**NOTE** *Clinical Pathology*

# *In vitro* Algaecide Effect of Disinfectants on *Prototheca zopfii* Genotypes 1 and 2

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(Received 31 May 2011/Accepted 26 June 2011/Published online in J-STAGE 8 July 2011)

ABSTRACT. Bovine mastitis due to *Prototheca zopfii* leads to reduced milk production and is difficult to cure. Therefore, prevention is the best approach and this is best achieved through the use of effective disinfectants. The aim of this study was to evaluate the *in vitro* algaecide efficacy of conventional disinfectants against strains of *P. zopfii* genotype 1 and 2. The minimal algaecide concentration (MAC) of alkyldiaminoethylglycine hydrochloride, chlorhexidine, dioxide chlorine, povidone iodine and sodium hypochlorous acid against 10 isolates and the type strain (SAG2063$^T$) of *P. zopfii* genotype 1 as well as 10 isolates and the type strain (SAG2021$^T$) of *P. zopfii* genotype 2 were examined using the micro dilution method. This *in vitro* study indicated that alkyldiaminoethylglycine hydrochloride, chlorhexidine, povidone iodine and sodium hypochlorous acid, but not dioxide chlorine, are effective against both genotypes of *P. zopfii*.

KEY WORDS: disinfectants, genotype, mastitis, *Prototheca zopfii*.

*J. Vet. Med. Sci.* 73(11): 1527–1529, 2011

Bovine mastitis due to *Prototheca zopfii* leads to reduced milk production and thin watery milk secretion containing white flakes. *P. zopfii* has been classified biochemically and serologically into genotypes 1 and 2, and *Prototheca blaschkeae* [1, 5, 6]. According to the latest genotypic classification, all isolates from bovine mastitis in Germany and Japan were *P. zopfii* genotype 2, suggesting that genotype 2 is the principal causative agent of bovine protothecal mastitis [2, 3].

In our previous *in vitro* study, the sensitivity of *P. zopfii* genotype 2 isolates was shown to be lower to conventional antimicrobial and antifungal drugs than that of genotype 1 [8]. Currently, bovine mastitis due to *Prototheca* is a chronic infection with no effective treatments. Therefore, suitable antimicrobial drugs or disinfectants are required to prevent bovine *Protothecal* mastitis. The aim of this study was to evaluate the *in vitro* algaecide effects of conventional disinfectants against *P. zopfii* genotype 1 and 2 strains.

The type strain of genotype 1 (SAG2063$^T$) and the type strain of genotype 2 (SAG2021$^T$) from *P. zopfii* were used for susceptibility tests.

In addition to the type strains, 10 isolates of *P. zopfii* genotype 2 from 10 cases of bovine protothecal mastitis in Japan and 10 isolates of *P. zopfii* genotype 1 from Japanese stock farm were examined [5, 8]. These isolates were previously identified by an 18S rDNA-based genotype-specific PCR assay [2, 3, 5] (Table 1).

The disinfectants alkyldiaminoethylglycine hydrochloride, chlorhexidine, dioxide chlorine, povidone iodine and sodium hypochlorous acid were analyzed for their minimal microbicidal concentration (MMC) (M27-A2) [9] using the micro dilution test with some modifications. The minimal algaecide concentration (MAC) of isolates was defined as the lowest disinfectant concentration able to prevent post-exposure algae growth in RPMI 1640 with compound 3-(N-morpholino) propanesulfonic acid (MOPS) [9].

Stock inoculum suspensions were prepared from 3-day-old cultures grown on yeast and mould agar (Oxoid, Ltd., Hampshire, UK) at 37°C, and were adjusted spectrophotometrically to optical densities that ranged from OD 0.8 to 1.0 ($OD_{600}$) in sterile 0.9% saline. The final concentrations of the inoculums were diluted 1,000 times ($2.3 \times 10^5$ to $3.1 \times 10^5$ CFU/m*l*) with RPMI 1640.

