (6.9)	1 (1.0)	92	3 (3.3)	2 (2.2)	2	0	0	17	0	0
9	220	0	0	244	2 (0.8)	0	581	4 (0.7)	0	15	0	0	104	1 (1.0)	0
10	315	36 (11.4)	0	333	35 (10.5)	1 (0.3)	308	31 (10.1)	1 (0.3)	104	5 (4.8)	0	53	3 (5.7)	0
11	86	3 (3.5)	0	84	3 (3.6)	0	78	3 (3.9)	1 (1.3)	3	0	0	61	0	0
12	152	2 (1.3)	1 (0.7)	149	4 (2.7)	1 (0.7)	165	2 (1.2)	1 (0.6)	70	3 (4.3)	2 (2.9)	123	1 (0.8)	0
13	131	8 (6.1)	0	146	7 (4.8)	0	146	6 (4.1)	0	68	1 (1.5)	0	117	5 (4.3)	0
14	343	10 (2.9)	0	326	6 (1.8)	0	374	13 (3.5)	0	63	7 (11.1)	0	268	0	0
15	345	4 (1.2)	2 (0.6)	418	2 (0.5)	<b>2 (0.5)</b>	464	3 (0.7)	2 (0.4)	165	4 (2.4)	1 (0.6)	288	1 (0.4)	<b>1 (0.4)</b>
16	374	6 (1.6)	0	401	6 (1.5)	0	399	10 (2.5)	0	162	20 (12.4)	1 (0.6)	362	11 (3.0)	0
Total	3,135	91	5	3,321	84	7	3,755	86	13	908	41	10	2,017	28	5

<sup>a</sup>Data are in bold where the prevalence of *S. agnetis* is greater than or equal to that of *S. aureus*. No coagulase-positive *S. hyicus* isolates were identified in this study.

<sup>b</sup>Herd size is the number of lactating cows present in the herd at the time of sample collection.

<sup>c</sup>*n* = total number of heifers or cows sampled at calving.

identified more than once were also identified on more than one herd (Fig. 3). A total of 3 strains were associated with persistent infections, with each of the 3 strains being isolated from at least one cow at more than one time point. The time between samplings and isolation of the same strain ranged from 41 to 264 days.

