

In vitro activity of an ear rinse containing tromethamine, EDTA, and benzyl alcohol on bacterial pathogens from dogs with otitis

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Objective—To evaluate the in vitro activity of an ear rinse (ER) containing tromethamine, EDTA, and benzyl alcohol on bacterial pathogens from dogs with otitis.

Sample Population—Organisms were collected from ear swab specimens from the external and middle ear and included *Staphylococcus* spp (n = 11; *Staphylococcus intermedius* [7] and *Staphylococcus* spp [4]), *Pseudomonas aeruginosa* (5), *Proteus* spp (5), β -hemolytic streptococcus (11), and 1 control strain of each organism.

Procedures—3 test solutions were evaluated including EDTA, tromethamine, and benzyl alcohol (ER); EDTA and tromethamine (ER without benzyl alcohol [ER – BA]); and purified water. Ten-milliliter aliquots of each test solution were transferred into 36 tubes and inoculated with one of the organisms. Samples were retrieved from each tube at 0, 15, 30, 45, and 60 minutes, transferred to Petri dishes, mixed with soybean-casein digest agar, and incubated. After incubation, plates were examined for growth, and the number of colonies was expressed as CFU per milliliter.

Results—ER significantly decreased bacterial growth in vitro of *P aeruginosa* and β -hemolytic streptococcal organisms within 15 minutes, *Proteus* spp within 30 minutes, and *Staphylococcus* spp within 60 minutes. Comparatively, the presence of benzyl alcohol in ER significantly decreased bacterial growth of β -hemolytic streptococcus and *Proteus* spp.

Conclusions and Clinical Relevance—On the basis of results of this study, future studies should be performed to evaluate the in vivo efficacy of ER alone as a treatment for otic infections caused by β -hemolytic streptococcus, *P aeruginosa*, and *Proteus* spp and of ER combined with an antimicrobial agent for otic infections caused by *Staphylococcus* spp. (*Am J Vet Res* 2006;67:1040–1044)

Otitis externa is the most common ear disease in dogs, with a reported prevalence of 10% to 20%.^{1,2} Fifty percent to 89% of dogs with chronic otitis externa may have concurrent otitis media.^{3,4} The most common

ABBREVIATIONS

ER	Ear rinse
ATCC	American Type Culture Collection
TML	Testing microbiology laboratory
BHI	Brain heart infusion
ER – BA	ER without benzyl alcohol

bacterial pathogens isolated from ears of dogs with otitis externa are *Staphylococcus intermedius*, *Pseudomonas aeruginosa*, *Proteus* spp, and β -hemolytic streptococcus.⁴⁻¹⁰ These same bacterial organisms have also been isolated from middle ears of dogs with otitis media.^{4,11}

Cell surfaces of gram-negative bacteria are damaged when exposed to EDTA.¹² Tromethamine is a synthetic buffer that enhances the chelating effect of EDTA.¹³ Gram-negative bacteria exposed to tromethamine-EDTA have increased permeability to extracellular solutes and leakage of intracellular solutes¹⁴; are sensitized to lysozyme, bactericides, and antimicrobials¹⁵⁻¹⁷; and release lipopolysaccharide, protein, phospholipids, and divalent cations from their cell walls.¹⁸ Cell walls of gram-positive bacteria are more resistant to the effects of tromethamine-EDTA than gram-negative bacteria.¹⁹ The in vitro activity of tromethamine-EDTA, with and without the addition of antimicrobial agents, against gram-positive and gram-negative bacterial organisms has been studied.^{17,19-23} However, few numbers of isolates were evaluated in each study, and no study evaluated the in vitro activity of a product containing tromethamine-EDTA against multiple clinical isolates of the 4 most common bacterial otic pathogens.

One commercially available ER^a contains benzyl alcohol in addition to tromethamine-EDTA. Benzyl alcohol has traditionally been used as an antiseptic, but presently is used as a preservative.²⁴ Benzyl alcohol has been found to have a greater inhibitory effect against gram-negative bacteria such as *P aeruginosa* and *Escherichia coli* than gram-positive bacteria.²⁵ However, the in vitro effect of benzyl alcohol in a solution of tromethamine-EDTA on gram-positive and gram-negative otic pathogens has not been evaluated.

