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Bovine *Staphylococcus aureus*: Dose response to iodine and chlorhexidine and effect of iodine challenge on antibiotic susceptibility

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ABSTRACT

Staphylococcus aureus is a gram-positive organism that is frequently associated with clinical or subclinical mastitis. The use of germicidal teat dips is one of the measures taken by the dairy industry to control mastitis. Iodine and chlorhexidine compounds are commonly used disinfectants in teat dips. We determined the minimum inhibitory concentrations (MIC) of iodine for 37 isolates of *Staph. aureus* and observed variations in MIC. Seven of these *Staph. aureus* isolates were selected as genotype group representatives based on their pulsed-field gel electrophoresis patterns. Dose responses against iodine and chlorhexidine were determined for the 7 genotype group representatives. The response of these isolates to iodine varied significantly, whereas all isolates were susceptible to chlorhexidine, even at concentrations as low as 0.0002%. We also evaluated whether exposure of *Staph. aureus* to sublethal levels of iodine influenced subsequent antibiotic susceptibility. No differences in antibiotic susceptibility of *Staph. aureus* were observed among cultures grown in brain heart infusion broth with and without supplemental iodine. The observed variation in iodine dose responses of *Staph. aureus* may have implications for the occurrence of *Staph. aureus* mastitis on dairy farms.
Key words: *Staphylococcus aureus*, disinfectant tolerance, mastitis, antibiotic stress

INTRODUCTION

Mastitis results in major economic losses in the dairy industry, primarily due to decreased milk yield. *Staphylococcus aureus*, a common cause of subclinical or chronic mastitis in dairy cows (Barkema et al., 2009), is a contagious udder pathogen that spreads within and among cows during milking (Barkema et al., 2009; Capurro et al., 2010). Investigations into the persistence of *Staph. aureus* in dairy herds have revealed that a limited number of *Staph. aureus* strains are usually harbored in individual herds, suggesting that these specific strains may possess resistance or virulence mechanisms that allow them to survive under the environmental or sanitation conditions of the particular dairy farms (Matthews et al., 1994; Sommerhäuser et al., 2003; Anderson and Lyman, 2006). Targeting these specific strains may be beneficial in controlling mastitis in a given herd (Kapur et al., 1995).

Several strategies are used on a dairy farm to minimize the risk of IMI. In previously described mastitis control programs (Bramley and Dodd, 1984; Sommerhäuser et al., 2003), measures to control *Staph. aureus* included postmilking teat disinfection, using individual towels for udder cleaning, and using proper milking technique to prevent new IMI. For the elimination of IMI, program recommendations include using antimicrobial therapy or culling (Bramley and Dodd, 1984; Sommerhäuser et al., 2003).

Many studies have indicated that teat disinfection is a beneficial practice for controlling mastitis (Galton et al., 1986; Rasmussen et al., 1991; Oliver et al., 1993; Magnusson et al., 2006; Gibson et al., 2008). Some commonly used teat disinfectants contain iodine or chlorhexidine as the active agent. Gibson et al. (2008) indicated that cleaning the teat with disinfectant and drying the teat before milking was the most effective approach in reducing teat-end microbial load on dairy farms.

Bacterial exposure to iodine can be considered a sublethal stress, but few reports describe how sublethal disinfectant stress affects mastitis-causing *Staph. aureus*. Of interest for this study was whether iodine exposure stress would have any effect on antibiotic resistance. A considerable amount of literature details bacterial responses to sublethal stressors such as heat, cold, salt, pH, disinfectant exposure, and others. Stresses have been shown to induce genotypic and phenotypic changes in bacteria (Oliver et al., 1993; Azizoglu and Drake, 2007). McMahon et al. (2007) reported that sublethal levels of stress conditions affect the antibiotic resistance levels of foodborne pathogens. They indicated that *Staph. aureus* cells that were subjected to heat stress at 45°C had lower MIC for the tested antibiotics. On the other hand, when *Staph. aureus* cells were subjected to

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sublethal levels of NaCl (4.5%) or acid (pH 5.0), their MIC against gentamycin and erythromycin increased. In accordance with these findings, the use of disinfectants at dairy farms may affect the development of antibiotic resistance in bacteria.

