Occurrence of Potential Pathogens in Water Containing Ornamental Fishes

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The bacterial population of the water supplied with ornamental fish purchased from retail outlets was examined qualitatively and quantitatively. As many as $10^8$ viable aerobic organisms per 100 ml were present, with fecal coliform counts as high as $10^4$ per 100 ml. Citrobacter, Escherichia, Pseudomonas, and Vibrio were isolated from 75% or more of the samples, whereas Aeromonas, Alcaligenes, Enterobacter, Flavobacterium, and Streptococcus were isolated from 45 to 65% of the samples. Pseudomonas aeruginosa, Edwardsiella tarda, and Klebsiella pneumoniae were also isolated.

Considerable attention has recently been given to the role of small pet turtles and tortoises as vectors of Salmonella. Recent data have shown that, of the estimated two million cases of human salmonellosis per year in the United States, approximately 300,000 are associated with pet turtles (26). For this reason, the United States Congress recently enacted legislation to effectively prohibit the interstate shipment of turtles harboring Salmonella and Shigella (22). In Canada, the Province of Alberta has prohibited the sale of pet turtles and tortoises.

Despite the attention given to the terrapins, little consideration has been given to the role of other aquarium species as vectors of potential pathogens for man. It is particularly surprising that little attention has been given to the ornamental fish since large numbers of these fish are imported into North America from areas of the world where sanitation is often inadequate and where numerous diseases of man are endemic. Many of these fish are offered for sale to the public. It has been estimated that there are more than 20 million household aquaria in the United States (3). In addition, aquaria containing ornamental fish are often found in school classrooms, medical and dental offices, eating establishments, department stores, nursing homes, and even in hospital wards. It is apparent that the presence of potentially pathogenic microorganisms in these aquaria would present a risk to the public health.

When ornamental fish are sold by retail stores to the public, the fish and a volume of their aquarium water is generally transferred to a plastic container. Although this allows the fish to be transferred to the purchaser's aquarium, it also represents one means by which the public could be exposed to pathogenic organisms. Thus, the present study was initiated to determine qualitatively and quantitatively the bacterial population in the water supplied with ornamental fish purchased from retail stores. No attempt was made to assess the parasite population in the water.

MATERIALS AND METHODS

Water samples. Ornamental fishes were purchased from 13 retail outlets (Sources A to M, Table 1) in Victoria, British Columbia. The fish were supplied in plastic containers containing water taken from the aquaria in which the fish had been housed except for Source F when fresh water was supplied. Water samples were transported to the laboratory and sampled immediately. Sources A to D were pet shops, source E was a hobby store, source F was a feed distributor, and sources G to M were department stores. The fish from source N had been shipped directly from a supplier in Toronto, Ontario. In addition, water from an aquarium in a local shopping center was also examined, as were water samples from aquaria at the Department of Bacteriology and Biochemistry, University of Victoria. These were sources O and P, respectively. The numbers of samples from each source, the numbers of fish in each sample, and the species of fish in each water sample are shown in Table 1. The water samples were placed into two groups. The water in the first group contained goldfish (Carassius auratus) and was taken from aquaria maintained at room temperature. The water in the second group contained tropical species and was sampled from aquaria maintained at 22 to 27 °C.
Source N was supplied with fish from Florida and was the major supplier of fish to sources A, C, D, F, G, H, I, K, L, and M. Sources A, D, and E were supplied with fish from a distributor in Seattle, Wash., whose fish were obtained from Missouri. Source D also obtained fish from a third distributor in Miami, Fla. Sources G, J, and I were supplied with fish imported directly from Asia.

**Quantitative bacteriological examination.** Duplicate dilutions of the water samples were prepared in 0.1% (wt/vol) peptone water (pH 7.2), and the viable mesophilic bacteria present were enumerated on Trypticase soy agar (Baltimore Biological Laboratories) by the drop-plate method of Miles and Misra (16). Inoculated media was incubated aerobically at 20, 30, and 37 C and anaerobically at 37 C for 48 h. Anaerobiosis was obtained by using anaerobic jars with disposable hydrogen-carbon dioxide generator envelopes (BBL).