The purpose of the study reported here was to evaluate the in vitro activity of an ER^a containing tromethamine, EDTA, and benzyl alcohol on bacterial pathogens from dogs with otitis. We hypothesized that this ER would have antibacterial activity against gram-negative and gram-positive otic pathogens and that the addition of benzyl alcohol would further enhance its activity against gram-negative pathogens.

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Materials and Methods

Bacteria—*Staphylococcus* spp (n = 11; *S intermedius* [7] and *Staphylococcus* spp [4]), *P aeruginosa* (5), *Proteus* spp (5), β -hemolytic streptococcus (11), and 1 ATCC control strain of each organism (*S intermedius* ATCC 29663, *P aeruginosa* ATCC 9027, *Proteus vulgaris* ATCC 6380, and *Streptococcus pyogenes* ATCC 51339) were used. The sample size for each organism was generated from results of a preliminary study based on the expected difference in bacterial count of the organisms in solution (ER) between time 0 and 15 minutes; the level of significance was set at $\alpha = 0.05$ and the power of 80%.

Sample collection of clinical isolates—Samples for bacterial culture were obtained from client-owned dogs evaluated by The Ohio State University Veterinary Teaching Hospital dermatology or soft tissue surgery service for bacterial otitis externa or otitis media. Approval by the institutional review committee on the care and use of client-owned animals at The Ohio State University was obtained prior to obtaining samples for the study. Client consent was obtained for sample collection. To obtain samples of otic exudate from dogs with otitis, a sterile cotton-tipped applicator was used. In dogs with otitis externa, a sterile cotton-tipped applicator was inserted into the external ear canal, and a sample was obtained from the junction of the vertical and horizontal ear canal (Table 1). In dogs with otitis media, samples were obtained from the middle ear cavity via myringotomy or directly from the tympanic bulla during bulla osteotomy by use of a sterile cotton-tipped applicator. Thirty-two bacterial organisms were obtained from ears of 17 dogs. Ear swab specimens were placed into sterile tubes and transported within 1 hour to the veterinary teaching hospital microbiology laboratory, plated on sheep blood agar and MacConkey's agar, and incubated at 35°C for 18 to 24 hours. Organisms were identified morphologically and by routine biochemical testing. *Staphylococcal* organisms were identified by use of the coagulase test and a commercial identification system.^b

Testing of solutions and bacterial organisms was performed at an off-site good manufacturing practice microbiology laboratory, designated as the TML. To store organisms for shipping to the TML, bacterial growth of each organism from the pure culture was lifted off the plate via a sterile cotton-tipped applicator, transferred to a tube containing BHI broth, and mixed until a turbid suspension of the organism was formed. Each organism was frozen in the tube containing BHI broth at -80°C prior to shipment to the TML. Once all organisms had been collected and frozen, organisms were prepared for shipment to the TML as follows: individual BHI broth tubes containing organisms were thawed in an incubator, mixed to resuspend organisms, streaked for isolation individually on blood agar plates, and placed back into an incubator for 18 to 24 hours at 35°C. Plates were removed from the incubator, wrapped with laboratory film,^c and shipped with ice packs via 2-day delivery to the TML. After arrival at the TML, bacterial strains were prepared on soybean-casein digest agar^d plates. The prepared microbial cultures were harvested by use of sterile 0.9% NaCl solution and adjusted to approximately 10⁸ organisms/mL.

Test solutions and antimicrobial testing—Solutions evaluated in the study included the ER² containing EDTA, tromethamine, and benzyl alcohol; EDTA and tromethamine (ER - BA); and purified water (water base of ER; control [H₂O]). For each test solution, 10-mL aliquots were transferred into 36 sterile, capped tubes. Each tube was inoculated with 0.1 mL of one of the test organisms to give a final microbial concentration of 10⁵ to 10⁶ microorganisms/mL. Samples were kept at 22.5 ± 2.5°C during the study.

