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RECOVERY OF *PASTEURELLA MULTOCIDA* FROM EXPERIMENTALLY-EXPOSED FRESHWATER SNAILS

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ABSTRACT: We determined how long *Pasteurella multocida* could survive in experimentally-exposed freshwater snails. *Physa virginea* were collected from the Sacramento National Wildlife Refuge, Glenn County, California (USA), an enzootic site for avian cholera. Exposure to water containing up to 10^7 *P. multocida* per ml did not produce observable changes or mortality in snails. A minimum of 84 *P. multocida* per snail was necessary for detection among the normal snail bacterial flora. When snails were exposed to *P. multocida* in vials containing 10^7 bacteria per ml, *P. multocida* was detected for up to 72 hours in snails. When uninoculated snails were placed in aquaria containing 10^6 *P. multocida* per ml, *P. multocida* was not detected within the snails; further, *P. multocida* was detected in the water for only 24 hours at this level. Based on these results, we propose that *P. virginea* is not an effective reservoir for *P. multocida*.

Key words: *Pasteurella multocida*, avian cholera, *Physa virginea*.

INTRODUCTION

Pasteurella multocida is a bacterium which causes illness and mortality in humans, domestic animals, and wildlife (Brogden and Rhoades, 1983). Among waterfowl, *P. multocida* causes avian cholera, a virulent disease that can kill affected birds within hours after the appearance of symptoms. Avian cholera has been observed in all major flyways since first being reported in North America in 1944 (Rosen and Bischoff, 1950) and is an important cause of waterfowl mortality (Botzler, 1991). Two reservoirs currently are considered for *P. multocida*, soil and water of enzootic sites and carrier birds; conclusive evidence has not been found for either possibility (Botzler, 1991).

To date, no one has determined the potential of freshwater invertebrates as hosts for *P. multocida* (Botzler, 1991). Other zoonotic bacteria, *Yersinia enterocolitica* and *Listeria monocytogenes*, have been isolated from freshwater snails, including members of the Physidae (Botzler et al., 1973, 1976).

Snails from the genera *Physa* and *Lymnaea*, which can live from 1 to 5 yr (Pennak, 1978), both were found at two enzootic avian cholera sites in California (USA): the Centerville Gun Club, 10 km west of Ferndale (40°35′N, 124°23′W), and the Sacramento National Wildlife Refuge, Willows (39°22′N, 122°09′W).

Pasteurella multocida has been isolated from water for as little as 2 to 3 days following an avian cholera epizootic (Price and Brand, 1984), and up to 30 days in water adjacent to duck carcasses (Titche, 1979). The feeding habits and gas exchange method used by the snails provide a potential for contact with, and assimilation of, bacteria present in the water column. The life histories of freshwater snails found at the Centerville Gun Club and Sacramento National Wildlife Refuge, and the consumption of snails by waterfowl, could implicate snails in avian cholera epizootics.

Our objectives were to determine the maximum concentration of *P. multocida* that caused no observable effects on these snails (Experiment 1), the minimum concentrations of *P. multocida* necessary for detection among natural snail bacterial flora (Experiment 2), the length of time *P. multocida* could be detected in snails after exposure to the bacteria (Experiment 3), and the survival time of *P. multocida* in the water of aquaria containing snails (Experiment 4).

METHODS

Physa virginea was used for all experiments. Snails were collected from October to December in 1988, 1989, 1990, and in January and February 1991, from marshes at the Sacramento National Wildlife Refuge in which high numbers of avian carcasses were collected during an avian cholera epizootic in 1988. The snails were identified according to the keys of Baker (1913), and 24 specimens were deposited at the University of Colorado Museum (UCM No. 37242), Boulder, Colorado (USA). Snails had a mean (±SE) shell length of 11.3 (±0.6) mm, with a range of 5 to 14 mm (n = 30).

Snails were rinsed in dechlorinated tap water and placed in 38-l glass maintenance aquaria to allow them to acclimate to laboratory conditions. The 38-l maintenance aquaria contained a plastic corner filter. Filters used throughout the study contained sterilized river gravel (approximately 2.5 to 10 mm diameter), activated charcoal (First Flight Marineland Aquarium Products, Simi Valley, California), and polyester filter material (Flexifiber, aquarium filter floss, Flexi-Mat Corporation, Chicago, Illinois USA).