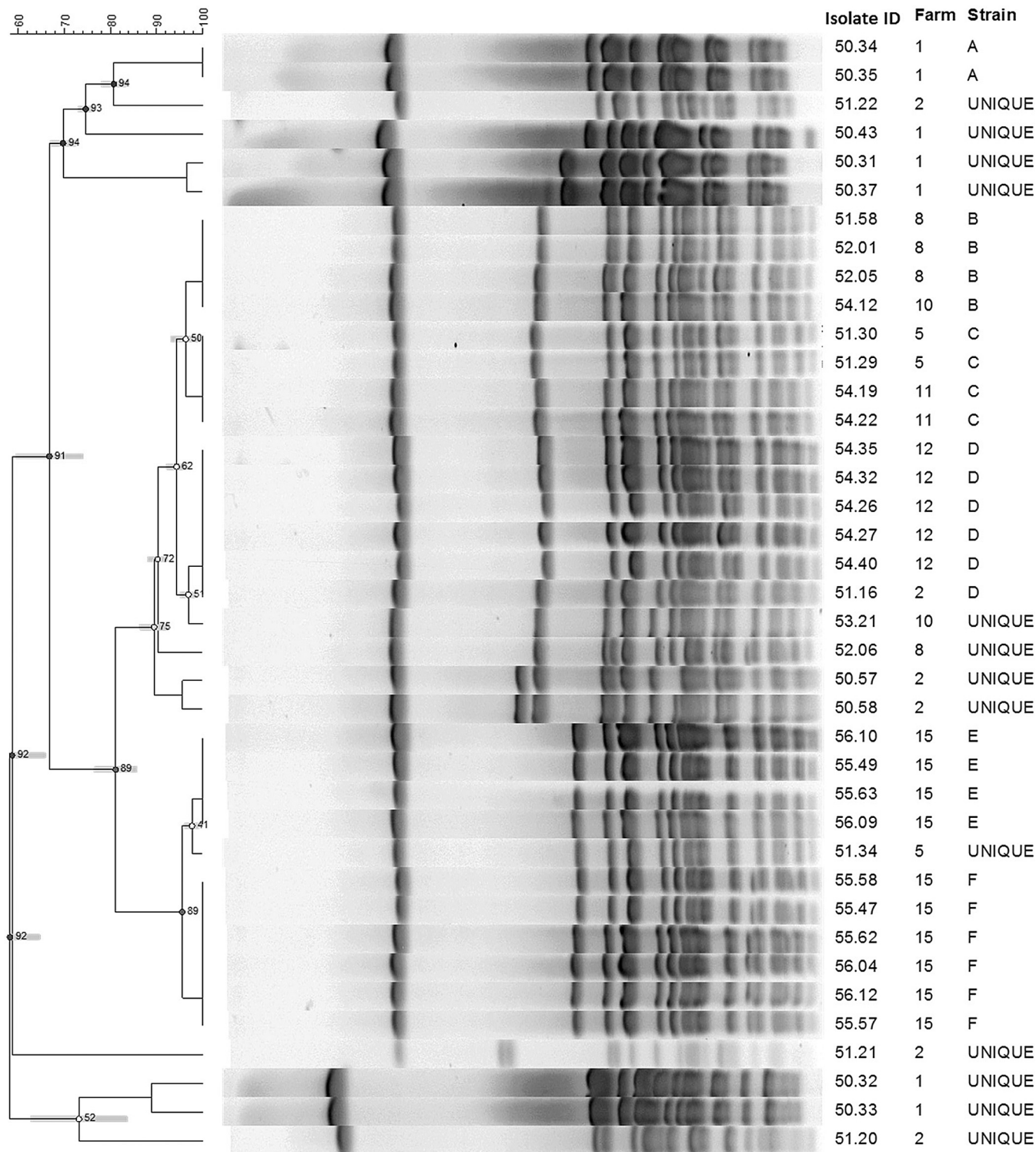
## DISCUSSION

To our knowledge, this is the first study to characterize a collection of coagulase-positive *S. agnetis* isolates from dairies in the United States and to develop a simple method to differentiate *S. hyicus*, *S. agnetis*, and *S. aureus*. In the original description of *S. agnetis*, 13 isolates originating from 13 cows on 8 dairies in southern Finland were characterized, including 12 isolates from bovine subclinical or mild clinical mastitis and one isolate from a teat apex (10). Of those original 13 *S. agnetis* isolates, 3 isolates were found to have a delayed coagulase-positive reaction, and the remaining 10 isolates were coagulase negative. The delayed coagulase reaction of the previously reported *S. agnetis* isolates (10) matched the coagulase description originally given to *S. hyicus* (5). In contrast, in the present study, most (88%) of the coagulase-positive isolates were

**TABLE 2** Summary of pulsed-field gel electrophoresis results for all strains identified more than once on a herd, including number of cows infected with each strain and number of cows identified with the strains at multiple sampling points

Herd no. <sup>a</sup>	PFGE strain typing ID	No. of isolates within strain type/ total <i>S. agnetis</i> isolates within herd (% of total <i>S. agnetis</i> isolates)	No. of cows with that strain type	No. of cows with strain type at multiple time points	Days between sample collections
1	A	2/8 (25)	2		
8	B	3/4 (75)	2	1	177
5	C	2/3 (67)	2		
11	C	2/2 (100)	2		
12	D	5/5 (100)	4	1	264
15	E	4/10 (40)	4		
15	F	6/10 (60)	4	2	41 and 153, respectively

<sup>a</sup>Herds were included here only if a PFGE strain type was identified more than one time. If a herd is not listed, all strains within that herd were unique based on a PFGE electrophoretic pattern with at least one band difference.



