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Antimicrobial Resistance in *Aeromonas salmonicida*

Furunculosis, a major cause of mortality in salmonid fishes, is caused by the Gram-negative bacterium *Aeromonas salmonicida*. The disease is systemic and may be characterized in several forms: an asymptomatic form; an acute form resulting in severe internal organ involvement, with high numbers of mortalities; and a chronic form that may involve internal pathology, but with lesser numbers of mortalities over longer periods of time. Furunculosis is treated with antimicrobial therapy; three such agents are approved by the Food and Drug Administration (FDA) for use in salmonids. Because the disease is systemic, the agent is administered as a food additive.

The most recent FDA-approved antimicrobial is Romet™ (1984), a potentiated sulfonamide comprised of sulfadimethoxine and ormetoprim (both affect the folic acid pathway of prokaryotic DNA synthesis). Sulfonamide-resistant strains of *A. salmonicida* were first described in the late 1950s. In recent years, some fish husbandry personnel have noted that treatment of furunculosis using Romet™ has been ineffective in reducing mortality or controlling disease. Furthermore, multiple antimicrobial resistance has been demonstrated in this bacterium, and this resistance can be encoded on transferrable R-factors by conjugational mating to recipient strains of *Escherichia coli*.

Our studies evaluated the widespread nature of resistance to Romet™ among strains of *A. salmonicida* and explained the molecular basis for this resistance.

Isolation of *Aeromonas salmonicida* and Screening For Resistance To Romet™

Primary bacterial isolation of *A. salmonicida* was conducted at 12 salmonid-rearing facilities (sites): five in New York, three in Vermont, and one each in Maryland, New Hampshire, Pennsylvania, and West Virginia. A total of 585 strains were presumptively identified as *A. salmonicida*, based on production of brown, water-soluble pigment on tryptic soy agar and a positive oxidase. Six hosts were represented: Atlantic salmon (*Salmo salar)*, cutthroat trout (*Oncoryhnchus clarki*), lake trout *(Salvelinus namaycush)*, brook trout *(S. fontinalis)*, brown trout *(S. trutta)*, and rainbow trout *(O. mykiss)*.

Three hundred fifteen (53.8%) of 585 isolates displayed resistance to Romet™. Resistant strains were isolated from 10 of 12 sites and all hosts. Using the Bauer-Kirby disk sensitivity method for determining resistance, three phenotypes of resistance to Romet™ were noted: (1) no zone of inhibition, (2) confluent but light bacterial growth within a zone of inhibition, and (3) individual resistant colonies within a zone of inhibition. Strains representing each of these phenotypes were selected for further study.

Comparison of Romet™-resistant Strains To Sensitive Strains

Fifty-one Romet™-resistant strains of *A. salmonicida* were compared to Romet™-sensitive strains in an effort to detect phenotypic characters in addition to resistance to Romet™. Biochemical

comparisons were made in 22 different test media, and utilization of 26 carbohydrates was evaluated. All strains were taxonomically confirmed to be *A. salmonicida*. Test results noted no differences between resistant and sensitive strains. Antimicrobial sensitivity comparison assays also were done to determine if the R-factor possessed multiple resistances. Sensitivity testing to 42 antimicrobial agents was done using the disk diffusion method.

Both resistant and sensitive strains were resistant to four antimicrobials and were sensitive to 12 others; the remaining agents demonstrated variable results. Variability of some antimicrobials was explained by differences between resistant and sensitive strains. The Romet™-resistant strains were resistant to additional antimicrobials, indicative of multiple resistance associated with the R-factor. In addition to being resistant to Romet™, most resistant strains also were resistant to tetracycline, sulfa drugs, trimethoprim, Tribrissen, and oxytetracycline. Oxytetracycline is one of the FDA-approved antimicrobials for treatment of furunculosis in salmonids. One resistant strain from Pennsylvania was unique in that it possessed resistance to an additional 13 antimicrobial agents, whereas none of the other resistant strains did.

R-plasmid DNA studies

Romet™-resistant strains representing each of the three resistance phenotypes were evaluated for their ability to transfer antimicrobial resistance and to encode DNA via conjugational mating to recipient strains of *E. coli*. Test results, along with screening for additional plasmid DNA following agarose gel electrophoresis (AGE) and staining with ethidium bromide, would indicate any plasmid-mediated resistance.

The R-plasmids and associated antimicrobial resistances of only two Romet™-resistant isolates were transferrable to *E. coli*. Using AGE, we demonstrated nonnative plasmid DNA in many of the resistant strains, indicating encoding of resistance on nontransferable R-plasmids. Interestingly, those strains with no zone of inhibition were noted to have an R-plasmid either 50 or 55 kilobases (Kb) in size; the slightly smaller 50-Kb R-plasmid was noted in those strains with confluent but light growth within a zone of inhibition. We detected no R-plasmid from two strains with this phenotype. Strains having individual resistant colonies within a zone of inhibition demonstrated only the 50-Kb R-plasmid. Studies are under way to determine and compare the DNA sequences of the two R-plasmids.

The results of our studies show that antimicrobial resistance of *A. salmonicida* is widespread. Use of a potentiator in developing Romet™ was intended to reduce the risk for in vivo development of resistant strains of bacteria; however, the combination of the bacterium, an antimicrobial agent creating selection pressure, and a source of an R-factor has led to resistant strains developing and subsequently being selected for. Although we did not demonstrate high frequency of transfer of R-plasmids with *A. salmonicida* as the donor strains, based on the prevalence of resistant strains we isolated, this bacterium appears to be a ready recipient of resistance factors. We have initiated additional studies that will attempt to determine the source of resistance factors from the environment.

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