The concentrations of the disinfectants were $5 \times 10^{-9}$ to 5 $\mu$g/m*l* for alkyldiaminoethylglycine hydrochloride and chlorhexidine, 0.094 to 48 $\mu$g/m*l* for dioxide chlorine ($ClO_2$), 0.012 to 10 $\mu$g/m*l* for povidone iodine and $6 \times 10^{-9}$ to 6 $\mu$g/m*l* for sodium hypochlorous acid (NaClO). The concentrations of the disinfectants are according to general use as disinfection at hospitals and dairy farms.

After a 48-hr incubation at 32°C, visual reading of wells was performed and the growth of each strain at various concentrations of the disinfectants was recorded. As the control, each of the 22 isolates was grown in drug-free medium and resultant growth recorded. The MAC of an isolate was defined as the concentration of the agent at which 90% of growth was inhibited ($MAC_{90}$). The experiments were performed in triplicate for each isolate on separate occasions. Agreement was evaluated by MAC endpoint discrepancies of no more than two dilutions.

For all isolates of genotype 1, the $MAC_{90}$ was $3.13 \times 10^{-3}$ $\mu$g/m*l* (range, $5.0 \times 10^{-4}$ to $5.0 \times 10^{-3}$ $\mu$g/m*l*) for alkyl-

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Table 1. Strains used in this study

| Strains | Origin | Genotype | Strains | Origin | Genotype |
|---|---|---|---|---|---|
| NUBS5 | Bovine mastitis | 2 | NUBS68 | Feces | 1 |
| NUBS19 | Bovine mastitis | 2 | NUBS88 | Feces | 1 |
| NUBS26 | Bovine mastitis | 2 | NUBS133 | Feces | 1 |
| NUBS44 | Bovine mastitis | 2 | NUBS150 | Feces | 1 |
| NUBS50 | Bovine mastitis | 2 | NUBS163 | Feces | 1 |
| NUBS51 | Bovine mastitis | 2 | NUBS21 | Drinking water | 1 |
| NUBS89 | Bovine mastitis | 2 | NUBS41 | Drinking water | 1 |
| NUBS101 | Bovine mastitis | 2 | NUBS70 | Drinking water | 1 |
| NUBS114 | Bovine mastitis | 2 | NUBS178 | Rat | 1 |
| NUBS184 | Bovine mastitis | 2 | NUBS73 | Sewage | 1 |

diaminoethylglycine hydrochloride, $3.13 \times 10^{-3}$ $\mu$g/m*l* (range, $2.5 \times 10^{-4}$ to $2.5 \times 10^{-2}$ $\mu$g/m*l*) for chlorhexidine, 60 $\mu$g/m*l* (range, 30 to 120 $\mu$g/m*l*) for dioxide chlorine, 1.17 $\mu$g/m*l* (range: 0.78 to 3.13 $\mu$g/m*l*) for povidone iodine, and 0.069 $\mu$g/m*l* (range: $3 \times 10^{-3}$ to 0.3 $\mu$g/m*l*) for sodium hypochlorous acid.

For all isolates of genotype 2, the $MAC_{90}$ was $1.65 \times 10^{-3}$ $\mu$g/m*l* (range, $5.0 \times 10^{-4}$ to $5.0 \times 10^{-3}$ $\mu$g/m*l*) for alkyldiaminoethylglycine hydrochloride, $1.72 \times 10^{-3}$ $\mu$g/m*l* (range, $2.5 \times 10^{-5}$ to $2.5 \times 10^{-2}$ $\mu$g/m*l*) for chlorhexidine, 51.5 $\mu$g/m*l* (range, 15 to 120 $\mu$g/m*l*) for dioxide chlorine, 1.63 $\mu$g/m*l* (range: 0.39 to 3.13 $\mu$g/m*l*) for povidone iodine, and 0.12 $\mu$g/m*l* (range: $3 \times 10^{-3}$ to 0.3 $\mu$g/m*l*) for sodium hypochlorous acid.

These *in vitro* findings indicate that alkyldiaminoethylglycine hydrochloride, chlorhexidine, povidone iodine and sodium hypochlorous acid are effective against both genotypes of *P. zopfii* while dioxide chlorine is not. We recognized that alkyldiaminoethylglycine hydrochloride, chlorhexidine, povidone iodine and sodium hypochlorous are algaecidal against *P.zopfii* (data were not shown).

