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REAL TIME MULTIPLEX PCR ASSAY FOR THE DETECTION OF *STAPHYLOCOCCUS AUREUS* USING *NUC* AND *EAP* GENES

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Abstract

Mastitis is a major economic concern for the dairy industry. Costs incurred from mastitis infections come from reduced production, increased culling rates, and the cost of treatment. *Staphylococcus aureus* is a gram positive bacterium that is a common cause of mastitis in dairy cattle. *S. aureus* is part of normal skin flora, but it can migrate into the mammary tissue during milking causing infection and inflammation. A high throughput diagnostic assay with high sensitivity and specificity is needed for timely and accurate diagnosis of infection. A multiplex real time PCR assay was developed for the detection of *S. aureus* thermonuclease (*nuc*) and extracellular adherence protein (*eap*) encoding genes. These two genes are highly prevalent in all *S. aureus* strains, but not in other *Staphylococcus* species. The assay was standardized with American Type Culture Collection reference strains and field strains provided by the Pennsylvania Animal Diagnostic Laboratory (University Park, PA). Of these 40 field strains, 38 were positive for *eap* and 36 positive for *nuc* based on the PCR assay. Sensitivity of the assay was found to be 95.8% for *eap* and 89.6% for *nuc*. Specificity was 100% for both genes. The assay was validated with quarter milk samples from mastitis cases provided by the Pennsylvania Animal Diagnostic Laboratory (University Park, PA). However, the assay was unsuccessful at detecting *S. aureus* in DNA extracted from milk samples. Once standardized and validated, PCR is a faster, more accurate diagnostic tool than traditional culture methods.

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Introduction

Staphylococcus aureus is a gram-positive bacterium that is a major cause of illness and infections in humans and animals. It causes a wide spectrum of disease, including superficial skin infections, abscesses, septicemia, and several toxin-mediated illnesses. Additionally, it is a leading cause of bovine mastitis, which is considered the most prevalent and costly disease in dairy herds worldwide (Seegers et al., 2003). *S. aureus* is part of normal skin flora, but it can migrate into the mammary tissue during milking, causing inflammation and infection. When bacteria enter the mammary gland, they can multiply in the milk and adhere to the epithelium, where they can produce toxins and trigger an immune response. The accumulation of white blood cells in milk appears as a high somatic cell count, resulting in poorer milk quality. Due to the ability to adhere to the epithelium and evade the host immune response, many mastitis infections are chronic, and persist at the subclinical level (Cucarella et al., 2004).

Mastitis is a major economic concern for the dairy industry. Costs incurred from mastitis infections come from reduced production, increased culling rates, and the cost of treatment. Clinical mastitis causes an estimated 5% decrease in production. While mastitis has a low mortality rate, the clinical mastitis or an elevated somatic cell count increases the relative risk of culling by 1.5 to 5, depending on severity (Seegers et al., 2003).

A high throughput diagnostic assay with high sensitivity and specificity is needed for rapid and accurate diagnosis of infection. Traditional biochemical assays often lack the desired sensitivity and specificity of a diagnostic test, are time consuming, and can be interpreted ambiguously (Hussain et al., 2008). Molecular methods like Polymerase Chain Reaction (PCR) offer high specificity and sensitivity and more rapid detection. A multiplex real time PCR assay for detection of *S. aureus* was designed for this purpose. A benefit of real time PCR is that gel electrophoresis is not needed to obtain results. This

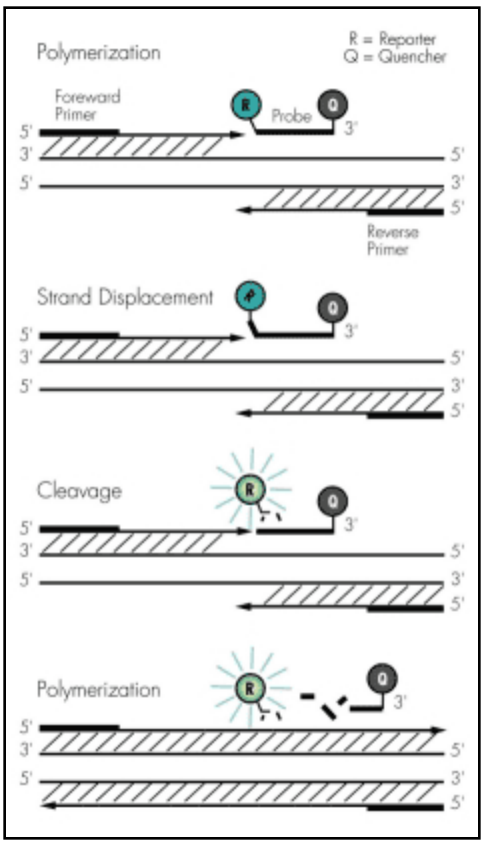

Figure 1: Principle of Real Time PCR. Primers and probes hybridize to target DNA. As DNA replication proceeds, the probe is cleaved, releasing the reporter dye (R) from the quencher dye (Q). **From Heid CA, Stevens J, Livak KJ, Williams PM, 1996. Real time quantitative PCR. Genome Res. 6:986-994.**

is also a quantitative PCR (qPCR) assay, which means that the relative amount of DNA present can be determined.

Real time PCR involves fluorescent signals to detect DNA amplification. Figure 1 gives a schematic of the rt-PCR process. Forward and reverse primers are used, in the same manner as regular PCR, to initiate replication of the specific DNA target. A specific probe, with a fluorescent dye attached at the 5' end and a quencher dye at the 3' end, hybridizes to the DNA target sequence. As replication proceeds, the probe is cleaved, releasing the fluorescent reporter dye from the quencher dye. This fluorescent signal is detected by the real time PCR instrument. As DNA proliferation continues, the amount of fluorescent dye increases. When the amount of fluorescent signal crosses above the threshold fluorescence level, this indicates a positive for that PCR target. This point is called the threshold cycle (C_t).

