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Potential Pathogens in the Environment: Isolation, Enumeration, and Identification of Seven Genera of Intestinal Bacteria Associated with Small Green Pet Turtles[1]

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Bacteriological analyses were performed on fecal swabs and the aquarium water of 27 individually purchased specimens of the small green pet turtle, *Pseudemys scripta elegans*. Representatives of *Aeromonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Proteus*, *Salmonella*, and *Serratia* were isolated. *Enterobacter*, *Klebsiella*, and *Salmonella* were encountered in 20% or more of the specimens, whereas *Aeromonas* was isolated from 63%. *Klebsiella pneumoniae* counts ranged from 10^3 to 10^4 per milliliter of aquarium water, whereas *Aeromonas* routinely exceeded 10^4 per milliliter. *Aeromonas* cultures from turtles were identical to 7 human isolates in some 29 biochemical tests. On the basis of our findings, we question whether the *Salmonella*-free certification program alone is sufficient to render these reptiles as safe pets.

An association of the human pathogen *Salmonella* with reptiles has been a bacteriological fact for over a quarter of a century (14). *Salmonellae* have been isolated from turtles, snakes, and even from lizards (4, 14). Recently, Congress enacted federal legislation to effectively prohibit the interstate shipment of turtles harboring *Salmonella* and *Arizona* (26).

Our interest in the microflora of pet turtles results from an interest in the ecology and taxonomy of *Aeromonas* bacteria. Aeromonads are gram-negative, fermentative, and oxidase-positive bacteria which have classically been associated with diseases in fish, frogs, and reptiles (2). In recent years, these opportunistic pathogens have also been isolated from numerous diseased animals from insects to man (1, 6, 9, 20, 24). During the course of acquiring a collection of *Aeromonas* cultures for ongoing taxonomic studies, one culture was received from R. M. Wood of the California State Department of Public Health. This isolate, designated CPHL in this paper, had been isolated from aquarium water containing a small green pet turtle which we believed to be *Pseudemys scripta elegans*. The owner of the turtle (female, 6 years) had been suffering gastroenteritis for 3 months. Unfortunately, the etiological agent, whether *Aeromonas*, *Klebsiella*, or *Salmonella*, was never determined. Knowledge of this situation precipitated the present survey for further *Aeromonas* isolates from *P. scripta elegans*. In this study we report the high incidence and cell numbers of *Aeromonas* which are commonly associated with this pet. During the survey, we encountered other genera of enteric bacteria and now document their incidence and biochemical properties. This paper was presented, in part, at the Annual Meeting of the American Society for Microbiology (Philadelphia, 23-28 April 1972).

MATERIALS AND METHODS

Sampling procedures. In the present study 27 small green turtles (*P. scripta elegans*) were obtained from separate merchants in California and Oregon. Upon arrival in the laboratory (maximum 48 h from purchase) the anal region of each turtle was rubbed with a sterile swab. Each turtle was placed into a separate sterile aquarium containing 25 to 35 ml of sterile frog Ringer saline. Samples for bacteriological examination, except *Klebsiellae*, were immediately prepared from the swabs or from dilutions of the saline in which the turtle had been immersed. A second saline sample was removed for examination

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after 5 days. Turtles were maintained in the laboratory (23 to 25 C), and, after the original 5 days, nonsterile tap water was used to cleanse aquaria at 7- to 14-day intervals. *Klebsiellae* were isolated and enumerated after turtles were maintained for several months.

Isolation and enumeration of bacteria. Salmonellae were enriched in tetrathionate broth at 41.5 C by using the protocol of Cheng, Boyle, and Goepfert (3). After incubation, a sample of tetrathionate broth was streaked onto Brilliant Green sulfa agar (Difco). Suspect colonies were picked from the Brilliant Green agar and subjected to biochemical and serological examination. It seems worth mentioning that the tetrathionate enrichments often gave rise to high counts of aeromonads and pseudomonads as well.

Attempts to isolate *Enterobacter* and *Klebsiella* were made from 18 turtle specimens. Appropriately diluted saline samples were plated onto the nitrogen-deficient medium of Hino and Wilson (10) but containing 2% mannitol and 5 μg of yeast extract (Difco) per ml. Plates were incubated in an atmosphere containing 80% N_2 and 20% argon. After several days of incubation at room temperature, *Klebsiellae* colonies were recognized by their large, glassy, and mucoid appearance.