The procedures of the American Public Health Association, Inc. (2) were followed for most probable number (MPN) estimates. Presumptive coliforms were estimated by the 10:1:0.1 multitube method by using MacConkey broth (BBL) and brilliant green bile broth (BBL), and were confirmed by separate loop transfers from the above positive tubes to MacConkey broth. Completed coliforms were estimated by loop transfers from positive confirmed coliform tests to MacConkey broth. Fecal coliforms were estimated by separate loop transfer from positive presumptive coliform tubes to MacConkey broth and incubated at 44.5 ± 0.5 C. The MPN estimation of enterococci was carried out in ethyl violet azide broth (BBL) and was confirmed by separate loop transfers to fresh ethyl violet azide broth. The MPN estimation of *Citrobacter* required incubation at 37 C in selenite-F broth (BBL) followed by separate loop transfers to Salmonella-Shigella agar (BBL).

**Qualitative bacteriological examination.** Representatives of the most numerous colonial types from all sources were selected from plates containing the highest countable dilution and from dilutions of the MPN enrichment broths. The isolates were then purified and maintained by weekly transfer on Trypticase soy agar and storage at 4 C. Selenite-F broth and tetraathionate broth (BBL) were also inoculated with water samples and incubated at 37 and 41.5 C. After 48 h, the broths were streaked on Salmonella-Shigella agar, brilliant green agar (BBL), and bismuth sulfite agar (BBL) and incubated at 37 and 41.5 C. The tests used to partially characterize the isolates were those described by Edwards and Ewing (9), Skerman (19), and Smith et al. (20).

Identifications were facilitated by examination of colonial morphology and pigmentation, as well as examination of the shape, arrangement, staining characteristics, flagellar arrangement, and motility of the cells. In addition, the ability of the isolates to produce oxidase and catalase, to ferment lactose, and to metabolize glucose fermentatively or oxidatively was tested. Gram-negative species were further separated on the basis of carbohydrate utilization, growth in the presence of bile salts, production of urease, and the indole, methyl red, Voges-Proskauer, and citrate utilization reactions. Other tests used were the ortho-nitrophenyl-β-d-galactopyranosidase (ONPG) test, the lysine and ornithine decarboxylase tests, and the arginine decarboxylase and dihydrolase tests. The ability to liquefy gelatin and to change litmus milk was also ascertained. *Vibrio* species were confirmed by testing the sensitivity of the culture to 2,4 diaminobenzidine, 7-diisopropylphtheride. Final identification of the isolates were based on the schemes of Breed et al. (6), Shewan et al. (18), Bain and Shewan (4), Cowan and Steele (8), and Edwards and Ewing (9). Identifications within the Enterobacteriaceae were confirmed by using the API System (Analytab Products, Inc., N.Y.).
RESULTS

Qualitative bacteriological examination. A total of 869 isolates were partially characterized from the 42 water samples. The results are shown in Table 2. The isolates comprised 23 genera of bacteria; 84% of these isolates were gram-negative rods. The most common species isolated belonged to the genus Citrobacter. These Citrobacter isolates comprised 26% of the total isolates and were demonstrated to be present in 38 of the water samples. Of the Citrobacter isolates, 188 were identified as Citrobacter freundii, 33 were Citrobacter diversus, and seven were identified as Citrobacter intermedium. The large number of Citrobacter isolated is in part due to the fact that H₂S-producing colonies on SS agar plates were tested biochemically to determine the presence of Salmonella and Arizona. No Salmonella or Arizona were isolated; however two isolates identified as Citrobacter were ONPG-negative and were agglutinated by Salmonella polyvalent antiserum and Salmonella group C₁ antiserum. These isolates when submitted to a reference laboratory were classified as Citrobacter freundii rather than Salmonella because they gave a delayed positive reaction in sucrose. Similarly, two isolates were identified as H₂S-producing Enterobacter hafniae rather than Arizona because they were Voges-Proskauer positive and unable to ferment sorbitol. Edwardsiella tarda was also isolated on five occasions.