A multiplex PCR enhances the accuracy of the test by targeting more than one gene. Two genes were chosen based on their uniqueness to S. aureus and the ubiquity of these genes in all *S. aureus* strains (Brakstad et al., 1992, Chesneau et al., 1993, Hussain et al., 2008). The *nuc* gene (270 bp) encodes a thermostable extracellular nuclease (Brakstad et al., 1992, Harraghy et al., 2003). The ubiquity of the thermonuclease gene in *S. aureus* has been well documented, as have its uses as a PCR target (Brakstad et al., 1992, Chesneau et al., 1993, Kim et al., 2001). It has been shown however that a few other *Staphylococci*, including *S. intermedius* which is only found in veterinary samples, produce a thermonuclease, which may reduce the specificity of an assay using this target (Chesneau et al., 1993,

Gudding, 1983). The 230 bp *eap* gene encodes the extracellular adherence protein, important for binding to eukaryotic cells, initiating infection, and evading host defenses (Harraghy et al., 2003, Hussain et al., 2008). Little documentation exists of *eap*'s role as a target for identifying *S. aureus*; however it has been documented to be highly prevalent among *S. aureus* strains. Hussain et al. (2008) report on a singleplex PCR analysis that is 100% sensitive and 100% specific for the *eap* gene in *S. aureus*. Thus, it is proposed that *eap* would make a good genetic marker for molecular identification of *S. aureus*, and the combination with *nuc* gene would increase specificity. Both genes are highly prevalent in *S. aureus* but not in other *Staphylococci*. The goals of this study are to develop and standardize a multiplex real time PCR assay for the detection of S. aureus and to validate this assay using DNA extracted directly from milk samples.

Background and Review of Literature

Mastitis is a serious economic concern for the dairy industry. Seegers et al. (2003) report that this is the most prevalent production disease in dairy herds worldwide. Costs come from primarily from reduced production, as clinical mastitis is associated with a 5% decrease in milk production and a 1.5 to 5.0 relative risk of premature culling. Estimated annual losses due to mastitis are approximately $2 billion in the US (Donovan et al., 2005).

Staphylococcus aureus, a gram-positive, aerobic, non-sporeforming cocci, is one of several etiological agents of mastitis. *S. aureus* has the ability to produce chronic infections resulting in subclinical mastitis, which does not elicit clinical signs but is responsible for elevated somatic cell counts and lower quality milk. One study of several New York dairies indicates that 28.5% of clinical mastitis cases are caused by gram-positive organisms including *S. aureus*. It was also found that 31.8% of cases were caused by gram-negative organisms, 15.0% by other organisms, and 24.8% with no organism identified. This study indicated that losses were greater due to gram-negative mastitis; however, this study did not include data from subclinical cases (Schukken et al., 2009). Subclinical cases are harder to detect due to the intermittent shedding of the pathogen. *S. aureus* mastitis is difficult to control with antibiotics. Antibiotic therapy is often unsuccessful in eradicating the infection, and thus animals are often culled prematurely, resulting in production loss (Cucarella et al., 2004). Bacteria enter through the streak canal especially during milking. Due to the ability to adhere to the epithelium, the bacteria can establish an infection in the mammary gland where they can multiply and produce toxins. *S. aureus* has the ability to produce biofilms, large aggregations of adherent cells and exopolysaccharide matrix. Because of their size, these biofilms are able to resist phagocytosis and the actions of some antimicrobial products (Cucarella et al., 2004).

Rapid and accurate diagnosis of *S. aureus* is important for timely and appropriate therapy, especially in light of the emergence of antibiotic resistant strains (Martineau et al., 1998). Traditional

detection methods, including microbial culture and biotyping, have produced unsatisfactory sensitivity and specificity, can be interpreted ambiguously, and are not timely enough (Hussain et al., 2008). These methods rely on phenotypic expression, which varies due to environmental conditions.

Genomic detection of *S. aureus* has been shown to be equally or more sensitive than conventional methods, while also being more rapid (Brakstad et al., 1992, Kim et al., 2001). Kim et al. (2001) report detection *S. aureus* by PCR of culture negative samples due to the ability of this method to detect lower levels of the organism, which may be attributed to intermittent shedding in subclinical cases. This finding was correlated with higher SCC in these animals. Phuektes et al. (2003) and Taponen et al. (2009) also report that genotypic methods were able to positively identify *S. aureus* strains that were not detected by culture methods.

The 270 bp thermonuclease gene (*nuc*), which encodes a thermostable exonuclease, has been commonly used as a target for genotypic diagnostic assays for *S. aureus* (Brakstad et al., 1992, Chesneau et al., 1993, Kalorey et al., 2007, Kim et al., 2001, Ramesh et al., 2002, Yamagishi et al., 2007, Yang et al., 2007). It has been documented however that other species, including several coagulase-negative *Staphylococci*, also produce a thermonuclease. Antibody detection for this thermonuclease has been used as a diagnostic test for *S. aureus*, but this has been shown to not be specific enough due to variations in phenotypic expression (Chesneau et al., 1993).

The 230 bp extracellular adherence protein gene (*eap*) has not been widely used as a target, but has been shown by Hussain et al. (2008) to be highly conserved in *S. aureus* strains, even more so than *nuc*. *Eap* is a potent virulence factor which enhances the ability of *S. aureus* to invade eukaryotic cells and inhibit wound healing. The gene does have some polymorphism, but a successful PCR was developed which detected the gene in all 597 *S. aureus* strains (including human, veterinary, and reference strains) used in that study. Not only was the sensitivity of this assay 100%, but it also had 100% specificity, as the gene was not detected in any of the 216 non-aureus *staphylococci* or any of the

other gram-positive cocci used. This gene is only expressed in 70% of the isolates tested in vitro, further suggesting the power of genotypic diagnostic tests. *Eap* was shown to be restricted to *S. aureus* and present in all isolates of the extensive collection tested in this study, giving it significant promise as a diagnostic marker for *S. aureus*.