Sampling—Samples were retrieved from each tube at 0, 15, 30, 45, and 60 minutes after inoculation. Samples were diluted with product diluent (soybean-casein digest broth with supplemental 4% polysorbate-20 and 1% lecithin) such that 1 mL was expected to yield between 30 and 300 colonies. The number of viable microorganisms was determined by use of the standard plate count technique.^{26,27}

Standard plate count technique—One milliliter of appropriately diluted product was transferred onto each of 2 sterile Petri dishes. Fifteen to 20 mL of soybean-casein digest agar that had previously melted and cooled to approximately 45°C was added. Petri dishes were covered, mixed by tilting or rotating dishes, and allowed to solidify at 22.5 ± 2.5°C. Petri dishes were inverted and incubated for 48 to 72 hours at 32.5 ± 2.5°C. After incubation, plates were examined for growth, colonies were counted, and the mean of the 2 plates was expressed as the number of CFU per milliliter.

Statistical analysis—The mean number of CFU per milliliter from 2 plates for each organism in each solution obtained at 0, 15, 30, 45, and 60 minutes was calculated. Because the initial (time 0) inoculum varied from isolate to isolate, the outcome in analysis was the proportion of bacterial growth in CFU per milliliter at each time relative to time 0. Making everything proportional to time 0 adjusted for the variation at the start. The proportion was modeled by use of a repeated-measures analysis in a commercially available statistical software package.^e Time and solution were factors, and an interaction term between time and solution was also included in the model. The analysis accounted for nonindependence by use of an autoregressive correlation structure for measurements obtained on the same solution with time. Separate models were constructed for each of the 4 organisms. Within an organism model, all pairwise comparisons of solution-time combinations were evaluated by use of the Tukey-Kramer adjustment for multiple comparisons. Values of $P < 0.05$ were considered significant.

Results

In all 4 analyses, including the control strains, the time-solution interaction was significant, which indicated that various solutions had different effects with time. For *Staphylococcus* spp, a significant decrease in

Table 1—Number of bacterial isolates obtained from the external ear canal or the middle ear cavity from 17 dogs with otitis.

Organism	External ear canal	Middle ear—myringotomy	Middle ear—bulla osteotomy
<i>Staphylococcus</i> spp	6	3	2
<i>Pseudomonas aeruginosa</i>	3	1	1
<i>Proteus</i> spp	3	0	2
β -Hemolytic streptococcus	10	0	1

In dogs with otitis externa, samples were obtained from the junction of the vertical and horizontal ear canal. In dogs with otitis media, samples were obtained from the middle ear cavity via myringotomy or directly from the tympanic bulla during bulla osteotomy.

bacterial growth was detected for ER at 60 minutes ($P = 0.001$), compared with time 0. Neither ER – BA nor H_2O had any effect on growth of *Staphylococcus* spp (Figure 1).

For β -hemolytic streptococcus, there was a significant decrease in bacterial growth for ER at all times (15, 30, 45, and 60 minutes; $P < 0.001$); for ER – BA at 60 minutes ($P < 0.005$); and for H_2O at 30 ($P = 0.002$), 45 ($P = 0.002$), and 60 minutes ($P < 0.001$), compared with time 0. Ear rinse was significantly more effective in decreasing bacterial growth ($P < 0.001$) at all times (15, 30, 45, and 60 minutes), compared with both ER – BA and H_2O (Figure 2).