**FIG 3** Comparison of all *S. agnetis* isolates ( $n = 39$ ) from 8 commercial dairy herds in which more than one *S. agnetis* isolate was identified from bovine milk samples. A total of 6 different strains were identified (strains A to F). Strains B, C, and D include isolates from more than one herd. ID, identification.

tube coagulase positive at 4 h. The isolates used in the present study originated from a study in which the primary goal was to identify *S. aureus* (26), but the study protocol included isolation and storage of all coagulase-positive staphylococci during the initial screening of all milk samples. Coagulase-negative staphylococci were not saved for further characterization and thus could not be evaluated in the present study. In the

original study, the banked CPS isolates were identified to the species level using biochemical methods, and only those isolates identified as *S. aureus* were utilized for further study at the time.

The data presented here demonstrate that although the overall prevalence of coagulase-positive *S. agnetis* was determined to be low, it can be prevalent among CPS isolated from milk in some herds and could be confused with *S. aureus*, especially since the majority of isolates clotted rabbit plasma after 4 h of incubation similar to *S. aureus* and differently from the delayed coagulation typically described for *S. hyicus* (5, 10). Misclassification could impact management decisions, such as unnecessary culling based on the belief that the cow has *S. aureus* and is a contagious reservoir for infection of other cows.

Based on the original phenotypic species identification of the isolates used in this study, 18 isolates were originally misclassified as *S. hyicus*. When genotypic species identification was performed, the 18 isolates were found to be staphylococcal species other than *S. hyicus* or *S. agnetis*. Some of this error could be due to storage error or loss of the initial organism during the recovery and subculturing of the isolates. Some of the error is also likely due to misclassification reported to occur with phenotypic species identification, as such methods can lead to a higher number of unidentified or misidentified staphylococcal isolates (27). When using phenotypic methods, *S. chromogenes* can be misidentified as *S. hyicus* (28), as was seen with 8/18 (44%) of the misclassified isolates in this study. Although phenotypic methods have been shown to be inaccurate for non-*aureus* *Staphylococcus* species (29), previous reports have shown the API system to be satisfactory in the identification of *S. aureus* (30). Therefore, in this study, the *S. aureus* isolate identities were not further confirmed using genotypic methods.

The *aroD* gene encodes 3-dehydroquinate dehydratase, an enzyme involved in the shikimate pathway used to synthesize chorismic acid, a central precursor for aromatic compounds (31). This is a housekeeping function for the cell and therefore, the gene should be highly conserved. Selection of a highly conserved gene as a target for species identification is important to allow for broad applicability and consistency in results. Based on the gene sequence analyses reported herein, the *aroD* gene fragment used for the multiplex PCR appears to be highly conserved, with over 99% homology found within all *S. agnetis* isolates studied, including isolates from 10 different U.S. herds that were composed of 23 different PFGE strains types, as well as four isolates from Finland.

Because *S. hyicus* was rarely found in this study, we are unable to determine how conserved the *aroD* gene is in this species. However, when the single study isolate was compared to a type strain isolate of swine origin, it was found to have 99% sequence homology, suggesting that a conserved region was selected. It should be further noted that the only *S. hyicus* isolate harvested from cow's milk was not coagulase positive, but it was included in the analyses to demonstrate that the multiplex PCR could differentiate a field *S. hyicus* strain from *S. agnetis* and *S. aureus*. Further, it was included in the PFGE analyses to demonstrate that the electrophoretic banding pattern was different from all of the *S. agnetis* isolates. Similarly, the coagulase-negative *S. agnetis* isolate was included in the PFGE analysis to demonstrate that it was genotypically different from the coagulase-positive variants. Note that the data in Table 1 do not include the coagulase-negative *S. agnetis* isolate or the *S. hyicus* isolate.