While genotypic methods have been shown to have advantages over phenotypic methods, extracting DNA directly from milk without bacterial culture has been shown to be problematic (Phuektes et al., 2003). Eliminating bacterial culture greatly reduces the time required to complete a diagnostic test; however, complications have arisen when DNA extracted from milk or other body fluids is used for downstream applications such as PCR. Specific substances in mastitic milk have been shown to inhibit the *Taq* DNA polymerase commonly used in PCR assays (Kim et al., 2001). This study found that the use of a different DNA polymerase (derived from *Thermus thermophilus*) was able to raise sensitivity from 65% to 80%, and the use of a chelating agent in the DNA extraction procedure raised sensitivity to 100%. PCR sensitivity has been shown to be lower from bacteria in body fluids than in saline or water (Brakstad et al., 1992, Kim et al., 2001, Phuektes et al., 2003), presumably due to interfering compounds.

Materials and Methods

Isolates and samples

Eighteen American Type Culture Collection reference strains were used (8 *Staphylococcus aureus*, 8 non-aureus *Staphylococci* [coagulase negative *Staphylococci*], and 2 non-*Staphylococcus* species) in this study. Table 1 lists all species used. Forty *S. aureus* field isolates (Table 2) and 72 quarter milk samples from mastitis cases were obtained from the Pennsylvania Animal Diagnostic Laboratory (University Park, PA). The PADL indentified several bacterial species, including *S. aureus*, as the etiological agent of the mastitis cases. To confirm the findings of the PADL, these milk samples were cultured onto 5% sheep blood agar, grown at 37^{o}C for 24 hours, and biochemically analyzed using the API Staph strip test (bioMérieux, Durham, NC), according to manufacturer's instructions. One sample was excluded from the study as the confirmed identification as *S. aureus* was only 77.7% confident, and two other samples were also excluded as they were identified as *S. lentus* (62.9% confidence) and *S. chromogenes* (86.8%), respectively.

Table 1: American Type Culture Collection reference strains.

Reference Strain	ATCC
Staphylococcus aureus	10832
S. aureus	12600
S. aureus	13565
S. aureus	13709
S. aureus	19095
S. aureus	27664
S. aureus	29213
S. aureus	51651
S. chromogenes	43764
S. cohnii	35662
S. epidermidis	14990
S. hominis	27844
S. intermedius	49051
S. simulans	27898
S. warneri	27836
S. xylosus	35663
Streptococcus uberis	9927
Escherichia coli	43889

Table 2: Field isolates. Accession numbers refer to the Pennsylvania Animal Diagnosis Laboratory, University Park, from the year 2008.

Sample No.	Accession No.	Origin	Confirmed Species	Percent Confidence
1	7367	Porcine lung	*S. aureus*	97.80
2	8000	Quarter sample	*S. aureus*	98.10
3	8000	Quarter sample	*S. aureus*	98.10
4	8000	Quarter sample	*S. aureus*	98.10
5	8004	Quarter sample	*S. aureus*	97.80
6	8004	Quarter sample	*S. aureus*	97.80
7	8143	Porcine lung	*S. aureus*	97.80
8	8464	Quarter sample	*S. aureus*	97.80
9	8549	Quarter sample	*S. aureus*	97.80
10	8868	Quarter sample	*S. aureus*	97.80
11	9082	Quarter sample	*S. aureus*	97.80
12	9952	Quarter sample	*S. aureus*	98.10
13	10205	Quarter sample	*S. aureus*	97.80
14	10417	Quarter sample	*S. aureus*	97.80
15	10682	Quarter sample	*S. aureus*	97.80
16	10776	Quarter sample	*S. aureus*	77.70
17	11316	Quarter sample	*S. aureus*	96.90
18	11427	Quarter sample	*S. aureus*	97.80
19	12159	Quarter sample	*S. aureus*	98.10
20	13371	Quarter sample	*S. aureus*	97.80
21	14371	Unknown	*S. aureus*	97.80
22	14537	Quarter sample	*S. aureus*	97.80
23	14985	Quarter sample	*S. aureus*	98.10
24	14985	Quarter sample	*S. aureus*	98.10
25	14985	Quarter sample	*S. aureus*	98.10
26	15701	Unknown	*S. aureus*	97.80
27	16246	Bulk Tank	*S. aureus*	97.80
28	16578	Quarter sample	*S. aureus*	98.10
29	17346	Quarter sample	*S. aureus*	97.80
30	17952	Liver	*S. aureus*	97.80
31	17952	Spleen	*S. aureus*	97.80
32	17958	Hock joint	*S. aureus*	97.80
33	17958	Foot pad	*S. aureus*	97.80
34	18144	Quarter sample	*S. lentus*	62.90
35	18498	Quarter sample	*S. aureus*	97.80
36	18572	Quarter sample	*S. aureus*	98.10
37	21007	Quarter sample	*S. aureus*	97.80
38	21012	Quarter sample	*S. aureus*	97.80
39	21012	Quarter sample	*S. aureus*	97.80
40	22901	Quarter sample	*S. aureus*	97.80
41	23107	Quarter sample	*S. aureus*	98.10
42	23728	Quarter sample	*S. aureus*	96.60
43	23048	Lung	*S. chromogenes*	86.80

Culture

Reference strains and field isolates were grown on 5% sheep blood agar, and incubated for 48 hours at 37^{o}C. The quarter milk samples were grown on 5% sheep blood agar for 48 hours at 37^{o}C. Any suspect colonies showing β-hemolysis were subcultured on Baird-Parker Agar (BPA), which is selective for *Staphylococci*, and incubated at 48 hours at 37^{o}C. Any colonies on the BPA that demonstrated a lipase reaction were considered *S. aureus*. For bacterial counts in the milk samples, 50 µl were spread on a plate using aseptic technique and incubated at 37^{o}C for 24 hours.