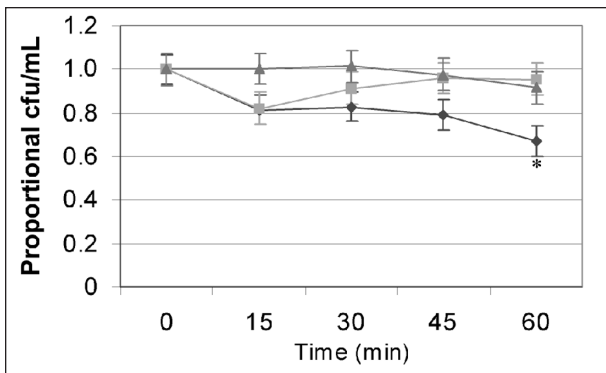


Figure 1—Bacterial growth of *Staphylococcus* spp in 3 solutions (ER containing EDTA, tromethamine, and benzyl alcohol, diamond; ER – BA, square; and purified water [water base of ER, control], triangle) with time. The means of the number of CFU per milliliter for each *Staphylococcus* spp organism ($n = 11$; *Staphylococcus intermedius* [7] and *Staphylococcus* spp [4]) in each solution at each time were calculated. Because the initial (time 0) inoculum of each *Staphylococcus* spp organism varied from isolate to isolate, the outcome in the analysis was the proportion of bacterial growth in CFU per milliliter (proportional CFU per milliliter) at each time relative to time 0. Values are reported as mean \pm SD proportional CFU per milliliter. *Within a solution, value differs significantly ($P < 0.05$) from the value at time 0.

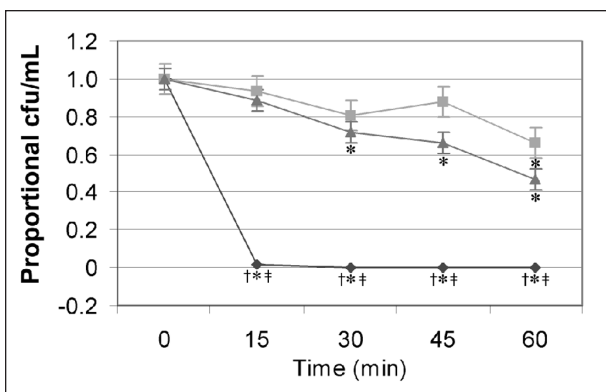


Figure 2—Bacterial growth of β -hemolytic streptococcus in 3 solutions with time. The means of the number of CFU per mL for each β -hemolytic streptococcal organism ($n = 11$) in each solution at each time were calculated. Because the initial (time 0) inoculum of each β -hemolytic streptococcal organism varied from isolate to isolate, the outcome in the analysis was the proportion of bacterial growth in CFU per milliliter (proportional CFU per milliliter) at each time relative to time 0. Values are reported as mean \pm SD proportional CFU per milliliter. †Within a time, ER value differs significantly ($P < 0.05$) from ER – BA value. ‡Within a time, ER value differs significantly ($P < 0.05$) from H_2O value. See Figure 1 for remainder of key.

For *P aeruginosa*, there was a significant decrease in bacterial growth for ER at all times (15, 30, 45, and 60 minutes; $P < 0.001$) and for ER – BA at 30, 45, and 60 minutes ($P < 0.001$), compared with time 0. Purified water (H_2O) had no effect on growth of *P aeruginosa*. Ear rinse significantly decreased bacterial growth at all times (15, 30, 45, and 60 minutes), compared with H_2O ($P < 0.001$; Figure 3).

For *Proteus* spp, there was a significant decrease in bacterial growth for ER at 30 ($P = 0.003$), 45 ($P < 0.001$), and 60 ($P < 0.001$) minutes, compared with time 0. Neither ER – BA nor H_2O had any effect on growth of *Proteus* spp. Ear rinse significantly decreased bacterial growth at 30, 45, and 60 minutes, compared with ER – BA ($P < 0.001$) and H_2O ($P = 0.007$; Figure 4).

