Bioencapsulation of the Antibacterial Drug Sarafloxacin in Nauplii of the Brine Shrimp Artemia franciscana

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Abstract—Nauplii of the brine shrimp Artemia franciscana were enriched with the antibacterial drug sarafloxacin hydrochloride to determine if levels effective against four pathogenic strains of Vibrio spp. would accumulate in brine shrimp. Three vibrio strains were Vibrio anguillarum, the fourth was V. vulnificus. Concentrations of sarafloxacin ranging from 1 to 40% (weight: volume) were incorporated into the oil phase of Super Selco brine shrimp diets. Enriched nauplii and controls were sampled at 2-h intervals for 24 h. Antibiotic sensitivity testing was evaluated with a modification of the Kirby-Bauer disk diffusion method. A microbiological assay with Escherichia coli was used to determine the concentration of sarafloxacin per sample of nauplii. The results indicate that sarafloxacin is rapidly taken up by larval brine shrimp and can be detected at 2 h of enrichment by microbiological assay. The efficacy of sarafloxacin appears to depend on the strain of pathogen present.

The current method of administering antibiotic therapy for bacterial infections of food fish is incorporating an approved drug as a premix into the feed (Alderman 1986). The treatment of non-food fish is affected either through the use of medicated feed or, more commonly, by dispensing antimicrobial drugs directly into the water as bath treatments. Both of these approaches can be problematic. For example, formulated feeds may not be readily accepted by young stages of fish, particularly larval fish that are raised on cultured live foods such as nauplii of brine shrimp Artemia spp., rotifers Brachionus spp., and various microalgae. Drugs may leach out of the feed and affect the environment, decompose, or both. The practice of applying chemotherapeutants directly to the water raises important, and in some cases unanswered, questions such as how much drug is absorbed from the water, how water quality affects drug stability, how resistant bacteria develop, and what environmental impact results.

A promising new method for the successful prevention and treatment of bacterial diseases in fish is the administration of live food supplemented with therapeutic agents by the technique of bioencapsulation. The advantages of this method are the controlled delivery of a drug to the fish and the circumvention of problems associated with current treatment applications (Nelis et al. 1991). This study was initiated to determine if effective levels
of sarafloxacain hydrochloride would accumulate in brine shrimp nauplii via bioencapsulation.

**Methods**

**Animals**

*Artemia franciscana* nauplii were hatched from Great Salt Lake cysts at 28°C ± 1°C in natural seawater (35%) with continuous aeration and illumination. After 24 h, instar I nauplii were harvested, separated from hatching debris, and thoroughly rinsed. The nauplii were counted; their concentrations were adjusted to 300/mL, and they were resuspended in fresh seawater to which the commercial live food enrichment diet Super Selco (*Artemia Systems S.A., Baasrode, Belgium*) was added at a concentration of 0.6 g/L. (Leger et al. 1986, 1987). Concentrations of sarafloxacain hydrochloride (*Abbott Laboratories, North Chicago, Illinois*) at 1%, 5%, 10%, 15%, 20%, and 40% (weight : volume oil phase) were added to the enrichment medium. Controls consisted of nauplii in the enrichment medium without sarafloxacain. Nauplii were enriched with aeration at 28°C ± 1°C for 2-24 h. At 2-h intervals, samples were taken and nauplii were counted, rinsed in ambient freshwater to remove excess surface Super Selco, concentrated to 3,000/mL, and resuspended in 2 mL of sterile saline. Suspensions were macerated in the stomacher for 3 min and filter-sterilized through a 0.2-μm-pore filter. The sterile filtrate was used for subsequent determination of bacterial activity and concentration.