DNA Extraction

Several procedures were used for DNA extraction. MO BIO Ultra Clean Microbial DNA Isolation (Mo Bio Laboratories, Inc., Carlsbad, CA) was used to extract DNA from the ATCC reference strains and the ADL field strains, using colonies from blood agar, according to manufacturer's instructions. The QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) was used to extract DNA from milk samples, according to the manufacturer's instructions. The stool kit was chosen based on its ability to remove PCR inhibitors that may be present in milk.

A chelating agent, Chelex-100 (400 Minus Mesh, Bio-Rad, Richmond, CA), was added as an additional step for the extraction of DNA from milk samples, according to the method described by Kim et al. (2001).

In another procedure, a phenol-chloroform extraction was performed on milk samples, modified from Phuektes et al. (2001). An additional lysis step was performed to improve DNA yield by breaking down the *staphylococcal* cell wall. Lysostaphin (Sigma-Aldrich, Saint Louis, MO), a lytic enzyme derived from *Staphylococcus staphylolyticus*, was used in this step because this enzyme degrades the *staphylococcal* cell wall. One ml of each milk sample was placed in an Eppendorf tube, centrifuged, and washed in phosphate buffered saline solution. 400 µl of a lysis solution containing lysozyme, lysostaphin, EDTA, and tris buffer was added, and samples were incubated at 37^{o}C for 30 minutes. After

heating for 10 minutes at 95°C, 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The aqueous phase was recovered. DNA was pelleted by the addition of 1 ml absolute ethanol, then resuspended in water. All DNA samples were stored at -80°C.

Primers and Probes

Primers and fluorescent-labeled probes were developed for the two target genes using Premier Biosoft Beacon Design Software. These primers and probes are listed in Table 3. Fluorescent labels were attached to the 5' end of the probes. A HEX (6-carboxy-2', 4, 4', 5', 7, 7'-hexachlorofluorescein) fluorescent signal was attached to the *nuc* probe, and FAM (6-carboxy-fluorescein) was attached to the *eap* probe.

Table 3: Primers and probes developed with Premier Biosoft Beacon Design Software

	eap	***nuc***
Forward	5'-ATAACTGTAACTTTGGCACTGG-3'	5'-GTTGTAGTTTCAAGTCTAAGTAGC-3'
Reverse	5'-GCCGGTAGTTTGTCCTTTTC-3'	5'-GAAGTTGCACTATATACTGTTGG-3'
Probe	5'-FAM-ACCTCATTACCTGCCACAGTAGCG-3'	5'-HEX-TGCATCACAAACAGATAACGGCGT-3'

PCR

The Stratagene (La Jolla, CA) Mx 3000p thermocycler system was used. Real time qPCR conditions were set at 40 cycles of DNA denaturation at 95°C for 30 sec (initial denaturing at 95°C for 5 minutes), primer annealing at 53°C for 1 min, and DNA extension at 72°C for 30 sec. The reaction was terminated by a final extension at 72°C for 10 min. A reaction mixture was prepared with 2.5 µl of sample DNA, and 22.5 µl of a master mix containing the following: 13.2 µl Stratagene Brilliant II QPCR Multiplex buffer; 1.25 µl each of *eap* forward primer, and *nuc* forward and reverse primers; 0.55 µl *eap* reverse primer; 2.5 µl each of *eap* and *nuc* probes; and 0.375 µl of reference ROX dye. The primers and probes were used in 0.5 µM solutions. Two positive controls (ATCC 10832 and 29213) were used in each assay performed, as well as two negative controls with 2.5 µl deionized water instead of DNA in 22.5 µl

of the master mix. To account for possible pipetting errors, ROX (carboxy-X-rhodamine) was used as a reference dye and added to the master mix.

PCR Assay Standardization

Standard curves for each reaction were generated using 10-fold dilutions of DNA extracted from *S. aureus* pure culture. Dilutions in triplicate from 10^0 to 10^{-6} were used. Initial DNA concentration was approximately 2000 units per microliter. Efficiency was for the reaction was found to be 81.1% for *nuc* and 74.3% for *eap*. The reaction was able positive to the 4^{th} dilution, which was approximately 2 units per microliter of DNA.

To determine the amount of DNA needed in milk samples for detection by the PCR assay, skim milk was inoculated with known concentrations of *S. aureus*. Tryptic soy broth was inoculated with ATCC *S. aureus* strain 29213 and grown overnight at 37^{o}C overnight. After being diluted to 0.5 McFarland, 300 µl of the inoculated broth was added to 10 ml of skim milk. Serial dilution in milk was performed to the 6^{th} power. The initial concentration of *S. aureus* in the first sample was 1.5×10^8 CFU/ml based on the accepted value for 0.5 McFarland.

Gel Electrophoresis

To confirm results, DNA bands were visualized using gel electrophoresis. A 1% agarose gel was prepared and stained with 5 µL ethidium bromide. A 1 kb DNA ladder (New England BioLabs, Ipswich, MA) was used as a reference. 5 µl of a 6X blue-orange loading dye (Promega, Madison, WI) was added to each sample.

Results

Reference Strains

A standard curve (Figure 2) was developed for the PCR assay using DNA dilutions from one of the *S. aureus* reference strains (ATCC 29213). The R^2 values for both reactions were greater than 0.93.

The multiplex PCR assay was able to detect the *eap* gene in 8 (100%) of the 8 American Type Culture Collection strains and the *nuc* gene in 7 (87.5%) of these. Neither gene was detected in any of the non-aureus *Staphylococci*. The two non-*staphylococcus* species, *E. coli* and *Streptococcus uberis*, were also negative for both genes. Thirty-eight of 40 *S. aureus* field strains (provided by the Pennsylvania Animal Diagnostic Laboratory, University Park) were positive for *eap,* while 36 were positive for *nuc*. These results are summarized in Table 4. Combining these results, the specificity of this assay was determined to be 95.8% for *eap* and 89.6% for *nuc*. Sensitivity was found to be 100% for both *nuc* and *eap*.