Species of Pseudomonas comprised 20% of the total isolates and were isolated from 39 water samples. The majority of these Pseudomonas isolates were nonpigmented species, with 67 isolations from goldfish water and 66 isolations from tropical fish water. Many of these nonpigmented isolates were biochemically similar to Pseudomonas aeruginosa and were able to grow at 42°C on Pseudocel agar (BBL). Typical pyocyanin-producing P. aeruginosa was also isolated on three occasions from goldfish water and on 21 occasions from tropical fish water. In addition, fluorescein-producing pseudomonads were isolated on two occasions from goldfish water and on 19 occasions from tropical fish water. Species of Alcaligenes, Flavobacterium,

| Table 2. Frequency of isolation of bacterial species from ornamental fish water |
|----------------------------------|------------------|------------------|------------------|------------------|
| Species isolated                | No. of isolates | No. of samples from which isolated |                   |                   |
|                                 | Goldfish water  | Tropical fish water | Goldfish water  | Tropical fish water |
| Achromobacter                   | 7               | 8                | 6               | 5                |
| Acinetobacter                   | 19              | 7                | 10              | 5                |
| Aeromonas                       | 27              | 19               | 9               | 11               |
| Alcaligenes                     | 21              | 14               | 16              | 12               |
| Bacillus                        | 7               | 7                |                 |                  |
| Brevibacterium                  | 4               | 3                | 2               |                  |
| Chromobacter                    | 4               | 2                | 4               | 2                |
| Citrobacter                     | 121             | 107              | 20              | 18               |
| Clostridium                     | 7               | 16               | 4               | 9                |
| Cytophaga                       | 9               | 5                | 8               | 5                |
| Edwardsiella                    | 1               | 4                | 1               | 3                |
| Enterobacter                    | 25              | 15               | 11              | 6                |
| Escherichia                     | 13              | 13               | 23              | 13               |
| Flavobacterium                  | 14              | 30               | 9               | 14               |
| Klebsiella                      | 4               |                  | 3               |                  |
| Micrococcus                     | 10              | 15               | 8               | 9                |
| Micrococcus                     | 1               |                  | 1               |                  |
| Proteus                         | 8               | 8                | 5               | 5                |
| Pseudomonas                     | 72              | 106              | 21              | 18               |
| Staphylococcus                  | 11              | 5                | 9               | 5                |
| Streptococcus                   | 27              | 21               | 12              | 12               |
| Vibrio                          | 21              | 19               | 12              | 10               |
| Xanthomonas                     | 2               |                  | 1               |                  |
| Yeast                           | 5               |                  | 2               |                  |

* Total number of samples was 24.
* Total number of samples was 18.
* Total number of samples was 10.
and Vibrio were also common isolates and were recovered from 28, 23, and 22, respectively, of the water samples.

Among the other isolates were species of Aeromonas, Escherichia, and Streptococcus. Aeromonads were isolated from 20 water samples and comprised 5% of the total isolates. Aeromonas hydrophila was the most common species isolated, whereas Aeromonas salmonicida was isolated on only one occasion. Escherichia coli was detected in 36 of the water samples and comprised 4% of the isolates. Streptococcus was also isolated from 24 water samples and all 48 of these streptococcal isolates were identified as belonging to the enterococcus group. Species of Enterobacter and Klebsiella also comprised 5% of the total isolates. Enterobacter cloacae was isolated on 11 occasions from tropical fish water and on five occasions from goldfish water, whereas Enterobacter hafnia and Enterobacter aerogenes were isolated nine times and 11 times, respectively, from goldfish water. The Klebsiella isolates were identified as Klebsiella pneumoniae, and the Proteus isolates included Proteus vulgaris, Proteus morgani, and Proteus mirabilis. Only two of the Staphylococcus isolates were identified as Staphylococcus aureus, whereas nine of the isolates were identified as Staphylococcus epidermidis. The yeast isolates included species of Shizosaccharomyces and Zygosaccharomyces.