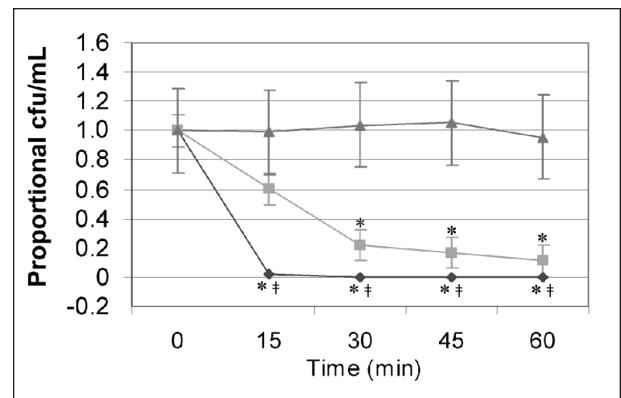


Figure 3—Bacterial growth of *Pseudomonas aeruginosa* in 3 solutions with time. The means of the number of CFU per milliliter for each *P aeruginosa* organism ($n = 5$) in each solution at each time were calculated. Because the initial (time 0) inoculum of each *P aeruginosa* organism varied from isolate to isolate, the outcome in the analysis was the proportion of bacterial growth in CFU per milliliter (proportional CFU per milliliter) at each time relative to time 0. Values are reported as mean \pm SD proportional CFU per milliliter. See Figure 1 and 2 for remainder of key.

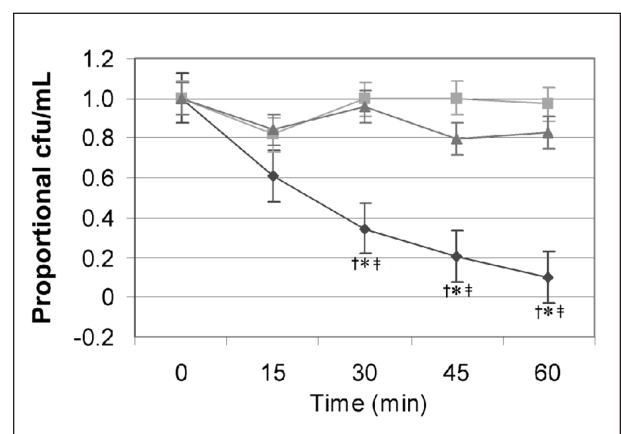


Figure 4—Bacterial growth of *Proteus* spp organisms in 3 solutions with time. The means of the number of CFU per milliliter for each *Proteus* spp organism ($n = 5$) in each solution at each time were calculated. Because the initial (time 0) inoculum of each *Proteus* spp organism varied from isolate to isolate, the outcome in the analysis was the proportion of bacterial growth in CFU per (proportional CFU per milliliter) at each time relative to time 0. Values are reported as mean \pm SD proportional CFU per milliliter. See Figures 1 and 2 for remainder of key.

Discussion

In the study reported here, ER significantly decreased in vitro bacterial growth of *P aeruginosa* and β -hemolytic streptococcal organisms within 15 minutes, *Proteus* spp within 30 minutes, and *Staphylococcus* spp within 60 minutes. In an in vitro study by Wooley and Jones,¹⁷ by use of tromethamine-EDTA without benzyl alcohol, *P aeruginosa* was rapidly lysed; β -hemolytic streptococcus and *P vulgaris* were also lysed, although not as rapidly as *P aeruginosa*; and there was no decrease in *Staphylococcus aureus* numbers.

Other studies^{28,29} have evaluated the in vitro effect of tromethamine-EDTA and lysozyme as well as tromethamine-EDTA and SDS, both of which were added to enhance the antimicrobial effects of tromethamine-EDTA. The addition of lysozyme to tromethamine-EDTA resulted in a rapid decrease in bacterial numbers of *P aeruginosa*; however, there was no effect on *S aureus* or *Streptococcus equi*. The combination of tromethamine-EDTA and SDS had synergistic antimicrobial activity in vitro against *P aeruginosa* as well as *P vulgaris* and additive antimicrobial activity against *S aureus*. In our study, addition of benzyl alcohol significantly increased the antimicrobial activity of tromethamine-EDTA on β -hemolytic streptococcus and *Proteus* spp organisms.