All experiments were conducted in 4.5-l glass experiment aquaria with Plexiglas covers into which 30-mm diameter air supply tubing was inserted. The 4.5-l experiment aquaria contained heat- and steam-sterilized dechlorinated tap water, sterilized beach sand, sterilized oyster shell fragments as a source of $CaCO_3$, and a 250-ml plastic filter (Rolf C. Hagen Corp., Mansfield, Massachusetts, USA) using an air-pump system. Water conditions in all experiment aquaria were checked before the addition of snails and monitored weekly thereafter. Measurements included pH, as measured with a Corning Model 10 pH meter (Corning Scientific, Corning, New York, USA), and temperature. Ammonia, nitrite, and nitrate concentrations were measured with a commercial aquarium test kit (TetraWerke). Snails were fed rinsed red-leaf and iceberg lettuce, and TetraMin® commercial fish food (TetraWerke, Melle, Germany) as needed. Lettuce was thoroughly rinsed in tap water before being fed to the snails.

The *P. multocida* strain used in this study was isolated from a snow goose (*Chen caerulescens*) carcass collected in 1987 at the Sacramento National Wildlife Refuge. Stock cultures were checked for purity before each experiment. To develop the stock cultures for inoculations, we inoculated *P. multocida* into 6 ml of Bacto Brain Heart Infusion (BHI) (Difco Laboratories, Detroit, Michigan, USA) broth in a test tube cuvette (Bausch and Lomb, Rochester, New York) and incubated the culture at 37 C. Cultures were mixed and checked for transmittance in a Spectronic® 20 spectrophotometer (Bausch and Lomb), using a red bulb and red-sensitive photocell, until a reading of 47% transmittance was reached. Based on viable counts (Brock et al., 1984), the bacterial rate of reproduction was at a maximum at this transmittance, and the culture contained approximately 2.0×10^8 *P. multocida* per ml. Unwashed *P. multocida* cells were used in the four experiments to minimize damage to the bacterial cells and enhance *P. multocida* survival during the experiments. During the first week of each experiment, *P. multocida* identification was verified for at least one suspect *P. multocida* colony from each sample.

For Experiment 1, concentrations of 10^5, 10^6, 10^7 and 10^8 *P. multocida* per ml each were tested to evaluate possible pathogenic or toxic effects of *P. multocida* on snails. For each concentration tested, five snails were placed in a 60-ml glass bottle containing *P. multocida* diluted in 50 ml sterile, dechlorinated tap water. After 1.5 hr of exposure to *P. multocida*, the snails were removed from the glass bottle, rinsed individually with gentle agitation in a vial containing sterile dechlorinated tap water, placed on a sterile watchglass where excess moisture was removed with a sterile swab, and then placed in a 60-ml glass bottle containing 50 ml sterile, dechlorinated tap water and two 20 x 20-mm squares of lettuce. Three hours after removal from *P. multocida* suspensions, two snails from each exposure concentration again were cleaned by rinsing and tested for the presence of *P. multocida*.

To test for the presence of *P. multocida*, snails were aseptically placed in a test tube with 0.06 to 0.11 g sterile beach sand and 0.1 ml of sterile 0.5% saline and ground with the sand; 0.9 ml saline was added and the entire contents were mixed using a vortex mixer. One-tenth ml of snail suspension from each concentration was spread on a plate of clindamycin agar, containing 500 μg clindamycin hydrochloride (Upjohn Industries, Kalamazoo, Michigan), 6.56 g dextrose starch agar (Difco), 0.003 g Congo red dye (Allied Chemical Company, Morristown, New Jersey, USA), and 100.0 ml distilled water. Inoculated plates were incubated at 37 C and examined for *P. multocida* colonies at 24 and 48 hr using a dissecting microscope and obliquely-transmitted light (Henry, 1933). At least one suspect *P. multocida* colony from each plate was verified as *P. multocida* using the API 20E system (API Analytab Products, Plainview, New York); other Gram-negative bacteria, including those isolated from dead snails, also were identified with the API 20E system.

The three remaining snails at each concentration were observed periodically for 9 days.

Any dead snails were tested for the presence of *P. multocida*. On day 9, all remaining snails were tested for the presence of *P. multocida*.