Quantitative bacteriological examination.

The results in Table 3 show that goldfish water contained as many as $10^7$ viable mesophilic aerobic bacteria per ml with as many as $5 \times 10^4$ organisms capable of anaerobic growth. The mean counts obtained when plated dilutions of tropical fish water were incubated at 30 and 37°C were significantly higher than the goldfish water. The total viable anaerobic count was also markedly higher in the tropical fish water.

The results of the most probable number assays are shown in Table 4. Countable numbers of coliforms were present in all but one sample each of goldfish water and tropical fish water. Both groups of water samples contained similar numbers of coliforms with the mean completed coliform count being $3 \times 10^4$ per 100 ml. Only five samples of goldfish water contained countable numbers of fecal coliforms, whereas countable fecal coliforms were demonstrated in 12 of the samples of tropical fish water. The mean fecal coliform count was also significantly higher in the water samples containing tropical fish. In addition to the coliforms, countable numbers of Citrobacter were present in all but one of the water samples tested. Both groups of water samples contained similar numbers of Citrobacter, and the mean Citrobacter count from all water samples was 2 $\times 10^5$ per 100 ml.

**DISCUSSION**

The present findings show that the aquarium water supplied with ornamental fish purchased at retail outlets contains significant numbers of a wide variety of bacteria. These bacteria likely originate from the fish and often include coliforms and fecal coliforms in significantly higher numbers than those allowed for recreational and bathing waters in the United States and Canada. A survey of regulations reveals that, depending on the state or province in question, coliform numbers are not to exceed 50 to 5,000 per 100 ml, whereas fecal coliforms are not to exceed 70 to 1,000 per 100 ml (15, 17). These upper limits were exceeded in 28 of the water samples, whereas the fecal coliform levels were exceeded in nine of the water samples. The mean coliform count in the fish water exceeded this upper allowable coliform limit by a factor of

| Assay            | Incubation conditions | No. of estimates | Estimated no. of viable organisms x 10^9/ml | Mean value | Range of values |
|------------------|----------------------|-----------------|--------------------------------------------|------------|----------------|
| Goldfish water   |                       |                 |                                            |            |                |
| Total aerobes    | 20 C, O_2            | 13              | 440                                        | 38 to 8,000|                |
|                  | 30 C, O_2            | 21              | 5,300                                      | 16 to 120,000|               |
|                  | 37 C, O_2            | 20              | 440                                        | 70 to 70,000|               |
|                  | 37 C, AnO_2          | 8               | 31                                         | 3 to 520   |                |
| Total anaerobes  | 37 C, AnO_2          | 8               | 31                                         | 3 to 520   |                |
| Tropical fish water |                   |                 |                                            |            |                |
| Total aerobes    | 20 C, O_2            | 15              | 14,800                                     | 26 to 88,000|               |
|                  | 30 C, O_2            | 18              | 3,739                                      | 8 to 27,000|                |
|                  | 37 C, AnO_2          | 18              | 2,061                                      | 3 to 22,000|                |
| Total anaerobes  | 37 C, AnO_2          | 18              | 2,061                                      | 3 to 22,000|                |
600, whereas the mean fecal coliform count exceeded the allowable limit by a factor of 27.

Significant numbers of Aeromonas, Citrobacter, Enterobacter, enterococcus, and Pseudomonas were often present, whereas Alcaligenes, Flavobacterium, and Vibrio were frequently isolated. This bacterial flora in water containing ornamental fish is similar to the bacterial flora found in water housing small pet green turtles (14, 26). Two genera found in the turtle water but not found in water holding ornamental fish are Salmonella and Arizona. The methods used in this study should have allowed the recovery of both Salmonella and Arizona; however, the large number of H₂S-producing Citrobacter and Enterobacter colonies on the SS agar plates may have effectively masked any Salmonella colonies present. Although Salmonella was not recovered, species that were serologically and biochemically very closely related to Salmonella were isolated, as was Edwardsiella tarda which is capable of producing disease patterns similar to those caused by Salmonella (11). Similarly, although Arizona was not isolated, species that were biochemically very similar to Arizona were demonstrated to be present.