Understanding the effect of disinfectants on the persistence of *Staph. aureus* on dairy farms might provide information concerning the source of antibiotic-resistant or chronic *Staph. aureus* strains. First, we determined the inhibitory effects of iodine and chlorhexidine on *Staph. aureus* isolates with different pulsed-field gel electrophoresis (**PFGE**) patterns. Next, we evaluated the effect of *Staph. aureus* growth under sublethal levels of iodine on subsequent antibiotic susceptibility.

MATERIALS AND METHODS

Bacterial Isolates and Culture Conditions

In all experiments, the selected *Staph. aureus* isolates were grown on trypticase soy agar plus 5% sheep blood (Becton Dickinson and Co., Sparks, MD) at 37°C overnight; a single colony was transferred and grown in 5 mL of brain heart infusion (**BHI**) broth (Becton Dickinson and Co.) at 37°C for 24 h before the respective studies.

Iodine MIC

Iodine MIC were determined for 37 *Staph. aureus* isolates selected from a library of approximately 4,000 milk isolates of *Staph. aureus* (Figure 1). The 37 isolates were representative of the range and prevalence of PFGE-determined genotypes present in the isolate library.

The MIC of iodine for the 37 *Staph. aureus* isolates was determined by spotting 5 μL of overnight culture of *Staph. aureus* onto BHI agar plates supplemented with different concentrations of iodine. The concentrations of titratable iodine tested were 0, 0.01% (100 mg/L), 0.05% (500 mg/L), 0.1% (1,000 mg/L), 0.15% (1,500 mg/L), 0.2% (2,000 mg/L), and 0.25% (2,500 mg/L). The plates were incubated overnight at 37°C, and the lowest iodine concentration that inhibited growth of *Staph. aureus* was recorded as the iodine MIC for that isolate.

PFGE

Preparation of bacterial genomic DNA, digestion by *Sma*I, and PFGE were performed according to the Centers for Disease Control (CDC) methods (CDC, 2001), with minor modifications. Digested fragments were separated by PFGE with an increasing pulse of 5 to 40 s at 200V for 21 h at 14°C (Chef-DR II Pulsed-Field Gel Electrophoresis system, Bio-Rad Laboratories Inc., Hercules, CA). Gels were stained in 0.1% ethidium bromide solutions for 20 min followed by destaining in distilled water for 1 h. Stained gels were digitally photographed and the photographs processed and dendrograms made using gel analysis and comparison software (BioNumerics, Applied Maths Inc., Austin, TX). Similarity coefficients were calculated and dendrograms constructed using the Dice coefficient and unweighted pair group method with arithmetic means, respectively, with an optimization value of 1.00% and a position tolerance of 0.65%.

Iodine and Chlorhexidine Dose Responses

Seven *Staph. aureus* isolates were chosen as being representative of genotype groups based upon PFGE determinations and subsequent analysis of the PFGE results. Of these, 6 isolates were from major genotype groups within the library (Figure 1) and were isolated from different dairy farms in North Carolina at different times. The seventh isolate was strain ATCC25923, obtained from American Type Culture Collection (Manassas, VA), which was used as an antibiotic-susceptible control.

The iodine dose response of each of the 7 *Staph. aureus* strains was determined by inoculating bacteria grown at 37°C for 24 h into BHI broth with different concentrations of iodine. Commercially available topical 10% Povidone-Iodine Prep solution containing 1% titratable iodine (Triad Disposables Inc., Brookfield, NJ) was used in the preparation of iodine solution. The dilutions of iodine solution were prepared in BHI broth. The concentrations of titratable iodine tested were 0, 0.0001% (1 mg/L), 0.0005% (5 mg/L), 0.001% (10 mg/L), 0.005% (50 mg/L), 0.01% (100 mg/L), 0.05% (500 mg/L), and 0.1% (1,000 mg/L). Following inoculation of each dilution with tested strains, they were incubated at 37°C for 24 h. The growth of *Staph. aureus* in media supplemented with iodine was measured by monitoring optical density at 600 nm ($\mathbf{OD_{600}}$), using a UV-visible spectrophotometer (UV-1201, Shimadzu, Columbia, MD).