For Experiment 2, we determined the minimum number of *P. multocida* necessary for detection among the normal bacterial flora of the snails. Five snails were used to test each of six concentrations: 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 *P. multocida* per ml. Any attached egg masses were removed and each snail was rinsed in dechlorinated tap water, blotted on sterile filter paper, wiped clean with a sterile cotton swab, and ground in sand. Each ground snail was combined with 0.1 ml of *P. multocida* suspension and 0.9 ml saline; the contents were mixed and serial dilutions were evaluated on clindamycin agar plates incubated at 37 C.

Suspect *P. multocida* colonies and any new colony types encountered were transferred to clindamycin agar and incubated at 37 C for 24 to 48 hr to obtain single colony isolations. Suspect colonies were verified as *P. multocida*. The lowest detectable number was defined as the lowest concentration used which could be detected in at least three of the five snails inoculated with a given concentration of *P. multocida*.

As a control, we determined whether detectable levels of *P. multocida* occurred in the uninoculated snails. Five snails from the same group but without the addition of *P. multocida* were tested. As a further control, two pooled water samples from each maintenance aquarium, each composed of five 0.2 ml samples, similarly were tested for the presence of *P. multocida*.

For Experiment 3, we determined how long *P. multocida* could be detected in snails after inoculation. Snails were removed aseptically from maintenance aquaria with sterile forceps, cleaned in individual tubes, and placed in 5 ml dechlorinated tap water containing 10^7 *P. multocida* per ml. Viable counts were used to verify *P. multocida* concentrations. Two separate treatments were considered in Experiment 3.

In Treatment 1 of Experiment 3, three snails were placed in each of 50 exposure vials and exposed to 10^7 *P. multocida* per ml for a mean (±SE) of 2.7 (±0.25) hr (range = 2.1 to 3.5 hr). Snails were removed aseptically from exposure vials, cleaned, and systematically distributed among three experiment aquaria.

In Treatment 2 of Experiment 3, 119 snails were cleaned and one snail was placed in each of 119 exposure vials containing 10^7 *P. multocida* per ml. Two 20 by 20-mm pieces of lettuce were added to each exposure vial to encourage active feeding by the snails during exposure. Exposure vials were examined at 30-min intervals to determine if snails were on the lettuce, a presumption of feeding. Snails were seen on the lettuce during at least one examination in 89 (75%) of 119 of the exposure vials. The mean (±SE) exposure time for snails was 3.28 (±0.26) hr (range = 2.0 to 4.5 hr). After exposure to the lettuce and *P. multocida*, snails were cleaned and distributed among three other 4.5-l experiment aquaria.

To estimate the number of *P. multocida* inoculated into the snails, six inoculated snails each from Treatments 1 and 2 were cleaned and tested for *P. multocida* concentrations immediately after inoculation. Snails were removed from exposure vials and ground in sand. Two ten-fold dilutions were prepared from each ground-snail suspension. Numbers of *P. multocida* present in the 12 snails were estimated by viable counts on clindamycin agar after 22 to 30 hr of incubation at 37 C.

As controls, six snails were removed from maintenance aquaria on day 0 of Treatments 1 and 2, cleaned and tested for of *P. multocida*. An additional 120 uninoculated snails were cleaned and 40 of the snails were placed in each of three experiment aquaria. These control snails were maintained and sampled using the same methods as with inoculated snails.

Snails in Treatments 1 and 2 and the controls were tested systematically for *P. multocida*. Two live snails were tested per experimental aquarium on the day of inoculation (day 0); days 1, 3, 5, and 7 of week one; and then twice weekly at 3 and 4 day intervals, respectively, until no snails remained in the aquaria. Results were recorded as the presence or absence of detectable levels of *P. multocida*.

Snails found dead over the first 12 days were tested for the presence of *P. multocida*. After day 12, dead snails were removed, sterilized, and discarded.

For Experiment 4, we determined the length of time *P. multocida* could survive in the water of aquaria containing snails. Fifty snails were placed in each of three 4.5-l experiment aquaria; six additional control snails were tested to ensure that no *P. multocida* was present. *Pasteurella multocida* was grown in stock culture to a concentration of 10^9 *P. multocida* per ml (33% transmittance in the spectrophotometer). The water of the experiment aquaria was inoculated to attain a final concentration of 10^6 *P. multocida* per ml.