The resistance of gram-positive bacteria to tromethamine-EDTA without benzyl alcohol is expected, since tromethamine-EDTA damages bacteria by releasing divalent cations from cell walls. Gram-positive bacteria have teichoic acid, which functions as a binding agent for divalent cations, in their cell walls.³⁰ In our study, even with the addition of benzyl alcohol, a significant decrease in bacterial growth of *Staphylococcus* spp was detected only after 60 minutes of contact time with ER. On the other hand, ER significantly decreased growth of β -hemolytic streptococcus as early as 15 minutes. The difference in reduction of bacterial growth in vitro between β -hemolytic streptococcus and *Staphylococcus* spp (both gram-positive organisms) in ER may be attributable to a difference in the type of teichoic acid in the cell walls of these organisms.³¹

Tromethamine-EDTA in combination with antimicrobial agents in vitro has been evaluated for enhanced antimicrobial activity against gram-negative and gram-positive bacteria.^{17,19–23} A decrease in the minimum inhibitory concentration of *S aureus* was detected when tromethamine-EDTA was added to specific antimicrobial agents in vitro.²³ However, this decrease was not as marked as that reported against gram-negative organisms.²³ Synergistic activity in vitro was reported for combinations of tromethamine-EDTA and aminoglycoside antimicrobials against *S intermedius*, *Proteus mirabilis*, and *P aeruginosa*.¹⁹ The in vivo efficacy of tromethamine-EDTA in combination with a topically administered antimicrobial agent used in 8 dogs with bacterial otitis externa resulted in resolution of clinical signs of otitis and the bacterial infection within 7 to 15 days.³² Therefore, combining an antimicrobial with tromethamine-EDTA may improve its antibacterial efficacy, which is especially important for gram-positive bacterial ear infections caused by *Staphylococcus* spp.

In our study, sampling times of test solutions with the bacterial inoculum were at 15, 30, 45, and 60 minutes. For both β -hemolytic streptococcus and *P aeruginosa*, significant decreases in vitro of bacterial organisms in ER were detected at all times, beginning at 15 minutes. In a clinical situation, it is unlikely that the entire amount of any topically applied solution would remain in the ear for 15 minutes. However, samples were not obtained before 15 minutes, and the decrease in bacterial organisms in vitro may have occurred earlier than 15 minutes. In addition, although an ear canal will not remain completely full of a topically applied solution for extended periods, it is likely that some of the solution will remain in the canal in contact with bacterial organisms.

In our study, the ER formulation with benzyl alcohol had enhanced antiseptic activity against β -hemolytic streptococcus and *Proteus* spp, compared with a solution of tromethamine and EDTA alone. These results also support the role of benzyl alcohol as a preservative to protect the solution from contamination. Virtually all veterinary otic medications are packaged in multiple-dose containers in which contact of the applicator tip with the infected ear canal has the potential to contaminate the product. Thus, an effective preservative is important to minimize the possibility of spreading or perpetuating infection through the repeated use of the medication.

Ear rinse significantly decreased bacterial growth in vitro of *P aeruginosa* and β -hemolytic streptococcus within 15 minutes and *Proteus* spp within 30 minutes. The ER formulation with benzyl alcohol had significantly increased efficacy in reducing bacterial growth of β -hemolytic streptococcus and *Proteus* spp in vitro. On the basis of our results, ER may be useful in the treatment of infectious otitis. Future studies evaluating the in vivo efficacy of ER as a sole treatment for otic infections caused by β -hemolytic streptococcus, *P aeruginosa*, and *Proteus* spp and of ER in combination with an antimicrobial agent for bacterial otic infections caused by *Staphylococcus* spp are warranted.

- a. T8 Solution, IVX Animal Health Inc, St Joseph, Mo.
- b. ID 32 Staph, bioMerieux Inc, Durham, NC.
- c. ParafilmM, American National Can, Menasha, Wis.
- d. Soybean-casein digest agar, BD Diagnostic Systems, Sparks, Md.
- e. PROC MIXED in SAS, version 9.1, SAS Institute Inc, Cary, NC.

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