Table 4: Summary of multiplex real time PCR results

	n	***eap* (%)**	***nuc* (%)**
ATCC *S. aureus* strains	8	8 (100)	7 (87.5)
ATCC Non-aureus *Staphylococci*	8	0	0
ATCC Non-*Staphylococcus* species	2	0	0
ADL Field Strains	40	38 (95)	36 (90)
S. aureus positive quarter milk samples	5	0	0
S. aureus negative quarter milk samples	35	0	0

Table 5 presents the real time PCR results for 18 ATCC reference strains. Two negative controls are used. Based on these data, a threshold cycle (Ct) above 35 for *eap* and above 33 for *nuc* was determined to be negative. The higher Ct's are probably due to the breakdown of the fluorescent probes. This will release the fluorescent dye from the quencher dye, which will be recorded as above threshold fluorescence. However this does not indicate replication of the target gene. All ATCC strains were positive for *eap*. One strain (27664) was negative for *nuc*, but all others were positive. All coagulase negative *Staphylococci* and the two non-*Staphylococcus* species had very high or no Ct's. Negative results are indicated by shading.

Table 5: rt-PCR results for ATCC reference strains

Species	**ATCC**	**Ct (*eap*)**	**Ct (*nuc*)**
Staphylococcus aureus	10832	19.73	20.98
S. aureus	12600	27.74	27.92
S. aureus	13565	21.29	22.99
S. aureus	13709	22.08	22.24
S. aureus	19095	24.29	25.48
S. aureus	27664	25.95	36.45
S. aureus	29213	23.74	22.19
S. aureus	51651	20.10	21.44
S. chromogenes	43764	37.62	36.80
S. cohnii	35662	37.57	35.81
S. epidermidis	14990	35.50	33.64
S. hominis	27844	35.68	33.56
S. intermedius	49051	37.20	36.68
S. simulans	27898	No Ct	No Ct
S. warneri	27836	35.68	35.03
S. xylosus	35663	37.88	36.20
Streptococcus uberis	9927	No Ct	37.58
Escherichia coli	43889	38.10	36.14
Negative Control	N/A	No Ct	No Ct
Negative Control	N/A	No Ct	No Ct

The results of the real time PCR assay on the 40 ADL field isolates are presented in Table 6. A negative control is included as the last sample. Threshold cycles (Ct) are listed for both the *eap* and *nuc* genes. Thirty-eight are positive for *eap* and 35 are positive for *nuc*. Thirty-five isolates (87.5%) are positive for both genes. Of the negative samples, three are negative for *nuc* only, while two are negative for both genes. A summary of these data is found in Table 4. It should be noted that the Ct for *eap* is consistently lower than the Ct for *nuc*. This is true for 7 out of the 8 ATCC *S. aureus* strains and in all 35 field strains that were positive for both genes.

Table 6: rt-PCR results for 40 field isolates

Sample	Ct (*eap*)	Ct (*nuc*)			
1	21.74	25.65	22	17.69	21.80
2	No Ct	No Ct	23	19.54	22.98
3	27.24	No Ct	24	17.62	21.09
4	21.30	26.15	25	20.99	24.51
5	22.44	26.56	26	20.18	24.03
6	27.66	No Ct	27	19.01	23.18
7	24.58	28.59	28	17.09	20.96
8	24.92	29.91	29	20.50	24.58
9	No Ct	No Ct	30	19.60	22.65
10	22.07	25.90	31	20.83	24.41
11	16.96	20.23	32	23.70	25.11
12	16.74	21.03	33	22.96	23.92
13	16.49	20.56	35	18.29	22.22
14	16.35	20.82	36	17.94	22.46
15	16.31	20.23	37	19.39	24.15
17	18.19	21.22	38	20.08	23.26
18	18.33	21.43	39	18.40	21.19
19	18.07	22.25	40	26.22	No Ct
20	17.98	21.40	41	20.17	24.06
21	19.10	22.78	42	20.48	25.21
			N/A*	No Ct	No Ct

*** Denotes negative control. Equivalent amount of distilled water replaces DNA**

Validation

Forty of the 72 quarter milk samples from the ADL were used to validate the assay. All samples were from mastitis cases. Of these, 5 were *S. aureus* positive based on biochemical analysis. These samples (21, 25, 30-32) are highlighted in Table 7. Only one of these samples crossed the fluorescence threshold at all (sample 31 for *nuc*), but this is not considered a positive reaction. All samples were

considered negative for both the *eap* and *nuc* genes on the PCR assay. The threshold cycles reported from the PCR assay are given in Table 6. These results are also summarized in Table 4. It should be noted that samples 1-20 and samples 21-40 were run in two different assays, but under the same conditions. For this reason, positive and negative controls appear twice.