Although *S. agnetis* was found at a low prevalence among all herds studied, it can be identified at a prevalence equal to or greater than that of *S. aureus* during some sampling periods. Based on PFGE, some strains of *S. agnetis* appear to act as contagious strains based on the fact that the same PFGE strain type was isolated from the milk of multiple animals in a single herd. Also, some strains appear to cause a persistent infection, lasting over 150 days in 3 cows. Cow-level somatic cell count and milk yield data were not collected in the original study; thus, it cannot be determined whether the reported strains have an important impact on udder health. The potential for contagious spread does raise concern that some strains of coagulase-positive *S. agnetis* may

be important udder pathogens in some herds, but further studies would be needed to confirm this finding.

The multiplex PCR described herein provides an inexpensive accurate method to differentiate three species of staphylococci that can be difficult to differentiate based on phenotype alone. Further, preliminary evidence is provided that suggests the multiplex PCR has promise for differentiating *S. chromogenes* from coagulase-negative variants of *S. hyicus* and *S. agnetis*. However, more coagulase-negative isolates need to be evaluated to confirm this finding.

One limitation of this study was the lack of a comparable number of *S. hyicus* field isolates and the lack of a coagulase-positive *S. hyicus* as a comparator for coagulase-positive *S. agnetis* in the PCR test. This may be due to *S. hyicus*, and in particular coagulase-positive *S. hyicus*, truly being a rare species in dairy herds or just rare in the herds within this study. Several other recent studies in which species identification was done using genotypic methods, have found the prevalence of coagulase-negative *S. hyicus* to be very low, ranging from 0.5% to 1.3% (32, 33). The data could also be biased, because the original protocol for isolate storage included only coagulase-positive isolates. Thus, the prevalence and importance of coagulase-negative *S. hyicus* and *S. agnetis* among staphylococcal species isolated from milk samples on these herds cannot be evaluated. The original misclassification of the two coagulase-negative isolates as coagulase positive (1 *S. hyicus* and 1 *S. agnetis*) may be due to the banked isolate not being the originally recorded CPS isolate or differences in interpretation of a weak or delayed coagulase test result. Although these isolates were misclassified, they were not removed from the study due to the fact that they were the staphylococcal species of interest and therefore useful to validate the multiplex PCR, as the total number of isolates in this study was low.

In conclusion, within these herds, *S. agnetis* was much more prevalent than *S. hyicus*. Based on PFGE, some strains of *S. agnetis* were shown to have contagious behavior and cause persistent infections. The use of the described multiplex PCR allows inexpensive and efficient differentiation of the common species of CPS isolated from milk without the need for housekeeping gene sequence analysis, and preliminary evidence suggests it may aid in differentiating CNS variants of *S. agnetis* and *S. hyicus* from each other and from *S. chromogenes*. Future studies will evaluate the utility of the multiplex PCR on CNS variants and also examine the effect of *S. agnetis* on the host, including somatic cell count.

## MATERIALS AND METHODS

**Isolate and phenotypic characterization.** Banked coagulase-positive non-*aureus* *Staphylococcus* isolates ( $n = 62$ ) collected from whole-herd milk samplings and milk samples of cows and heifers at calving on 15 dairy herds in Washington and Idaho as part of a previous study (26) to evaluate different cattle importation practices on the prevalence of *S. aureus* IMI were utilized for the present study. The original study used herds located in the Pacific Northwest (PNW) region of the United States that were identified as being willing to participate in the study by their veterinarian. Veterinarians were contacted by mail using a mailing list provided by the American Association of Bovine Practitioners of all members located in the PNW region. Sixteen herds were initially identified, enrolled, and grouped based on cattle importation practices. One herd (herd 3) was excluded due to lack of sample collection by the herd. In the original study, all lactating cattle had composite mammary foremilk samples (aliquots of milk from all four mammary quarters comingled in a single sample vial) aseptically collected three times at approximately 4-month intervals over a 1-year period. Additional composite foremilk samples were taken from cows and heifers at the time of calving. During sample processing to identify cows with *S. aureus* IMI, all coagulase-positive staphylococci were stored in phosphate-buffered glycerol at  $-20^{\circ}\text{C}$ ; no coagulase-negative staphylococci were saved. All coagulase-positive isolates were then identified to the species level using a commercial biochemical test strip (API Staph; bioMérieux, Inc., Hazelwood, MO, USA). Isolates were either identified as *S. aureus* or *S. hyicus* by this method. The focus of the present study was the isolates originally identified as coagulase-positive *S. hyicus*.

