Preparation of Ormetoprim–Sulfadimethoxine-Medicated Discs for Disc Diffusion Assay

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Abstract
Romet (a blend of ormetoprim and sulfadimethoxine) is a type A medicated article for the manufacture of medicated feed in the catfish industry. Recently, the commercial manufacture of ormetoprim–sulfadimethoxine susceptibility discs was discontinued. Ormetoprim–sulfadimethoxine discs were prepared at the Mississippi State University College of Veterinary Medicine Aquatic Diagnostic Laboratory according to Clinical and Laboratory Standards Institute guidelines. The performance of the laboratory-prepared discs was compared with that of commercially prepared discs in disc diffusion assays with 23 field isolates of Edwardsiella ictaluri. The a priori limits of agreement for the laboratory-prepared ormetoprim–sulfadimethoxine discs were ±5 mm of the zones for the commercial discs. The resulting zones of inhibition ranged from 33 to 50 mm and from 35 to 52 mm for the laboratory-prepared and commercially prepared discs, respectively. The data comparing the two sets of discs were analyzed to determine the level of agreement. The analysis demonstrated that the laboratory-prepared discs produced a slightly smaller zone of inhibition than the commercially prepared ones. The observed limits of agreement were within the a priori established limits of agreement, indicating that the laboratory-prepared ormetoprim–sulfadimethoxine discs were acceptable replacements for the commercially prepared discs.

Antimicrobial medicated feeds are administered to sick fish to control mortality from bacterial diseases. Romet, Terramycin, and Aquaflor are the only type A medicated articles commercially available for the manufacture of food-fish medicated feeds in the United States. To ensure efficacy, it is important to monitor bacterial susceptibility to these antimicrobials.

Diagnostic laboratories routinely monitor bacterial susceptibility using in vitro assays. The disc diffusion test is a semiquantitative assay that is rapid and simple to perform (Forbes et al. 2002). In this assay, an agar plate is uniformly streaked with a pure bacterial culture isolated from a fish lesion and a disc impregnated with a known concentration of antimicrobial is placed on the plate. During incubation, the antimicrobial drug diffuses out from the disc and inhibits the growth of the susceptible bacteria. After incubation for 24–48 h, the zone of inhibition around the disc is measured. Although interpretive criteria are not yet uniformly established for antimicrobial susceptibility testing of aquatic bacteria (CLSI 2006; Miller and Reimschuessel 2006), each laboratory establishes criteria for disc-diffusion zone sizes it considers to be indicative of sensitive, intermediate, or resistant for their locale. These criteria are based on the measured diameter of inhibited growth (zone of inhibition) and the clinical response of fish treated with the antimicrobial.

Within the last year, the commercial manufacture of ormetoprim–sulfadimethoxine (the active ingredients of Romet) discs was discontinued, which hampered diagnostic laboratories from monitoring bacterial susceptibility to this antimicrobial. The Mississippi State University College of Veterinary Medicine Aquatic Diagnostic Laboratory prepared ormetoprim–sulfadimethoxine discs for disc diffusion assays with 23 field isolates of Edwardsiella ictaluri. The resulting zones of inhibition ranged from 33 to 50 mm and from 35 to 52 mm for the laboratory-prepared and commercially prepared discs, respectively. The data comparing the two sets of discs were analyzed to determine the level of agreement. The analysis demonstrated that the laboratory-prepared discs produced a slightly smaller zone of inhibition than the commercially prepared ones. The observed limits of agreement were within the a priori established limits of agreement, indicating that the laboratory-prepared ormetoprim–sulfadimethoxine discs were acceptable replacements for the commercially prepared discs.
Veterinary Medicine Aquatic Diagnostic Laboratory (MSU CVM ADL) receives over 600 accessions annually that must be monitored for antibacterial susceptibility. In order to assess susceptibility of bacteria to Romet, a popular antimicrobial in the U.S. catfish industry, we manufactured our own ormetoprim–sulfadimethoxine discs based on the method described in the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2006). This manuscript describes the preparation of discs using the same formulation of ormetoprim–sulfadimethoxine that is in Romet (a blend of 1.25 and 23.75 µg of ormetoprim and sulfadimethoxine, respectively) and compares the in vitro susceptibility of bacteria to Romet, a popular antimicrobial used in the U.S. catfish industry, with that of commercially prepared discs.