**Bacteria**

The bacteria used in the antibiotic sensitivity assays were three strains of *Vibrio anguillarum* originally isolated from hatchery disease outbreaks. Strains 6) and 261 were isolated from European bass *Morone labrax* at hatcheries in Italy and France, respectively, and strain 43 was isolated from gilthead bream *Sparus auratus* at a hatchery in Greece. All strains were initially identified by biochemical methods (Grizez et al. 1991), serologically confirmed, and maintained frozen in the Laboratory for Microbiology and Microbial Genetics, University of Ghent. A fourth bacterial strain was isolated from thiosulfate citrate bile salt and blood agar from a hatchery outbreak among larval turbot *Scophthalmus maximus* at the Artemia Reference Center, University of Ghent. This sucrose- and oxidase-positive, beta-hemolytic strain was biochemically identified by both the API 20E and Nonfermenter Test multitest strip systems (bioMerieux, Marcy-l’Etoile, France) as *Vibrio vulnificus*; serological analysis was not performed. Stock cultures of all strains were maintained on tryptic soy agar supplemented with 2% sodium chloride.

**Sensitivity assay.**—A modification of the Kirby-Bauer disk diffusion method was used to determine bacterial sensitivity to sarafloxacain. A saline suspension of 20-24-h colonies of each test strain was adjusted to match a 0.5 McFarland turbidity standard (approximately 1.5 × 10⁶ colony-forming units per milliliter) and swabbed onto a Mueller-Hinton agar plate supplemented with 2% sodium chloride. A disk containing 5 μg of sarafloxacain hydrochloride (*Abbott Laboratories, North Chicago, Illinois*) was used as the standard control. Duplicate blank sterile disks inoculated with 0.2 μL of each sample suspension were used to determine efficacy of the test samples. The plates were incubated at 25°C for 24 h, and the zones of inhibition were measured with a mechanical zone reader.

**Concentration assay.**—The concentration of sarafloxacain per sample was determined by microbial assay according to the methods for biological assays in the U.S. Pharmacopeia (1990). Antibiotic assay medium 9 (adjusted to pH 7.2) was used as the assay medium. *Escherichia coli* ATCC (American Type Culture Collection) 10536 was used as the test organism at a concentration of 0.05%. A stock solution of sarafloxacain hydrochloride was prepared in sterile water and 1 M sodium hydroxide. Dilution standards were prepared from the stock solution without further addition of sodium hydroxide. Three samples and three dilution standards were used per plate. The wells were filled with 50 μL of either the standard dilution or sample preparation and incubated at 35°C for 24 h. Zone sizes were measured by a mechanical zone reader.

**Sulfamethoxazole-trimethoprim assay.**—Prior research on antibacterial drug enrichment of brine shrimp was primarily focused on the use of potentiated sulfonamides. The uptake of sulfamethoxazole-trimethoprim by nauplii was demonstrated in vitro by liquid chromatographic techniques (Nelis et al. 1991; Verpraet et al. 1992). A microbiological assay of potentiated sulfonamides (sulfadimethoxine-trimethoprim) from enriched brine shrimp nauplii was reported by Mohney et al. (1990). We used sulfamethoxazole-trimethoprim to determine its efficacy against pathogenic strains of *Vibrio* in comparison with sarafloxacain. Nauplii were enriched in Super Selco, as described, at a concentration of 15% sarafloxacain. A
mixture of sulfamethoxazole and trimethoprim (5: 1) was used for enrichment (Verpraet et al. 1992). All samples were handled in a manner similar to that already described for sarafloxacin samples. A standard disk (23.75 μg sulfamethoxazole and 1.25 μg trimethoprim) was used as the control for sensitivity assays. Concentration assays were not performed.

Results and Discussion

The optimum nauplii enrichment time for sarafloxacin efficacy in the sensitivity assay against all four strains of pathogenic Vibrio was 6 h at a concentration of 15% sarafloxacin. Under these conditions, nauplii extracts produced zones of inhibition that equaled or exceeded 19 mm, the minimum zone size indicating sensitivity to sarafloxacin. A zone size of 19 mm or greater is approximately equivalent to a minimum inhibitory concentration of 1 μg/ml (J. Stamm, Abbott Laboratories, personal communication). Zones were obtained at a 10% enrichment concentration; however, these zones were smaller, indicating intermediate sensitivity or resistance. This was particularly true for V. anguillarum strains 61 and 261 and the isolate identified as V. vulnificus. These strains consistently required slightly higher concentrations of sarafloxacin (15%) to be effective. Enrichment for longer periods did not increase zone sizes; indeed, samples enriched for 24 h produced slightly smaller zones. Control samples enriched with Super Selco alone did not produce zones.