The same protocol was used in determination of the chlorhexidine dose response for each of the 7 *Staph. aureus* strains. A commercially available chlorhexidine solution (Nolvasan, Fort Dodge Animal Health, Fort Dodge, IA) was used in the preparation of the BHI broth with varying concentrations of chlorhexidine. This solution contained 2% chlorhexidine diacetate as its active ingredient. The concentrations of chlorhexidine diacetate tested in this study were 0, 0.0002% (2 mg/L), 0.001% (10 mg/L), 0.002% (20 mg/L), 0.01%

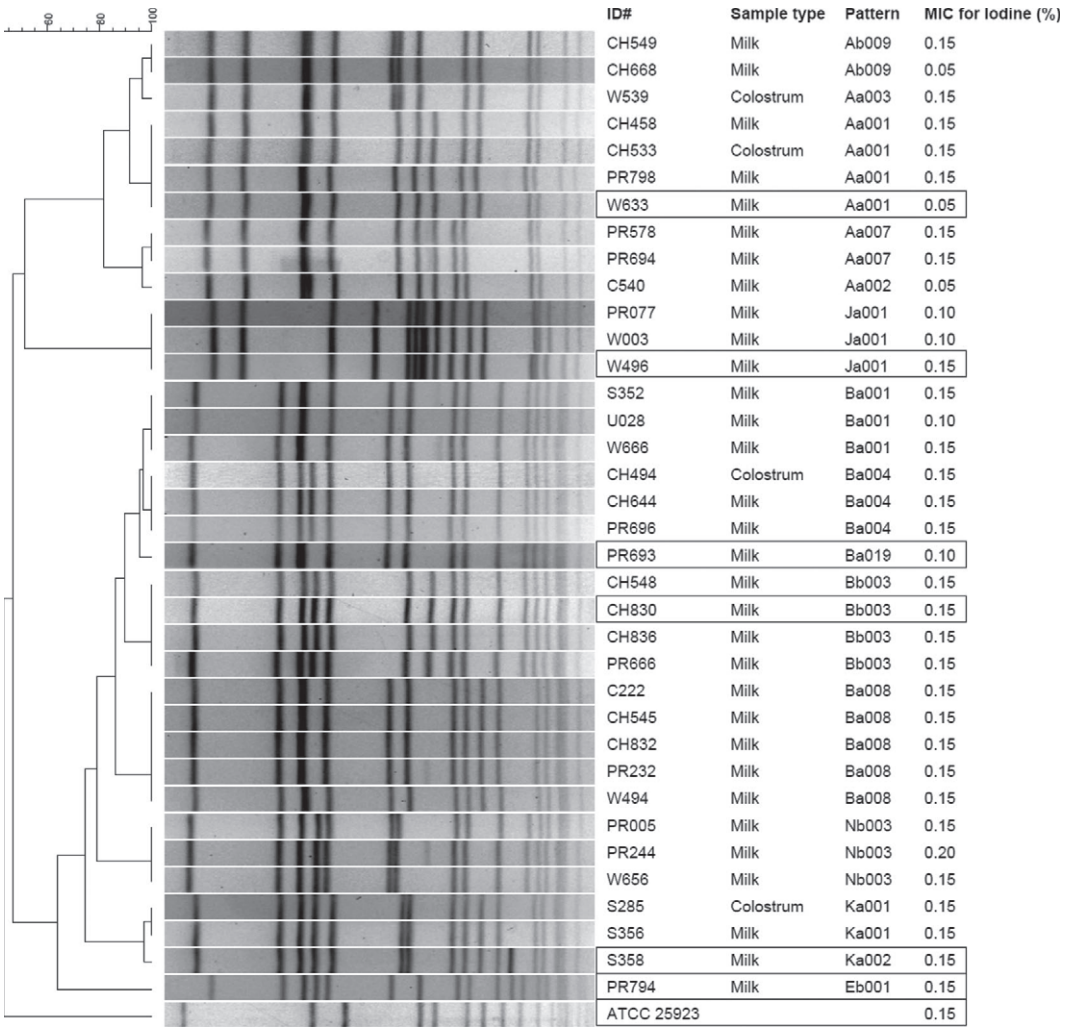


Figure 1. Composite gel of all pulsed-field gel electrophoresis (PFGE) patterns, isolate identity (ID), MIC of iodine of *Staphylococcus aureus* isolates, and a dendrogram showing the relatedness of patterns. Seven isolates selected for dose response tests are presented within rectangular boxes.