Table 7: rt-PCR results for 40 quarter samples

Sample	Ct (eap)	Ct (nuc)	Sample	Ct (eap)	Ct (nuc)
1	No Ct	No Ct	21	No Ct	No Ct
2	No Ct	No Ct	22	No Ct	No Ct
3	No Ct	No Ct	23	No Ct	37.63
4	No Ct	No Ct	24	No Ct	No Ct
5	No Ct	37.92	25	No Ct	No Ct
6	No Ct	No Ct	26	No Ct	No Ct
7	No Ct	No Ct	27	No Ct	No Ct
8	No Ct	39.8	28	39.72	No Ct
9	38.6	No Ct	29	No Ct	No Ct
10	No Ct	No Ct	30	No Ct	No Ct
11	No Ct	No Ct	31	37.67	No Ct
12	No Ct	No Ct	32	No Ct	No Ct
13	37.61	No Ct	33	No Ct	No Ct
14	37.85	No Ct	34	No Ct	No Ct
15	No Ct	No Ct	35	No Ct	No Ct
16	No Ct	No Ct	36	No Ct	No Ct
17	35.85	37.93	37	No Ct	No Ct
18	37.45	38.27	38	No Ct	No Ct
19	No Ct	No Ct	39	No Ct	38.72
20	No Ct	No Ct	40	No Ct	No Ct
ATCC 10832	19.48	20.89	ATCC 10832	19.37	19.98
ATCC 29213	18.8	18.79	ATCC 29213	19.32	18.42
N/A*	No Ct	No Ct	N/A*	No Ct	38.83
N/A*	37.39	No Ct	N/A*	No Ct	No Ct

*** Denotes negative control. Equivalent amount of distilled water replaces DNA**

For the inoculated skim milk dilutions, the PCR assay was able to detect *S. aureus* to the third dilution (10^{-2}, or approximately 6,000 cells/ml). It should be noted that this is a weak positive result, as the threshold cycle was above 30 for all but the undiluted sample, while the reference samples all had Ct's less than 30. Two positive controls were used. ATCC 12600 had Ct's of 23.74 and 26.48, while ATCC 29213 had Ct's of 16.87 and 17.93 for *eap* and *nuc* respectively. Two negative controls used in this

assays had Ct's of 38.62 and No Ct for *nuc*, and No Ct for *eap*. The results of both the culture on Baird-Parker Agar and the PCR for these samples are presented in Table 8.

Table 8: Culture and rt-PCR results for inoculated milk dilutions

	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
eap							
Ct	29.29	33.56	35.32	37.33	36.7	38.4	37.95
Interpretation	+	+	+	-	-	-	-
nuc							
Ct	29.53	33.68	35.88	37.75	39.03	39.28	No Ct
Interpretation	+	+	+	-	-	-	-
***S. aureus* (cfu/ml)**	600,000†	60,000†	6,000†	590.5	69.5	6	1

†Estimate. Colonies on plates were too numerous to count

DNA Extraction modifications

Neither the addition of Chelex-100 (a chelating agent), nor the modified phenol-chloroform DNA extraction method, caused any improvement of PCR results. Table 9 shows the real time PCR results with and without Chelex-100. There is no major difference between the two conditions. Some of the Ct's are actually higher with Chelex-100.

Table 9: rt-PCR results for inoculated milk dilutions with and without Chelex-100

	Without Chelex-100		With Chelex-100	
Dilution	**Ct (*eap*)**	**Ct (*nuc*)**	**Ct (*eap*)**	**Ct (*nuc*)**
10^{0}	29.29	29.53	31.13	29.90
10^{-1}	33.56	33.68	34.85	33.67
10^{-2}	35.32	35.88	36.73	36.75
10^{-3}	37.33	37.75	36.40	37.55
10^{-4}	36.70	39.03	38.36	37.16
10^{-5}	38.40	39.28	35.96	37.40
10^{-6}	37.95	No Ct	37.64	37.25
ATCC 12600	23.74	26.48	23.60	25.89
ATCC 29213	16.87	17.93	18.62	17.61
N/A*	No Ct	38.62	39.27	37.26
N/A*	No Ct	No Ct	No Ct	36.82

Table 10 displays the PCR results after a modified phenol-chloroform extraction compared to the QIAamp DNA Stool Mini Kit extraction. The rt-PCR data from the 7 inoculated milk dilutions, from Table 8, is compared to the same PCR protocol performed on DNA from a modified phenol-chloroform

extraction procedure. These data indicate that this change also did not improve PCR results. A different positive control was used in the initial procedure (ATCC 12600) than in the later procedure with the DNA extraction modification (ATCC 13565).

Table 10: rt-PCR results for two different DNA extraction methods

	QIAamp DNA Stool Mini Kit extraction		Modified phenol-chloroform extraction	
Dilution	**Ct (*eap*)**	**Ct (*nuc*)**	**Ct (*eap*)**	**Ct (*nuc*)**
10^{0}	29.29	29.53	38.75	29.7
10^{-1}	33.56	33.68	36.43	32.67
10^{-2}	35.32	35.88	No Ct	34.44
10^{-3}	37.33	37.75	37.4	33.39
10^{-4}	36.70	39.03	39.75	39.64
10^{-5}	38.40	39.28	No Ct	No Ct
10^{-6}	37.95	No Ct	35.4	35.12
ATCC 12600	23.74	26.48	‡	‡
ATCC 13565	‡	‡	20.31	21.43
ATCC 29213	16.87	17.93	16.49	13.5
N/A*	No Ct	38.62	38.75	34.65
N/A*	No Ct	No Ct	34.23	35.29

In summary, the multiplex real time PCR assay developed in this study was able to detect *S. aureus* using eap and nuc genes as targets when using DNA extracted from bacterial culture. The assay was unsuccessful at detecting DNA extracted directly from milk samples. The findings of this study are summarized in Table 4.