Aeromonads were isolated and enumerated on the selective medium, peptone-beef extract-glycogen (PBG) agar. The medium is similar to that of Meeks (19) but contains 0.1 g of bromothymol blue, 0.1 g of sodium laurel sulfate, and 15 g of agar (Difco) per liter. Samples were prepared by the pour plate method and incubated for 4 to 5 h at room temperature. The PBG agar was then overlayed with 2% agar in water (unpublished data). At room temperature incubation, large yellow *Aeromonas* colonies appeared in 3 to 4 days. A detailed study of this selective medium will be published separately (McCoy and Pilcher, personal communication).

Commercial turtle food (three brands) was examined only for the presence of *Salmonella* and *Aeromonas* by using the above procedures. All samples were negative.

Identification of isolates. Biochemical examination of all enteric isolates was accomplished in the standard fashion as recommended in the *Manual of Clinical Microbiology* (1). It was considered essential to run the cytochrome oxidase test to differentiate oxidase-positive *Aeromonas* from oxidase-negative enteric bacteria. A modified Gaby and Hadley procedure was employed (23). In addition to the biochemical characterization, *Salmonella* were partially serotyped using commercially available polyvalent antisera (Difco).

The guanine plus cytosine base composition (% GC) of representative aeromonads was determined by the thermal melting procedure using equation 5 of Mandel et al. for calculations (17). Deoxyribonucleic acid from *Escherichia coli* B with a known base composition of 51% GC was used as a control.

Mouse pathogenicity tests. Adult white mice (20 to 30 g) of both sexes were maintained at room temperature on a 14 h light, 10 h dark cycle. Food was removed 24 h before inoculation and returned 12 h postinoculation. Water was supplied continuously.

Representatives of each group of aeromonads were grown overnight at 30 C in peptone-yeast extract broth (1% peptone, 0.3% yeast extract; Difco). Appropriate dilutions were prepared and 0.2 ml was inoculated intraperitoneally (i.p.) at the end of the light cycle.

RESULTS

Table 1 summarizes the incidences of the most commonly encountered intestinal bacteria, *Aeromonas*, *Enterobacter*, *Klebsiella*, and *Salmonella*. Whereas some 20% of the turtles harbored *Enterobacter*, *Klebsiella*, and *Salmonella*, *Aeromonas* was recovered from more than 60% of the specimens. This large incidence would indicate that the first isolation of *Aeromonas* from this species of turtle by California public health officials was not a unique or unusual situation. Further inspection of the table readily reveals that *Aeromonas* or *Salmonella* was carried by 70% of the specimens.

Occasionally an atypical colony was picked from the *Aeromonas* selecive medium and identified. In this manner, *Citrobacter*, *Proteus*, and *Serratia* were found. Cultures representing two types of *Citrobacter* were encountered. One isolate exhibited the classical biochemical pattern and was readily separable from *Salmonella* on the basis of lactose and sucrose fermentation and the negative decarboxylase reactions. Three atypical indole-positive *Citrobacter* were isolated, and, as in previ-

TABLE 1. *Incidence in turtles of Aeromonas, Enterobacter, Klebsiella, and Salmonella*[a]

Organism	No. of turtles tested	No. of turtles infected[b]
Aeromonas	27	17 (63%)
Enterobacter	18	5 (28%)
Klebsiella[c]	18	4 (20%)
Salmonella[d]	27	5 (20%)

[a] Additional genera identified from atypical colonies appearing on the *Aeromonas* selective medium include: *Citrobacter* (infected turtles, 4 of 27 tested); *Proteus* (infected turtles, 1 of 26 tested); and *Serratia* (infected turtles, 1 of 27 tested).

[b] Numbers in parentheses represent percentage of infected turtles.

[c] Of 18 turtles tested, 3 contained both *Aeromonas* and *Klebsiella*.

[d] Of 27 turtles tested, 3 contained both *Aeromonas* and *Salmonella*.

ous reports, these strains also were ornithine decarboxylase positive (1).

Although we did not isolate or examine the cultures in detail, a fluorescent *Pseudomonas* species was quite commonly observed on the Brilliant Green agar plates following tetrathionate enrichments for *Salmonella*.

Klebsiella pneumoniae cultures exhibited classical biochemical reactions (1). The decarboxylase pattern, urea hydrolysis, malonate fermentation, and lack of motility confirmed their identification. When the *Klebsiellae* were encountered, they were present in significant numbers since colonies picked for identification were taken from the 10^{-3} and 10^{-4} dilutions of aquaria water.

At least 5 of the 27 specimens harbored *Salmonella*. Four of these *Salmonella* agglutinated with O-group C2 antisera and one with B. In our modest sample there appeared to be no correlation between state of origin and the O-group encountered, since two C2 cultures came from each of the states sampled. The group B culture was isolated from a turtle shipped from California.