(100 mg/L), 0.02% (200 mg/L), 0.1% (1,000 mg/L), and 0.2% (2,000 mg/L).

Effect of Iodine Exposure on Antibiotic Susceptibility of Staph. aureus Isolates

We evaluated the effect of iodine exposure stress on antibiotic susceptibility using the 7 genotype group representatives and 2 approaches. In the first approach, we determined antibiotic susceptibility of the 7 *Staph. aureus* strains using disc assay. Each of the 7 *Staph. aureus* strains was grown at 37°C for 24 h in either BHI broth supplemented with a sublethal level of iodine or BHI broth without iodine. Then, each culture was diluted in BHI broth to a density equivalent to a 0.5 McFarland turbidity standard (Ieven et al., 1995) and spread evenly on blood agar plates (Becton Dickinson and Co.) using sterile cotton swabs. The disc for each antibiotic was placed on the surface of the plates. Following incubation at 37°C for 24 h, the diameter of the zone of inhibition was measured by ruler. The antibiotic susceptibility of an isolate was interpreted according to the Clinical and Laboratory Standards Institute (2006) standards. Antibiotics selected included those commonly used to treat bovine mastitis. The antibiotics tested (and their concentration on each disc) were as follows: ampicillin (10 μg), cephalothin (30 μg), erythromycin (15 μg), cefoxitin (30 μg), novobiocin (30 μg), penicillin (10 μg), ceftiofur (30 μg), streptomycin (10 μg), tetracycline (30 μg), sulfisoxazole (250 μg), pirlimycin (2 μg), and oxacillin (1 μg). All antibiotic discs were purchased from Becton Dickinson and Co.

In the second approach, we tested the antibiotic tolerance of the 7 *Staph. aureus* strains grown in me-

dium supplemented with iodine by dropping 5 μL of overnight culture on the surface of BHI agar plates supplemented with different concentrations of selected antibiotics. Antibiotics used for MIC tests included tetracycline, ampicillin, erythromycin, and cephalothin (Sigma-Aldrich, St. Louis, MO). These antibiotics were selected because they are or have been commonly used in treatment of mastitis. Stock solutions of each antibiotic were prepared in distilled water, and different concentrations of each antibiotic were prepared by adding stock solution to BHI agar before pouring. The concentrations of each antibiotic were 0.5, 1.0, 1.5, 2, 2.5, 5, or 10 μg/mL. Bacteria grown in BHI without iodine were used as the negative control. Growth of *Staph. aureus* strains on BHI agar plates supplemented with specific antibiotics was screened following incubation at 37°C for 24 h.

Statistical Analysis

All experiments were replicated 3 times. The changes in the dose response of the 7 genotype group representatives of *Staph. aureus* against iodine or chlorhexidine at different concentrations were tested by ANOVA (SAS software, version 9.1, SAS Institute Inc., Cary, NC). The significance of differences was determined at an unadjusted level of $\alpha < 0.05$.

RESULTS AND DISCUSSION

Iodine MIC

The iodine MIC of 37 *Staph. aureus* isolates showed some variation and results are shown in Figure 1. Twenty-nine *Staph. aureus* isolates had MIC of 0.15% (1,500 mg/L), 4 had MIC of 0.1% (1,000 ppm), 3 had MIC of 0.05% (500 mg/L), and 1 isolate had a MIC of 0.2% (2,000 mg/L). Three *Staph. aureus* isolates having MIC of 0.05% iodine showed >85.7% similarity to each other according to their PFGE patterns (Figure 1). Two of the 4 *Staph. aureus* isolates having MIC of 0.1% iodine were identical to each other, and the other 2 were 96.8% similar (but the 2 groups were only 47.7% similar; Figure 1).