Isolates were subcultured from the phosphate-buffered glycerol storage medium onto brain heart infusion agar slants (Remel, Lenexa, KS, USA) at Washington State University and shipped chilled to the University of Missouri for further characterization. At the University of Missouri, isolates were grown on Columbia blood agar (CBA) (Remel) for approximately 24 h at  $37^{\circ}\text{C}$ , and representative colonies of each isolate were coagulase tested using rabbit plasma (Thermo Scientific, Lenexa, KS, USA). Coagulase results were recorded after 4 h and 24 h of incubation at  $37^{\circ}\text{C}$ .



**TABLE 3** Primers used for the multiplex PCR to identify to the species level and differentiate *S. hyicus*, *S. agnetis*, and *S. aureus*

Gene <sup>a</sup>	Primer	Sequence (5' to 3')	Amplicon size (bp)	Positive species
<i>aroD</i>	aroD-HyF	TAT GGT GTC GAC CAA TCG AAG GCT	425	<i>S. hyicus</i>
<i>aroD</i>	aroD-HyR	ACC CTA TAG CCC GCT TAC TT		
<i>aroD</i>	aroD-AgF	CGC ATG AGA GAC CAA TAC GCT	293	<i>S. agnetis</i>
<i>aroD</i>	aroD-AgR	TAG GAC GTA TAG AGG TGG		
<i>nuc<sup>b</sup></i>	nuc-S	CTG GCA TAT GTA TGG CAA TTG TT	664	<i>S. aureus</i>
<i>nuc<sup>b</sup></i>	nuc-AS	TAT TGA CCT GAA TCA GCG TTG TCT		

<sup>a</sup>*aroD*, 3-dehydroquinate dehydratase gene; *nuc*, thermonuclease gene.

<sup>b</sup>See reference 35.

**Confirmation of species identification by housekeeping gene sequence analysis.** A single colony was picked from CBA and inoculated into 100  $\mu$ l of 1 $\times$  Tris-EDTA (Promega, Madison, WI, USA) for lysate preparation. Standard PCR, with the addition of an initial denaturation step of 15 min at 94°C, was used to amplify the *rpoB* (18) and *tuf* (22) genes. The PCR for *rpoB* gene identification and subsequent sequencing for species identification were done on all isolates, and the PCR for *tuf* gene identification and subsequent sequencing were conducted only on isolates not definitively identified as *S. agnetis* or *S. hyicus*. The resultant PCR products were purified using a commercial kit (Invitrogen, Carlsbad, CA, USA) and submitted to the University of Missouri DNA Core Facility for Sanger sequencing. Sequences were compared to the GenBank database using the publicly available nucleotide-BLAST algorithm ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). For *rpoB* analysis, species identity was confirmed if the sequence had  $\geq 97\%$  identity with a single species of *Staphylococcus* in the GenBank database (34). For *tuf* gene analysis, species were confirmed if sequence identity was  $\geq 98.0\%$  with  $>0.8\%$  separation between species (22).

**Multiplex PCR development.** A novel multiplex PCR was developed to differentiate *S. hyicus* from *S. agnetis* by comparing the whole-genome sequences for *S. agnetis* CBMRN 20813338 (24) and *S. hyicus* ATCC 11249<sup>T</sup> (23). The Artemis comparison tool (Wellcome Trust Sanger Institute, Hinxton, UK) was used to identify regions of difference between the two genomes. Based on these genome-wide comparisons, primer sets were designed to amplify a partial segment of the *aroD* gene for *S. hyicus* and *S. agnetis* (Table 3). Standard multiplex PCR, including sets of *aroD* gene primers to identify *S. hyicus* and *S. agnetis*, respectively, and a *nuc* gene primer set to identify *S. aureus* (35), was performed using the following thermocycler conditions: 94°C for 15 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The run was completed at 72°C for 5 min and then held at 4°C. After amplification, electrophoresis was completed using a 1% agarose gel, and gels were visualized by UV transillumination. A 100-bp ladder was used to determine PCR product size.