Two of the C2 *Salmonella* displayed classical biochemical reactions. The two aberrant cultures failed to ferment dulcitol, a trait typical of *Arizona* (1). On the other hand *Arizona* usually ferment malonate and lactose thereby biochemically confirming the *Salmonella* identification.

Our original interest was in the isolation and characterization of *Aeromonas* cultures, and Table 2 compiles the results of this survey. The key identifying characteristics of *Aeromonas* include a positive cytochrome oxidase test and a fermentative attack on carbohydrates, traits also shared with *Vibrio* (1). Differentiation from the latter genus is based on negative ornithine and lysine decarboxylase reactions and deoxyribonucleic acid with 57 to 61% GC, properties characteristic of *Aeromonas*. With the three exceptions noted in the methyl red—Voges-Probauer (MRVP) reaction, all turtle *Aeromonas* isolates reacted essentially the same in 29 biochemical tests as did the clinical cultures. Confirmation on identification of aeromonads representing all groups in Table 2 was obtained from the Center for Disease Control.

Representatives of all *Aeromonas* groups were beta-hemolytic on horse and sheep blood agar, and i.p. injections of 10^7 to 10^8 cells were lethal for mice in 12 to 36 h. One nonhemolytic isolate was not virulent.

By the use of the selective medium, it was possible to estimate the viable count of *Aeromonas* in the aquaria water. Representative counts from eight known positive specimens are compiled in Table 3. We estimate the range of viable counts to be from 10^4 to nearly 10^7 per milliliter. Counts in this range were routinely repeatable even after maintenance of turtles for 5 months in the laboratory.

DISCUSSION

Much attention has been given to the association of *Salmonella* with reptiles, especially with the pet turtle *P. scripta elegans*, as well as various genera of lizards (4, 12, 14). Indeed, *Salmonella* has been isolated from at least 10 genera of turtles including specimens caught in the wild as well as those sampled in zoos (11, 12, 13). The incidence of *Salmonella* for individually contained turtles is about 15 to 20% (12, 14), a range comparable to that reported by us. *Salmonella* have been isolated from turtle intestines, feces, ovaries of adults, and even from eggs (15). In light of these reports we believe that *Salmonella* may either be a member of the normal bacterial flora associated with reptiles, or, at the least, *Salmonella* readily forms a most compatible nondisease-associated relationship with reptiles. In this case we wonder how it would be possible to produce a "certified" *Salmonella*-free shipment of turtles. There is some evidence, however, which might seem to contradict this assertion. A *Salmonella* certification program in Seattle-King Co., Washington, has resulted in a 30-fold decrease in turtle-associated human salmonellosis (16). However, it is clear now that the drop in salmonellosis rates is not due to "pathogen-free" turtles but rather to a decline in the numbers of specimens imported and thus sold in that state.

As recently as 1970 (and at the 1972 Annual Meeting of the American Society for Microbiology) clinical microbiologists were still discussing simplified procedures to identify *Aeromonas* (1, 6, 7), while at the same time others stressed greater awareness of this waterborne potential human pathogen (20, 24). Nothing seems to have been reported, however, on the possible significance of *Aeromonas* in human turtle-associated infections. This is not too surprising since investigators specifically look to implicate *Salmonella* and *Arizona*. Furthermore, without the oxidase test, aeromonads can be easily dismissed as atypical enterics and therefore remain unrecognized.

It is obvious from the literature and our Table 2 that aeromonads possess tremendous

TABLE. 2. *Biochemical characterization of human and turtle-associated Aeromonas*[a]

Isolate	Biochemical Reaction																		
	Indole	MR	VP	Citrate	Urea	β-hemolytic	Mice	TSI Slant	TSI Butt	H_2S	Lactose	Sucrose	Aesculin	Sorbitol	Cellobiose	Salicin	Adonitol	Lys	Arg	Orn
CPHL[b]	+	+	−	+	−	+	+	A	A	−	−	+	−	−	−	−	−	−	+	−
Clinical[c] (6)	+	D	D	+	−	+	+	A	A	−	−	+	−	−	−	−	−	−	+	−
Turtle (14/17)[d]	+	−	+	+	−	+	+	A	A	−	D	+	−	−	−	−	−	−	+	−
Turtle (3/17)[e]	+	+	−	+	−	D	D	A	A	−	D	+	−	−	D	−	−	−	+	−

[a] All cultures are fermentative, motile, oxidase-positive; deoxyribonucleic acid base composition of representative cultures is 57 to 61% GC. Tributyrin and tyrosine (21) refer to reactions resulting in zones of clearing around colonies. Beta-hemolysis was detected on sheep blood agar plates. Mice refers to the death of mice within 36 h from inoculation. All cultures hydrolyzed gelatin, tributyrin, and tyrosine and fermented dextrin, glycogen, and starch. One MR+ *Aeromonas* which was not beta-hemolytic (D) also was not virulent for mice. Abbreviations: MR, methyl red; VP, Voges-Prosbauer; A, acid reaction; D, different reactions.