K. pneumoniae and P. aeruginosa were also isolated. Both these species are potential pathogens of man and can be of considerable clinical significance (25). Moreover, there are increasing numbers of reports of human infections in which fluorescent pseudomonads, aeromonads, Citrobacter and Enterobacteria cloacae, and Enterobacter hafnia are implicated (7, 12, 23, 24). These infections include genito-urinary tract infections, enterocolitis and diarrhea in children, bacteremia, and septicemia.

The association of these bacterial species with turtles has been suggested to have considerable public health significance (14). These workers have in fact questioned whether the Salmonella-free certification program alone is sufficient to render these poikilotherms as safe pets. The same may also be true in the case of ornamental fish. Conditions existing in aquaria are ideal for bacterial growth. The bacteria are provided with a liquid menstruum, added nutrients in the form of fish feed, surfaces for colonization, heated water, and aeration. A wide variety of bacterial species capable of causing disease of man are able to grow using commercial fish diets as sole source of nutrients (21). Moreover, some of these fish diets have also been shown to contain potential pathogens of man (21), whereas other studies have shown that human pathogens such as Vibrio cholerae, Vibrio parahaemolyticus, Erysipelothrix rhusiatophaiae, and Leptospira icterohaemorrhagiae are able to survive and multiply in the gut, mucus, and tissues of fish (10). Since bacteria present in an aquarium could readily be transferred to humans, the presence of potential pathogens in the aquarium could obviously present a hazard to human health. This has been shown with aquarium-borne mycobacteria as well as the cases of salmonellosis originating from aquaria housing turtles and tortoises (1, 5, 13). It should be noted that mycobacteria were not isolated in the present study, but this may have been due to the relatively short incubation times and the nonselective media used.

The etiological agents of eye, ear, nose, and throat, gastrointestinal, and genito-urinary infections in humans which could be fish or water borne are rarely identified accurately and more rarely traced to their source (10). It appears that

| Assay                  | Incubation conditions | No. of estimates | No. of positive samples | Most probable numbers × 10⁷/100 ml in positive samples |
|-----------------------|-----------------------|------------------|-------------------------|---------------------------------------------------|
| Goldfish water        |                        |                  |                         | Mean value                                         |
| Completed coliforms   | McC*, 37 C            | 22               | 21                      | 33,000                                            |
| Fecal coliforms       | McC, 44 C             | 23               | 5                       | 10                                               |
| Confirmed enterococci | EVA*, 37 C            | 24               | 11                      | 36                                               |
| Confirmed Citrobacter | Sel*, 37 C            | 11               | 11                      | 28,221                                            |
| Tropical fish water   |                        |                  |                         | 93 to 46,000                                      |
| Completed coliforms   | McC, 37 C             | 17               | 16                      | 25,990                                            |
| Fecal coliforms       | McC, 44 C             | 18               | 12                      | 375                                               |
| Confirmed Citrobacter | Sel, 37 C             | 18               | 17                      | 18,019                                            |

*McC, MacConkey broth.  
*EVA, Ethyl violet azide broth.  
*Sel, Selenite-F broth.
epidemiological studies of the relationship of bacteria in aquaria with clinical conditions in humans is worthy of investigation by public health officials, especially since so little consideration and less research has been devoted to the possibility that fish and aquaria may serve as vectors of human pathogens. For example, the incidence of enteric infections in clerks involved in the sale of aquarium species may be worthy of study. The current means of selling ornamental fish surely represents a unique situation where the public can purchase a mixed bacterial broth culture containing as many as $10^7$ cells per ml and including potential pathogens. The need to establish and enforce regulations similar to those currently in use for the control of turtle-borne disease is evident.

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