On the day of inoculation, two pooled water samples from each experiment aquarium were tested after inoculation. For each sample, we removed two 0.2 ml water samples from the bottom of the experiment aquarium, where snail fecal matter had accumulated, one 0.2 ml sample from the surface of the water, and two 0.2 ml samples from the top surface of the filter. For each experiment aquarium, the five 0.2 ml

samples were combined and mixed. Duplicate 0.1 ml samples from each pooled sample were cultured on clindamycin agar and checked for *P. multocida*.

Two snails from each inoculated aquarium were sampled 3 hr after inoculation. Snails and water were sampled daily from day 1 until day 7, then twice weekly from week 2 through week 4. The last two snails were sampled and the final water samples taken 36 days after inoclation. On each sampling day, two snails and one pooled water sample from each experiment aquarium were tested for detectable levels of *P. multocida*. Results were recorded as the presence or absence of detectable levels of *P. multocida*.

RESULTS

In Experiment 1, exposure to concentrations of 10^5, 10^6 and 10^7 *P. multocida* per ml produced no observable effects on the snails' behaviors. *Pasteurella multocida* was detected in each of the two snails tested from each concentration on day 0. The three remaining snails inoculated at 10^8 *P. multocida* per ml died on day 3 or day 4. *Pasteurella multocida* was not detected in the dead snails. *Pasteurella multocida* was not detected in any snails at the end of the experiment on day 9. Since 10^7 *P. multocida* per ml was the highest concentration not associated with an observable effect on the snails, this concentration was used for inoculations in Experiment 3.

In Experiment 2, *P. multocida* was detected in three of the five snail suspensions inoculated with a mean (±SE) of 84 (±7.2) *P. multocida* (range 71 to 96) and was detected in only one of 14 snail suspensions inoculated with mean (±SE) of 10 (±1.6, range = 7 to 15) or 2 (±0.2, range = 0 to 4) *P. multocida*. Thus, 84 *P. multocida* was estimated to be the minimum number of bacteria required for detection in snails. *Pasteurella multocida* was not detected in any of the five uninoculated snails, nor in the pooled water samples removed from maintenance aquaria.

The estimated dose to which snails were exposed in Treatment 1 of Experiment 3 ranged from 1.1 to 1.5 × 10^7 *P. multocida* per ml (mean = 1.3 × 10^7, SE = 0.98 × 10^6), for 2.7 hr. Based on the viable counts, we detected a mean of 4.0 × 10^4 *P. multocida* (SE = 3.5 × 10^4, range = 3.0 × 10^3 to 1.8 × 10^5) among the six snails tested immediately after exposure. On day 0, *P. multocida* was detected in all six snails sampled. By day 1, *P. multocida* was detected in three of six snails, one snail from each experiment aquarium. On day 3, *P. multocida* was detected in only one of six snails. No *P. multocida* was detected among 48 snails tested between days 5 and 28.

The estimated dose to which the snails fed lettuce in Treatment 2 of Experiment 3 were exposed ranged from 1.3 to 9.5 × 10^7 (mean = 4.3 × 10^7, SE = 0.76 × 10^7) *P. multocida* per ml for 3.3 hr. Based on the viable counts, we detected a mean of 3.8 × 10^4 *P. multocida* (SE = 2.1 × 10^4, range = 1.0 × 10^3 to 1.4 × 10^5) among the six snails tested immediately after exposure. On day 0, *P. multocida* was detected in five of six snails. On day 1, *P. multocida* was detected in four of six snails, including at least one snail from each experiment aquarium. On day 3, *P. multocida* was detected in only one of six snails. No *P. multocida* was detected among 48 snails tested between days 5 and 28.

In Experiment 4, the experiment aquarium water contained a mean of 1.0 × 10^6 (SE = 2.9 × 10^4, range = 0.97 to 1.1 × 10^6) *P. multocida* after inoculation. *Pasteurella multocida* was detected in all three pooled water samples taken on day 0, and again on day 1, but was not detected in any pooled water samples between days 2 and 36. Snails in the experiment aquaria were exposed to a mean of 10^6 *P. multocida* per ml. Since *P. multocida* also was detected in experiment aquarium water samples on day 1, exposure time of snails to these bacteria was at least 24 hr. We sampled 30 snails during the 36 days following inoculation; *P. multocida* was not detected on any day in any snails in Experiment 4.