In Japan, sodium hypochlorous acid disinfectant is widely used in dairy farms for the disinfection of floors, walls, utensil and equipment. In the present study, we found that sodium hypochlorous acid should be used at a concentration of > 0.3 $\mu$g/m*l* (0.03%) to be effective. Povidone iodine is generally used in pre- and post-dipping antisepsis of mammary glands, and was found to be effective at 0.39 to 3.13 $\mu$g/m*l* (more than 0.313%) in this study. Salerno *et al.* also reported that a low concentration of sodium hypochlorite (0.15625%) and iodine (0.625%) is effective against *P. zopfii* [7].

The antiseptic effect of chlorhexidine against both genotypes of *P. zopfii* was higher than that of sodium hypochlorous acid or povidone iodine. Our findings suggest that chlorhexidine at a concentration of > $2.5 \times 10^{-2}$ $\mu$g/m*l* (0.0025%) would provide effective antiseptic dipping.

The alkyldiaminoethylglycine hydrochloride has not been used in dipping antisepsis of mammary glands in Japan. However, this antiseptic at a concentration of > $5.0 \times 10^{-3}$ $\mu$g/m*l* (0.0005%) would provide effective against *P. zopfii* genotype 2. This antiseptic has been widely used in various fields including human and small animal medicines. It needs more consideration for this antiseptic in livestock.

In our previous study, *P. zopfii* genotype 2 was more resistant to amphotericin B, gentamicin and kanamycin than *P. zopfii* genotype 1 [8]. Moreover, genotype 2 isolates and 7 isolates of genotype 1, including the type strain, were resistant to itraconazole (MIC > 10 $\mu$g/m*l*) [8]. Therefore, disinfectants should be further examined with a view to developing more effective antimicrobial and antifungal drugs for the control of bovine mammary protothecosis in dairy farms.

ACKNOWLEDGMENTS. This study was supported by Grants-in-Aid for Scientific Research (C) (22580368) from the Academic Frontier Project of the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT), and from Nihon University.

REFERENCES

1. Blaschke-Hellmessen, R., Schuster, H. and Bergmann, V. 1985. Differenzierung von Varianten bei *Prototheca zopfii* (Krüger 1894). *Arch. Exp. Veterinärmed.* **39**: 387–397.
2. Möller, A., Truyen, U. and Roesler, U. 2007. *Prototheca zopfii* genotype 2 -the causative agent of bovine protothecal mastitis? *Vet. Microbiol.* **120**: 370–374.
3. Osumi, T., Kishimoto, Y., Kano, R. *et al.* 2008. *Prototheca zopfii* genotypes isolated from cow barns and bovine mastitis in Japan. *Vet. Microbiol.* **131**: 419–423.
4. Pore, R.S. 1973. Selective medium for the isolation of *Prototheca*. *Appl. Microbiol.* **26**: 648–649.
5. Roesler, U., Möller, A., Hensel, A., Baumann, D. and Truyen, U. 2006. Diversity within the current algal species *Prototheca zopfii*: a proposal for two *Prototheca zopfii* genotypes and description of a novel species, *Prototheca blaschkeae* sp. nov. *Int. J Syst. Evol. Microbiol.* **56**: 1419–1425.
6. Roesler, U., Scholz, H. and Hensel, A. 2003. Emended phenotypic characterization of *Prototheca zopfii*: a proposal for three biotypes and standards for their identification. *Int. J. Syst. Evol. Microbiol.* **53**: 1195–1199.
7. Salerno, T., Garcia, M., Langoni, H., Siqueira, A.K. and da Costa, E.O., Melville, P.A., Bueno, V.F.F. 2010. In vitro algaecide effect of sodium hypochlorite and iodine based antiseptics on Prototheca zopfii strains isolated from bovine milk. *Res. Vet. Sci.* **88**: 211–213.
8. Sobukawa, H., Watanabe, M., Kano, R., Ito, T., Onozaki, M., Hasegawa, A. and Kamata, H. 2010. *In vitro* susceptibility of

*Prototheca zopfii* genotypes 1 and 2. *Med. Mycol.* **49**: 222–224.
9. Wayne, P.A. 2002. NCCLS; National Committee for Clinical and Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2, National Committee for Clinical Laboratory Standards.