Iodine and Chlorhexidine Dose Responses

The 7 genotype group representatives were tested for their dose response against iodine. The growth of the 7 isolates in BHI supplemented with iodine indicated significant variation in the dose response of the isolates ($P < 0.05$; Figure 2). Among the 7 isolates, W633 showed the greatest response to iodine, with growth severely impaired at iodine concentrations >0.001% and completely inhibited at ≥0.01%. The control strain, ATCC25923, also showed growth impairment at iodine concentrations ≥0.005%, but to a lesser degree. The growth of ATCC25923 was completely inhibited at an iodine concentration of 0.05%. Two tested *Staph. aureus* isolates, S358 and CH830, showed growth impairment at iodine concentrations >0.001% and >0.005%, respectively. However, complete inhibition of growth occurred at similar concentrations for these isolates and for isolates that were more tolerant of iodine. Isolates W496, PR693, and PR794 were the most iodine-tolerant isolates of the *Staph. aureus* panel screened in this study. Their growth did not show significant impairment until the concentration of iodine was increased to 0.05% ($P > 0.05$). The growth of all *Staph. aureus* isolates tested in this study was inhibited at iodine concentrations >0.05%. Teat dips used in the dairy industry contain titratable iodine at levels as low as 0.1%. Our findings indicate that this level is effective in eliminating the *Staph. aureus* in liquid media.

However, the MIC tests conducted on agar plates showed that most (28/37, 75.7%) of the *Staph. aureus* isolates were able to tolerate iodine concentrations as high as 0.1%. Previously, increased tolerance of agar-grown bacteria to stressful conditions has been shown (Azizoglu et al., 2009). It should be noted that the efficacy of iodine was tested in vitro, and other factors may play a role in the effectiveness of iodine in vivo, such as contact time, cleanliness of the teats, and reaction with the skin. In a recent study, Quirk et al. (2012) reported significant increases in IMI caused by CNS in quarters on which no postmilking iodine dip was used. However, they highlighted the species-specific differences of CNS in response to bacteriocidal effect of this disinfectant. Kassaify et al. (2007) previously reported the low bacteriocidal activity of iodophors in eliminating a panel of common dairy-associated bacteria, including *Staph. aureus*. Their in vitro tests indicated that disinfectants containing quarternary ammonium showed better bacteriocidal efficiency than iodophor-based disinfectants. In another study, Whist et al. (2006) indicated that using iodine as a teat-dip significantly reduced the chance of developing clinical mastitis compared with no teat-dip treatment. In their study, they used iodine concentrations at 0.15%, higher than that found to inhibit the growth of *Staph. aureus* isolates tested in this study. Interestingly, the variation of iodine tolerance of *Staph. aureus* isolates has not been reported previously and this might explain the observed variation of the efficacy of iodine in eliminating *Staph. aureus*.

All 7 isolates tested in this study were unable to grow in BHI broth supplemented with chlorhexidine at concentrations as low as 0.0002% (Figure 3). Chlorhexidine