None of 88 uninoculated control snails individually tested for *P. multocida* among

the four experiments had detectable levels of *P. multocida*. *Pasteurella multocida* was not detected in any snails found dead in any of the experiments. However, *Aeromonas hydrophila* consistently was isolated from dead snails; no other bacteria were detected consistently.

DISCUSSION

For the two treatments of Experiment 3, there was little difference in the results among snails exposed in vials with or without lettuce, even though in Treatment 2, 89 (75%) of the 119 snails were observed on the lettuce and, therefore, probably feeding. Thus, if the bacteria were ingested, probable feeding by the snails on the lettuce did not appear to increase the number of *P. multocida* ingested or the survival time of *P. multocida* in the snails.

Following the addition of snails, the experiment aquaria of Experiment 4 were maintained within the optimal ranges of temperature and pH known for *P. multocida* (Bredy and Botzler, 1989). However, the survival time in water of <3 days for *P. multocida* in Experiment 4 was minimal when compared to previous studies of *P. multocida* survival times as summarized by Botzler (1991).

The recovery of *P. multocida* from snails varied with the *P. multocida* concentration to which they were exposed. Over the first 3 days, *P. multocida* was detected in snails from Experiment 3 exposed to concentrations of 10^7 *P. multocida* per ml. In contrast, *P. multocida* was not detected in any snails from Experiment 4 which were exposed to a concentration of 10^6 *P. multocida* per ml, even though exposure times were a minimum of 24 hr in an experiment aquarium. Thus we believe that a minimum level of 10^7 *P. multocida* per ml is needed to infect snails; this is more than we would expect to occur in contaminated waters during an avian cholera epizootic.

The results of Experiment 3 were similar for both snail treatments. For the combined treatments, *P. multocida* was detected in 11 of 12 snails on the day of exposure, but was detected in only two of 12 snails by day 3. *Pasteurella multocida* was not detected in any samples after day 3. Thus, the rate of disappearance of *P. multocida* from the snails was similar to that with which the bacteria disappeared from water in Experiment 4.

The bacteria recovered from snails in these experiments could have been on the shell surface, in the mantle cavity, on the foot, or ingested by the snails. In some preliminary work, we determined whether the *P. multocida* might be adhering to the external shells of the snails. We exposed five snails to a concentration of 10^6 *P. multocida* and exposed six additional snails to a concentraction of 10^7 *P. multocida*; we then carefully swabbed their shells after they had been cleaned by our standard procedure. We were able to detect *P. multocida* on only one of the 11 snails (S. Miller, unpubl.). Thus, it seems likely that most bacteria recovered in these experiments were in the snail rather than on the shell. We did not attempt to distinguish ingested bacteria from those that might be associated with the mantle or the foot.

However, independent of whether the bacteria were ingested or associated with some other part of the snail, it was evident that *P. multocida* did not become established in the snails, despite the artificially high concentrations of *P. multocida* to which the snails were exposed. Based on our results, we propose that *P. multocida* cannot survive or multiply in these snails and that when infected, *Physa virginea* retain the bacteria for less than 5 days.

While *Physa virginea* may not serve as a stable reservoir for *P. multocida*, the snails may be important in the transmission of bacteria once an epizootic has started; 24 hr after exposure in Treatments 1 and 2 of Experiment 3, *P. multocida* still was detected in over half of the snails sampled. During preliminary trials for Experiment 2, we noted that very high numbers of *P. multocida* bacteria adhered to snail egg masses (data not shown). Because we

could not control for the presence or size of the egg masses in the various experiments, they were removed prior to inoculations. However, the possible role of snail egg masses in maintaining *P. multocida* should be explored further.

Snails feeding in the vicinity of carcasses shedding *P. multocida* could become infected with bacterial numbers sufficiently high that the snails might maintain the bacteria for 2 or 3 days and infect other wildfowl. *Pasteurella multocida* has been isolated from water associated with carcasses from avian cholera mortalities (Price and Brand, 1984). *Physa virginea* is capable of moving rapidly in water, apparently using air in the mantle cavity; snails can travel up to 1 m in under 5 sec (S. Miller, unpubl.). While the role of transmission by snails during an extensive avian cholera epizootic seems minimal, snails could enhance transmission of *P. multocida* after the arrival of a bird infected with *P. multocida* in an area. If the bird died, snails could provide a mechanism for initial transmission to other birds and thus enhance the potential for an epizootic.

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