METHODS

Twenty-three archived Edwardsiella ictaluri isolates from channel catfish Ictalurus punctatus affected with enteric septicaemia of catfish occurring from natural outbreaks in commercial catfish ponds from Mississippi, Louisiana, Alabama, and Arkansas were arbitrarily selected for disc diffusion susceptibility testing. The bacteria were identified as E. ictaluri using an abbreviated biochemical assay (BBL Crystal, Cockeysville, Maryland). Before testing, the archived bacteria were removed from the ultracold freezer (−60 ± 10°C), allowed to thaw to room temperature, cultured on Mueller–Hinton Agar (MHA) plates, and incubated at 27 ± 2°C for 2 d. Escherichia coli (ATCC 25922) was used for quality control for susceptibility testing.

Ormetoprim–sulfadimethoxine–medicated discs.— Ormetoprim (99.7% purity) and sulfadimethoxine (99.2% purity) standards were generously supplied by PHARMAQ (Oslo, Norway). Ormetoprim–sulfadimethoxine medicated discs were prepared in the laboratory according to CLSI guidelines (CLSI 2006). Briefly, 62.5 mg of ormetoprim were dissolved in 50 mL of 0.05 mol/L HCL followed by the addition of 450.0 mL deionized water. To prepare the sulfadimethoxine solution, 59.4 mg of sulfadimethoxine powder was dispersed into 20.0 mL of sterile, warm (35°C), deionized water followed by dissolution into 400 µL of 2.5 mol/L sodium hydroxide. The addition of 4.6 mL of sterile deionized water yielded 25.0 mL of a 23.75-µg/10 µL sulfadimethoxine solution. Equal volumes of the prepared ormetoprim and sulfadimethoxine solutions were combined to yield a solution that contained 0.25 µg ormetoprim and 23.65 µg sulfadimethoxine per 20 µL. In a sterile hood, 20 µL of the prepared ormetoprim–sulfadimethoxine solution were pipetted onto blank, 6-mm diameter, sterile paper discs (Becton Dickinson, Sparks, Maryland) and allowed to dry for 4 h. The dried discs were individually collected with sterile forceps and placed in lots of 50 into a sterile vial containing a molecular sieve desiccant pack (Impak Corp., Los Angeles, California). The vials of discs were stored in a monitored refrigerator at 0–8°C.

One tube of 50 commercially prepared ormetoprim–sulfadimethoxine–medicated discs (Becton Dickinson) was supplied courtesy of Aquatic Health Resources, Inc. (Minnetonka, Minnesota). The laboratory- and commercially removed discs were stored from the refrigerator and brought to room temperature 2 h prior to testing. Agar disc-diffusion susceptibility testing was performed as outlined in the CLSI performance standard guidelines (CLSI, 2006). Edwardsiella ictaluri colonies were removed from the agar plate with a sterile cotton swab and suspended in sterile media to the density of McFarland 0.5 barium sulfate turbidity standard (equivalent to 1–2 × 10⁶ colony-forming units (CFU)/mL). Sterile Mueller–Hinton agar plates were streaked with the swab over the entire agar surface while plates were rotating to provide an evenly distributed inoculum of E ictaluri. Each inoculated plate was divided into four quadrants, and a disc was placed in the center of each quadrant within 15 min of inoculation. A laboratory-prepared and commercially prepared ormetoprim–sulfadimethoxine disc was placed in quadrants 1 and 2, respectively, and quadrants 3 and 4 each contained a sterile blank filter paper disc. All isolates were tested in duplicate. Plates were stacked at four plates per stack and incubated for 48 h at 27 ± 2°C. The zone of bacteria growth inhibition (including disc diameter) was measured to the nearest millimeter with a certified caliper. For quality control, plates containing E. coli ATCC 25922 were incubated at 27 ± 2°C and observed after 16–18 h according to CLSI guidelines. The diffusion zones for the laboratory-prepared discs were compared with those for commercially prepared discs. The laboratory discs were periodically tested to ensure the zones were within the CLSI guidelines for quality control organisms (CLSI 2006) and to establish a shelf life.

Statistical analysis.—For each E. ictaluri isolate (n = 23) the zone of inhibition was determined for a commercially prepared ormetoprim–sulfadimethoxine disc, a laboratory-prepared ormetoprim–sulfadimethoxine disc, and two blank discs on each of two culture plates, thereby providing two replicates for each isolate. The data comparing the commercially and laboratory-prepared ormetoprim–sulfadimethoxine discs were analyzed according to methods described by Bland and Altman (1986) to determine the level of agreement between the two disc sources. It was established a priori that acceptable limits of agreement for the zones of inhibition for laboratory-prepared ormetoprim–sulfadimethoxine discs would be ±5 mm of the zones of inhibition for the commercial ormetoprim–sulfadimethoxine discs.