[b] Isolate received from R. M. Wood, California Public Health Laboratory, Berkeley, Calif.

[c] These six human clinical isolates were obtained from A. L. Rashad, University of Oregon Medical School, Portland.

[d] Of 17 turtles tested, 14 were infected with this type.

[e] Of 17 turtles tested, 3 were infected with this type.

TABLE 3. *Enumeration of Aeromonas from aquaria water containing the pet turtle, P. scripta elegans*[a]

Turtle no.	Dilution	No. of typical colonies per plate	No. of oxidase-positive colonies[b]
1	10^{-5}	25	2 (5)
2	10^{-5}	30	4 (6)
3	10^{-4}	100	4 (4)
9	10^{-2}	100	4 (4)
10	10^{-3}	25	3 (7)
13	10^{-4}	5	4 (4)
15	10^{-4}	100	5 (5)
20	10^{-5}	100	4 (7)

[a] Viable counts were made on *Aeromonas* selective medium. Dilutions were prepared directly from aquarium water which had been changed from 2 to 7 days prior to sampling. Typical colonies refers to the large yellow colonies formed on the selective medium.

[b] Numbers in parentheses represent the number of colonies tested.

biochemical versatility. However, to aid in differentiating aeromonads and to define the limits of their versatility, we have intentionally included five carbon sources which were not fermented by most of our isolates (aesculin, sorbitol, cellobiose, salicin, and adonitol). All aeromonads fermented the polysaccharides dextrin, glycogen, and starch (5).

There is information in the literature indicating a low virulence of *Aeromonas* isolates for mice (5, 22). The mean lethal dose of the most virulent strain studied by Shimizu was 10^6 to 2×10^6 cells injected i.p. (22). Eddy (5) demonstrated that for 12 out of 13 *Aeromonas* isolates, injection of 2×10^9 cells was lethal for mice in less than 48 h. These reported values are comparable to the high cell numbers found in the present study. Our only avirulent *Aeromonas* was nonhemolytic, a property shared with Eddy's single nonvirulent culture.

The viable counts for *Aeromonas* are obviously influenced by the frequency of cleansing the aquarium. The counts reported represent samples taken from 2 to 7 days after a water change. Outside of sewage and in the locale of infections, we know of no other natural habitat containing such high *Aeromonas* counts.

The association of *Enterobacter* and *Klebsiella* with pet turtles may have public health significance. Many reports appear in the clinical and public health literature confirming the dramatic increase in the incidence of genitourinary infections caused by *Enterobacter cloacae* and *Klebsiella pneumoniae* (18, 25). In addition, these bacteria have been implicated in enterocolitis and diarrhea in children and infants (8). In the absence of any specific epidemiological information we do not claim these diseases were ever associated with turtles. However, we do point out this potentially dangerous situation in which children have direct physical contact with turtles and aquarium water.

To the best of our knowledge, *Klebsiella*, *Enterobacter*, and *Serratia* have not previously been reported to be associated with this species of turtle although *Enterobacter hafniae*, *Serratia*, and *Citrobacter* have been isolated from other genera (11). It is biologically interesting that this reptile carries this many representatives of the enteric bacteria which have been classically associated with warm-blooded animals. This association of potential human pathogens is certainly not transient since *Klebsiella*, *Enterobacter*, and *Aeromonas* were readily isolated throughout the time specimens were kept in the laboratory (5 to 7 months).

We know of no studies examining causal relationships between the occasional *Aeromonas* infection (especially in children) and possession of reptiles, especially pet turtles. Such a study might be worthy of future investigations by appropriate public health officials. The same suggestion would be offered for genitourinary and other types of *Klebsiellae* infections. *Aeromonas* and *Klebsiellae* have been described as "opportunistic" pathogens and often act in concert with stress conditions. To have a concentrated focal point containing moderate to high numbers of at least 7 or 8 genera of potential pathogens is, to say the least, an undesirable habitat for human contact.

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