At 15% sarafloxacin enrichment for 6 h, zones for V. anguillarum strains were as follows; strain 43, 29–32 mm; strain 61, 33–35 mm; strain 261, 17.5–21 mm. Zones for the V. vulnificus isolate ranged from 14 to 19 mm. Zones for the sarafloxacin control disk were 43–46 mm, 33–36 mm, 33–35 mm, and 27–33 mm, respectively (Table 1). The V. vulnificus isolate consistently was the most resistant to sarafloxacin; V. anguillarum strain 43 was the most sensitive in control tests and strain 61 in the enrichment tests.

Enrichment with 15% sulfamethoxazole-trimethoprim was not efficacious for any of the four bacterial isolates; enrichment for 2 or 4 h did not produce zones against any of the three strains of V. anguillarum. A 6-h enrichment produced zones of 15.4–16.7 mm against strain 43 and 13.0–15.7 mm against strain 61; no zones were produced against strain 261 (Table 1). Control disks produced zones of 30.3 mm against strain 43, 29.6 mm against strain 61, and 27.9–29.3 mm against strain 261. A zone size of 16 mm or more is considered the minimum size indicating susceptible to sulfamethoxazole-trimethoprim. A 15% enrichment for 6 h also failed to produce zones against the V. vulnificus isolate; however, enrichment for 2 and 4 h produced zones ranging from 6.9 to 7.9 mm, well below that of the susceptible level. Control disks produced zones of 32–32.8 mm (Table 1).

In the concentration assay, extracts of approximately 75 nauplii enriched with 15% sarafloxacin for 6 h produced zone sizes corresponding to those produced by the 4-μg/ml concentration standard. These data seem to indicate that 75 nauplii could be enriched to contain approximately the same amount of drug as the control disk (5 μg) used in antibiotic sensitivity testing.

Other researchers have also tested the potential of drug bioencapsulation by Artemia. Trimethoprim and sulfamethoxazole in a ratio of 1:5 were used to enrich Artemia nauplii that were subsequently fed to larval turbot and European bass. Drug levels measurable by liquid chromatography were attained in fish tissue 30–60 min after feeding (Chair et al. 1991, 1993). Touraki et al. (1991) showed that trimethoprim was more easily bioencapsulated in nauplii than a sulfamethoxazole-trimethoprim combination, which accumulated at a
slower rate. However, these researchers did not perform any bacterial testing. Mohney et al. (1990) fed nauplii enriched with Rotem-30P (sulfadimethoxine-ormethoprim) to control disease in larvae of the penaeid shrimp *Penaeus stylirostris*. Enriched nauplii were readily accepted by the diseased shrimp, and treated larvae eventually exhibited a statistically greater overall survival than untreated animals. Mohney et al. (1990) also measured zone sizes resulting from inhibition of *Vibrio alginolyticus* in an in vitro test system similar to the method we used.

The results of this study and others indicate that oral delivery of antimicrobial drugs can be facilitated through bioencapsulation in brine shrimp nauplii. This methodology represents a potentially useful new tool for both the prophylactic and therapeutic treatment of larval fish and shrimp. Live *Artemia* nauplii are a primary food source for rearing many cultured freshwater and marine animal species. Many hatcheries or culture facilities routinely hatch and raise brine shrimp on site for larval feeding. Bioencapsulation would provide a means to medicate fish that could easily be incorporated into already existing procedures. However, more research is needed to better delineate dose concentrations, particularly in regard to antibiotic-resistant bacteria.

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References