RESULTS AND DISCUSSION

The resulting zones of inhibition against 23 E. ictaluri isolates ranged from 33 to 50 mm and from 35 to 52 mm for the laboratory-prepared and commercially prepared ormetoprim–sulfadimethoxine discs, respectively. The mean of the mean zone of inhibition for each of the isolates was 40.3 mm (SD = 3.56 mm) for the laboratory-prepared discs, and 40.6 mm (SD = 3.54 mm) for the commercially prepared discs. The diffusion zones for the quality-control organism were within
FIGURE 1. Zones of inhibition for commercially versus laboratory-prepared ormetoprim–sulfadimethoxine discs for 23 *Edwardsiella ictaluri* isolates. If the two disc sources gave exactly the same zones of inhibition for each isolate, the values would lie on the line of equality (the straight line in the plot). Zones of inhibition were determined in duplicate for each isolate for each disc source. Although 46 pairs of measurements were plotted, only 26 are visible due to the overlapping of data points.

CLSI limits for both the laboratory-prepared and the commercially prepared discs. The zones of inhibition for the laboratory-prepared and commercial ormetoprim–sulfadimethoxine discs are plotted against each other in Figure 1. If there was perfect agreement between the two sources, all of the measurements would fall on the line of equality that is also plotted. The data are presented in a Bland–Altman plot in Figure 2 in which the differences between the zones of inhibition of the two sources of discs (commercial minus laboratory) are plotted against the means of the zones of inhibition for the two sources of discs for each of the 23 isolates of *E. ictaluri*. The plot demonstrates that when the zones of inhibition are different, the commercial discs generally have larger zones of inhibition than do the laboratory-prepared discs. No relationship between mean zone of inhibition and the magnitude of the differences between the disc sources is evident. A histogram of the differences between the sources of discs, which confirms that the zone of inhibition of the commercial discs when different are usually larger than the laboratory-prepared discs, is presented in Figure 3.

The bias (i.e., the mean difference between the means for each method) was 0.3 mm, indicating that the zones of inhibition of the commercial discs were, on average, 0.3 mm larger than the corresponding zones of inhibition for the laboratory-prepared discs. The SD of the differences between the two disc sources, corrected for replication, was 1.18 mm. The 95% confidence interval (CI) for the bias ranged from −0.2 to 0.8 mm.

If the differences were normally distributed, we would expect 95% of the differences between the two disc sources to lie between −2.0 and 2.6 mm, the limits of agreement. As shown in Figure 3, the distribution of the differences was skewed to the right but generally followed a normal distribution. The SE of the limits of agreement was 0.43 mm. The 95% CI for the lower limit of agreement was −2.9 to −1.1 mm and the 95% CI for the upper limit of agreement was 1.7 to 3.5 mm.

The analysis demonstrated that the laboratory-prepared ormetoprim–sulfadimethoxine discs, on average, produced a slightly smaller zone of inhibition than those commercially prepared. With the given limits of agreement, we would expect the zone of inhibition for the laboratory-prepared discs to range from 2.6 mm less than to 2.0 mm greater than the commercial ormetoprim–sulfadimethoxine discs. The

FIGURE 2. Difference in zones of inhibition of ormetoprim–sulfadimethoxine (commercial minus laboratory) plotted against the mean zone of inhibition for the two disc types for 23 isolates of *Edwardsiella ictaluri*. Due to overlapping, only 18 of the 23 data points are visible in the plot.

FIGURE 3. Histogram of differences in zones of inhibition for ormetoprim–sulfadimethoxine (commercial minus laboratory) for 23 *Edwardsiella ictaluri* isolates.
observed limits of agreement were within the a priori established limits of agreement indicating the laboratory-prepared ormetoprim–sulfadimethoxine discs are acceptable replacements for the commercially prepared discs. After 8 months of monitoring, the zones of the laboratory-prepared discs remained within the CLSI guidelines established for quality-control organisms.

In conclusion, the laboratory-prepared ormetoprim–sulfadimethoxine discs yield accurate results for disc diffusion zones and will be useful in assessing the susceptibility of *E. ictaluri* to Romet type A medicated article. This will enable diagnosticians to reliably monitor antimicrobial susceptibility and choose the appropriate antimicrobial therapy when needed.

REFERENCES