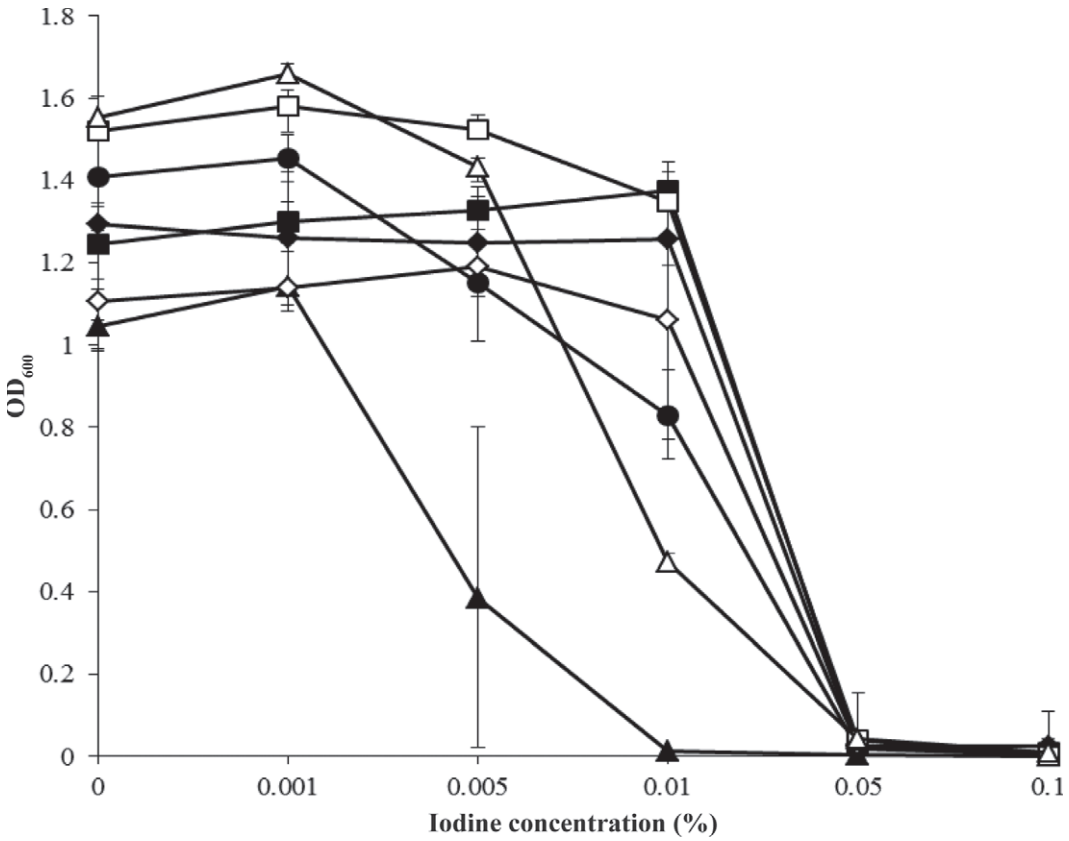


Figure 2. Growth of *Staphylococcus aureus* isolates (◆ = W496; ■ = PR693; ▲ = W633; ● = S358; ◊ = PR794; □ = CH830; Δ = ATCC25923) in brain heart infusion broth (optical density at 600 nm, OD_{600}) supplemented with varying concentrations of iodine following incubation at 37°C for 24 h. The data represent the means of 3 experiments; bars represent the maximum and minimum values at each point.

is commonly used in the dairy industry for multiple purposes, including teat dipping. Our findings showed some potential advantage to chlorhexidine as a teat disinfectant, at least considering the specific *Staph. aureus* isolates tested here. Hogan et al. (1995) tested the efficacy of using 0.55% chlorhexidine gluconate or 1% iodophor as a teat dip for 12 mo by periodically culturing milk from tested cows. No significant difference was observed between the tested disinfectants in decreasing the development of new IMI caused by *Staph. aureus* (Hogan et al., 1995). As that study was based on culturing milk samples from a dairy farm, other environmental factors could change the efficacy of each disinfectant, which might explain the differences in our findings.

Effect of Iodine Exposure on Antibiotic Susceptibility of Staph. aureus Isolates

All tested isolates of *Staph. aureus* were susceptible to the selected antibiotics and did not develop tolerance following growth in media supplemented with iodine. The *Staph. aureus* isolates screened in this study were susceptible to the 12 tested antibiotics based on the formation of zones of inhibition by antibiotic disc assay (Table 1). In addition, they were not able to grow on BHI agar plates supplemented with tetracycline, ampicillin, or cephalothin at concentrations as low as 0.5 μg/mL. These findings indicate that the tested isolates of *Staph. aureus* did not develop antibiotic tolerance. This is consistent with one previous report indicating that the majority of the *Staph. aureus* isolated from

Table 1. Mean (±SEM) of diameters of zones of inhibition of *Staph. aureus* PR794 for some of the tested antibiotics

	Diameter of zone of inhibition (mm)	
Antibiotic	With iodine	Without iodine
Penicillin (β-lactam)	42.00 ± 2.99	41.00 ± 0.99
Ceftiofur (β-lactam)	27.00 ± 0.99	29.00 ± 0.99
Erythromycin (macrolide)	28.50 ± 2.49	30.50 ± 0.50
Tetracycline	27.50 ± 1.49	32.50 ± 1.49
Pirlimycin (lincosaminide)	22.00 ± 0.00	24.50 ± 0.50
Novobiocin	25.50 ± 0.50	27.50 ± 0.50