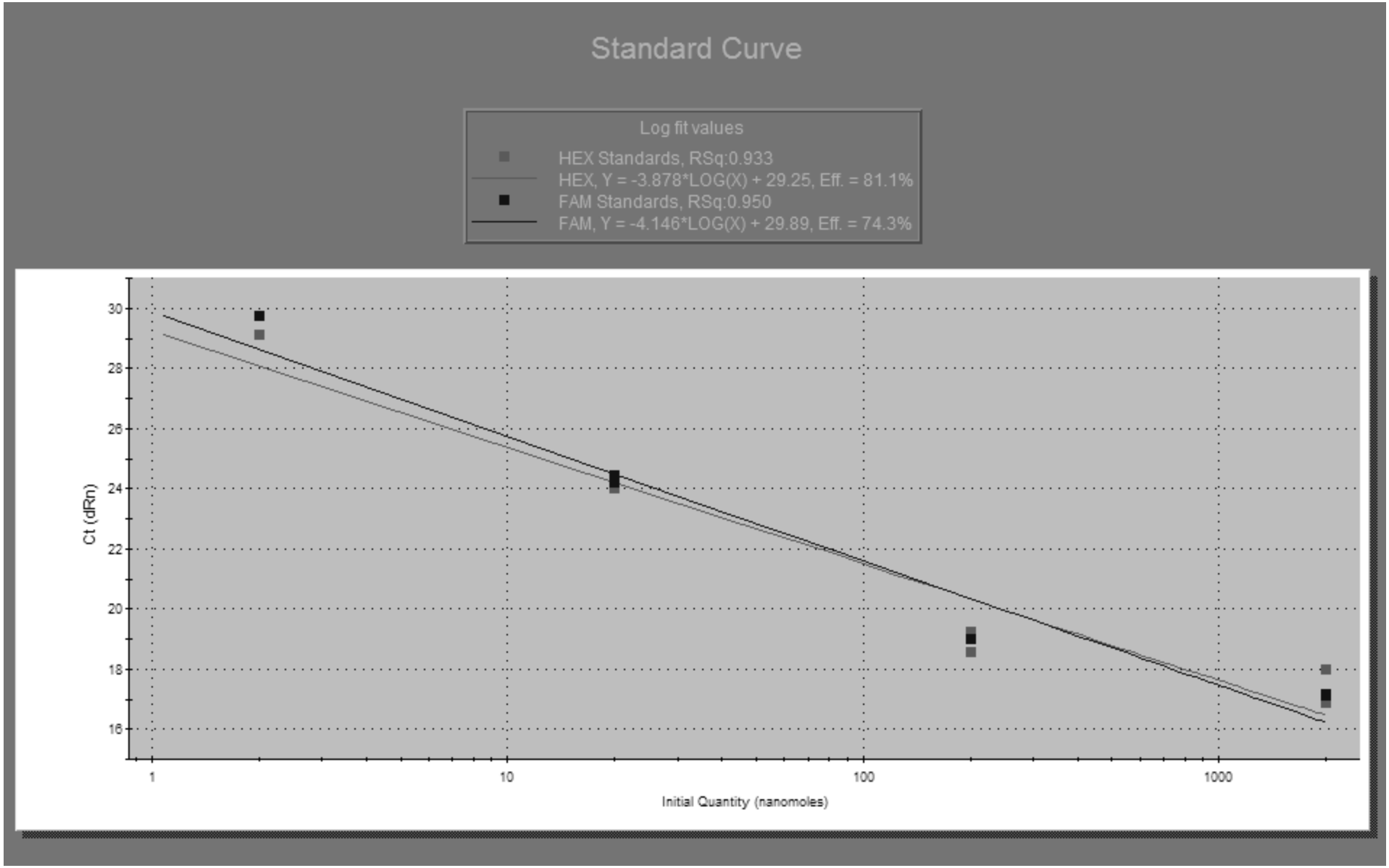

Figure 2: Standard Curve

Discussion

Due to the significance of *Staphylococcus aureus* as a human and animal pathogen, faster and more accurate diagnosis is important for timely and appropriate treatment. *S. aureus,* a leading cause of mastitis in dairy cattle, is a serious economic concern to the dairy industry. Costs incurred from mastitis infections come from reduced production, increased culling rates, and the cost of treatment (Seegers et al., 2003). A high throughput diagnostic assay with high sensitivity and specificity is needed for rapid and accurate diagnosis of infection. This will allow for more timely treatment with antibiotics.

Staphylococcus aureus is not only an animal pathogen, but also a major human pathogen, causing skin infections, bacteremia, as well as toxin-mediated illnesses (Brakstad et al., 1992, Cucarella et al., 2004, Martineau et al., 1998). Ultimately this assay could be used for rapid and accurate detection of human pathogens, leading to faster treatment time and improved recovery. Additionally, *S. aureus* is a food quality factor when evaluating dairy products. Enterotoxins produced by *S. aureus* are the third leading cause of food poisoning (Morandi et al., 2007).

Faster initiation of effective antibiotic therapy can reduce the use of broad spectrum antibiotic regiments, which are associated with high costs, toxicity, and the emergence of drug resistance (Martineau et al., 1998). Such a diagnostic test is in the process of being validated by this study. While this PCR assay shows high sensitivity and specificity for DNA extracted from culture, the number of false negatives for DNA extracted directly from milk makes it currently unsuitable for use when detecting *S. aureus* directly from milk samples. It is probable that inhibitors from the milk remain in the DNA product after the extraction procedure and interfere with the polymerase reaction (Kim et al., 2001). Besides milk, blood and other body fluids have been shown to inhibit PCR amplification or lower sensitivity (Brakstad et al., 1992, Kim et al., 2001). The assay was highly accurate for cultured samples. Reducing the number of PCR cycles from 40 to 30 was able to eliminate any false positives that appeared due to probe degradation. Specificity was 100% for both genes. For *eap*, sensitivity was

100%, but only 89.6% for *nuc*. These results suggest that *eap* may in fact be more accurate than *nuc* for the detection of *S. aureus* using genotypic methods. It is possible that there were false positives for *eap*; however, the results of Hussain et al. (2008) suggest otherwise. It is proposed that eap could replace *nuc* as a gene target for diagnostic tests for *S. aureus*. While this assay was successful at detecting these two target genes in *S. aureus*, it should be noted that the efficiency of the reaction could be improved. Efficiency was 81.1% for *nuc* and 74.3% for *eap* based on the standard curve developed with a reference strain. Modifications of PCR conditions, including changing annealing temperatures or modifying primer sequences, have the potential to improve this.