**Testing the specificity of the novel multiplex PCR assay to differentiate *S. hyicus*, *S. agnetis*, and *S. aureus*.** Isolates obtained from Washington State University that were identified as *S. agnetis* or *S. hyicus* by housekeeping gene sequence ( $n = 44$  of 62 isolates) were analyzed using the novel multiplex PCR described above. Positive controls included *S. agnetis* CBMRN 20813338 and DSM 23656<sup>T</sup> (6 VT), *S. hyicus* ATCC 11249<sup>T</sup>, and *S. aureus* ATCC 12600<sup>T</sup> and ATCC 29740. Additional *S. agnetis* strains were also included as controls, including the reference strain DSM 23658 (55 OE), and two strains (43 OE and 100 VT) reported in the original description of *S. agnetis* (10). Given that *S. chromogenes* is genotypically similar to both *S. hyicus* and *S. agnetis*, two strains of *S. chromogenes* (ATCC 43764<sup>T</sup> and MU 970) were tested to ensure that the multiplex PCR did not misclassify *S. chromogenes* as *S. agnetis*, *S. hyicus*, or *S. aureus*. All PCR products that amplified with the *aroD* gene primer sets were purified and sequenced to verify amplification of the targeted gene and to compare sequences.

**Pulsed-field gel electrophoresis.** To understand subspecies variation within the tested isolates, pulsed-field gel electrophoresis was performed as previously described (36). Briefly, Smal (Invitrogen, Carlsbad, CA, USA) digests of staphylococcal DNA were separated by PFGE in a 1% agarose gel (Pulsed Field Certified agarose; Bio-Rad, Hercules, CA) immersed in 0.5% Tris-boric acid-EDTA (TBE) buffer at 15°C for 20 h at 6 V/cm with a 5- to 50-s pulse time on a CHEF-DR III PFGE machine (Bio-Rad). *Salmonella* BAA-664 was used as a molecular weight size standard and was digested using XbaI (Invitrogen). Standards were included in the first, middle, and last lanes of each gel. Completed gels were stained with ethidium bromide and photographed using UV transillumination.

**Data analysis.** All data were descriptive, with isolate species and strain type information expressed as absolute numbers and percentages. For the purpose of comparing the prevalence of detection of *S. aureus* versus coagulase-positive non-*aureus* *Staphylococcus* spp. at the cow level, *S. aureus* prevalence data from the original study were utilized (Table 1). Using the definitive genus and species identification data for coagulase-positive non-*aureus* *Staphylococcus* spp. and the *S. aureus* data from the original study (26), the cow-level prevalences for each CPS species identified from the three herd cultures and cattle at the time of calving on each herd were calculated. Herd identification numbers from the original study (26) were maintained.

Sequenced products from *aroD* gene PCR amplification were aligned and compared through the construction of a neighbor-joining dendrogram based on the multiple alignment similarity matrix using computer software (BioNumerics, version 7.5; Applied Maths, Kortrijk, Belgium). For interpretation of PFGE results, isolates that had indistinguishable banding patterns based on having the same number of

bands and corresponding bands of the same apparent molecular weight were considered to be the same strain (37). Additionally, PFGE gel images from herds with more than one *S. agnetis* isolate were analyzed using BioNumerics 7.5 (Applied Math) using Dice similarity coefficient and an unweighted pair group method using average linkages (UPGMA) dendrogram type. Tolerance settings included optimization at 1.0% and position tolerance at 1.0%. Strain types were determined by using a threshold cut point of 100% similarity.

**Accession number(s).** The *aroD* gene fragment sequences from the isolates included in the study were submitted to NCBI GenBank, accession numbers [KT750097](https://doi.org/10.1128/JCM.41.2.767-771.2003) to [KT750140](https://doi.org/10.1128/JCM.41.2.767-771.2003).

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