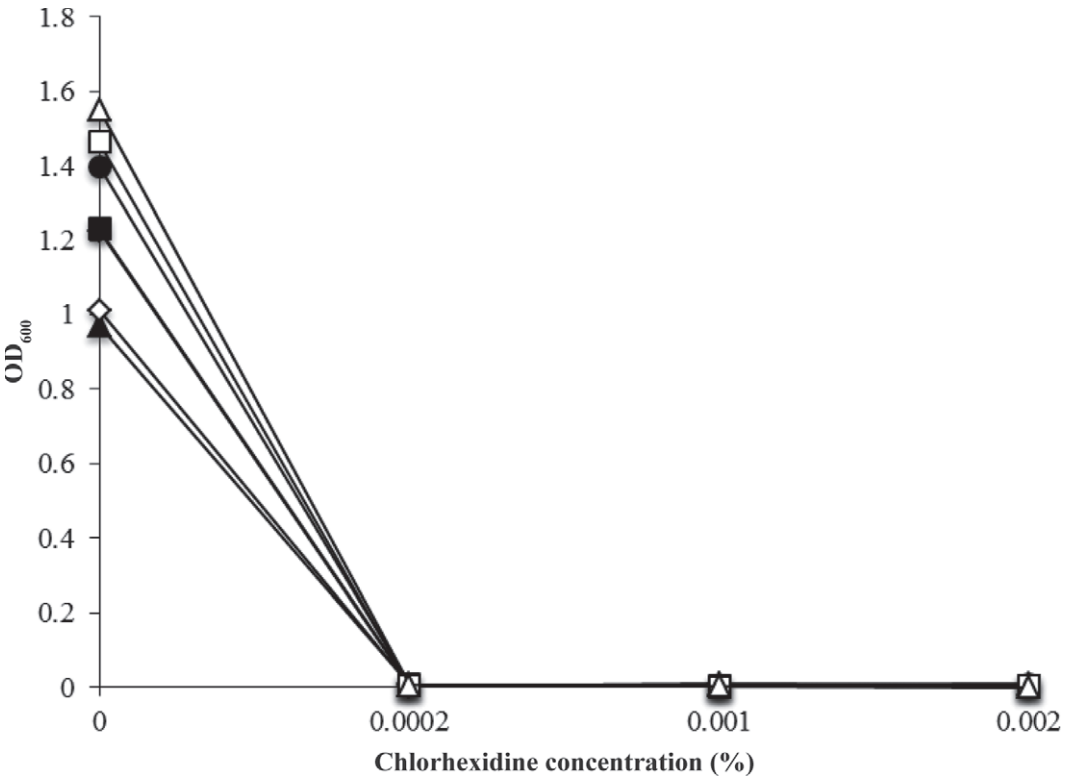


Figure 3. Growth of *Staphylococcus aureus* isolates (◆ = W496; ■ = PR693; ▲ = W633; ● = S358; ◊ = PR794; □ = CH830; Δ = ATCC25923) in brain heart infusion broth (optical density at 600 nm, OD_{600}) supplemented with varying concentrations of chlorhexidine following incubation at 37°C for 24 h. The data represent the means of 3 experiments.

milk are susceptible to several antimicrobials (Anderson et al., 2006).

The tolerance of *Staph. aureus* isolates to iodine showed variation. Therefore, we determined if the highest iodine concentrations that allowed growth of a specific *Staph. aureus* isolate provided any protection against subsequent antibiotic stress. As iodine-based teat-dips are commonly used in the dairy industry, such protection developed by more iodine-tolerant *Staph. aureus* might be problematic during treatment of clinical mastitis cases. In most of the tested isolate–antibiotic combinations, we did not observe any significant difference between the *Staph. aureus* grown in BHI broth supplemented with or without iodine. However, in isolate PR794, we repeatedly observed a decrease of approximately 5 mm in the zone of inhibition by tetracycline in the culture grown in BHI supplemented with 0.01% iodine, and the difference was statistically significant ($P < 0.0001$; Table 1). In addition, the difference in the zone of inhibition in PR794 by pirlimycin was less pronounced than that of tetracycline but the difference between the cultures grown in BHI broth supplemented with or without iodine was also significantly different ($P < 0.05$; Table 1). These findings indicate that exposure to iodine did not result in antibiotic-tolerant *Staph. aureus*; however, in some isolates it may provide additional protection against specific antibiotics.

CONCLUSIONS

Although the isolates tested in this study were selected according to different PFGE patterns, the genomic basis of the observed isolate-specific iodine tolerance is not known and has yet to be studied. This study revealed that the tolerance of *Staph. aureus* to iodine is variable and this may have implications for the occurrence of *Staph. aureus* mastitis on dairy farms..

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