Despite the fact that *S. aureus* was not detected in the mastitis milk samples, the assay was able to detect *S. aureus* in inoculated skim milk samples to a dilution of 10^{-2}, or 6,000 CFU/ml. Because this was pasteurized, homogenized, fat-free milk, there were likely fewer inhibitors, and less DNA from somatic cells and other bacteria. Phuektes et al. (2001) report a similar finding. The undiluted inoculated milk also contained a higher concentration of bacteria than the quarter milk samples. The results from the inoculated milk samples indicate that the assay is able to detect *S. aureus* in DNA extracted from milk, but some conditions may have to be modified to improve the sensitivity of the test.

To improve the results from this assay, more samples should be used to give greater statistical power. Additionally, more controls should have been used. While positive controls were used (*S. aureus* reference strains), and negative controls that contained PCR reagents only, DNA from non-aureus *Staphylococci* and non-*Staphylococcal* species should have also been used for comparison.

An improved rapid procedure for extracting DNA directly from milk samples needs to be developed. A commercial kit for extracting DNA from fecal samples (QIAamp DNA Stool Mini Kit) was largely unsuccessful, as well as a modified phenol-chloroform method. The use of a chelating agent was also unsuccessful at improving DNA quality. It is proposed that filters to remove inhibitors may be a useful tool for this purpose. A larger pore filter could be used to remove substances in milk such as fat

micelles and debris, and a smaller pore filter could be used to isolate bacterial cells from the remaining fluid. These steps could potentially yield cleaner DNA and thus better PCR results. Phuektes et al. (2001) reported PCR inhibition due to substances in milk, and found that the use of spin columns, which would allow additional filtering of inhibitory substances, was able to improve PCR sensitivity.

This assay is currently unsuccessful in detecting *S. aureus* in DNA extracted directly from quarter milk samples. Modifications to the PCR conditions or to the DNA extraction procedure to increase DNA yield or remove inhibitors may increase the diagnostic power of this test. Once validated for pathogen detection in milk samples, this assay could be used for pathogen identification from wounds, blood, or other tissues. It could also be used for identification of pathogens isolated from humans or other animals, not just dairy cattle.

Currently, biochemical assays based on phenotypic characterization are routinely used to identify *S. aureus*. This can cause ambiguity because of subjective interpretation of results, and because phenotypic characterization depends on gene expression, which can vary significantly depending on environmental conditions (Hussain et al., 2008). Time is another major limitation of conventional detection methods. *S. aureus* takes up to 48 hours to grow on culture media, while results can be obtained from this multiplex PCR in under 6 hours. A molecular detection method, such as real time PCR, offers a faster, unambiguous high throughput alternative to conventional methods. Additionally, the sensitivity and specificity of molecular methods are generally higher due to their reliance on genotype rather than phenotype.

This study attempted to design and validate a real-time, multiplex PCR assay for the detection of *S. aureus* from milk samples. The assay was successfully designed and standardized with reference cultures and field isolates of *S. aureus*. However, the assay was not validated for pathogen detection in milk samples. Due to the difficulty of extracting DNA directly from milk samples, accurate results were not obtained from the PCR for these samples. It is proposed that a better technique for extracting

staphylococcal DNA from milk would resolve this problem. If this assay were successfully validated for pathogen detection in milk samples, it could be used as a routine diagnostic test for mastitis.

Conclusions

A multiplex real time PCR was developed for the detection of *Staphylococcus aureus* using two gene targets, the 270 bp thermonuclease (*nuc*) gene, and the 230 bp extracellular adherence protein (*eap*) gene. The assay was 95.8% sensitive for *eap* and 89.6% for *nuc.* Specificity was 100% for both genes. It is proposed that the *eap* gene may be a more specific gene target for identification of *S. aureus* than the traditionally used *nuc* gene. The high sensitivity and specificity of this assay makes it a promising diagnostic tool. However, more work is needed for validation of the assay and improvement of protocol for rapid identification of *S. aureus* directly from milk samples.

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College Activities:

Women's Swimming and Diving Team **2006 – 2010**
The Pennsylvania State University
Competed at the Big Ten Championships 2007 – 2010. Team placed 3rd in 2007 and in 2009.

Undergraduate Researcher **2008 – present**
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Working on an honors thesis entitled *Real time multiplex PCR assay for the detection of* Staphylococcus aureus *using* nuc *and* eap *genes*

Lutheran Student Community **2006 – present**
The Pennsylvania State University
Actively involved in attending worship and fellowship activities.

Professional Experience:

Dairy Barn Worker **Summer 2007**
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Performed routine chores typical of a dairy farm, including milking, cleaning barns, and caring for calves. Lived at barn unit, and performed weekly night checks.
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Job Shadowing **Summer 2007 and 2008**
Winding Hill Veterinary Clinic, Mechanicsburg, PA
Observed examinations and surgical procedures. Assisted with handling animals.

Honors/Awards:

Academic All-Big Ten **2008 – 2010**

Big Ten Distinguished Scholar **2009**

Cynthia Earley Education Foundation Scholarship Recipient **2007, 2009**
Awarded to the highest student fundraiser in the annual Great Chesapeake Bay Swim.

College of Agricultural Sciences Outstanding Senior Award Finalist **2010**

Outstanding Freshman Honor **2007**
One of two students recognized by Dr. Graham Spanier at the 2007 Penn State New Student Convocation for making the most of their freshman year.

Laboratory Skills:

Molecular biology techniques: PCR, Gel Electrophoresis, rt-PCR, Pulse Field Gel Electrophoresis, DNA isolation, and plasmid isolation

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Camp Counselor **Summer 2007**
Harrisburg Area Confirmation Camp, Harrisburg, PA
Served in a leadership role at a week-long Christian camp. Supervised high school campers in daily activities, and led in small group sessions. Completed a leadership training program to be eligible.

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