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Pathogenicity of *Aeromonas hydrophila*, *Klebsiella pneumoniae*, and *Proteus mirabilis* to Brown Tree Frogs (*Litoria ewingii*)

Ermin Schadich* **and Anthony LJ Cole**

Bacterial dermatosepticemia, a systemic infectious bacterial disease of frogs, can be caused by several opportunistic gram-negative bacterial species including *Aeromonas hydrophila, Chryseobacterium indologenes*, *Chryseobacterium meningosepticum*, *Citrobacter freundii, Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa,* and *Serratia liquifaciens*. Here we determined the pathogenicity of 3 bacterial species (*Aeromonas hydrophila, Klebsiella pneumoniae,* and *Proteus mirabilis)* associated with an outbreak of fatal dermatosepticemia in New Zealand *Litoria ewingii* frogs. A bath challenge method was used to expose test frogs to individual bacterial species (2×10^7 cfu/mL in pond water); control frogs were exposed to uninfected pond water. None of the control frogs or those exposed to *A. hydrophila* or *P. mirabilis* showed any morbidity or mortality. Morbidity and mortality was 40% among frogs exposed to *K. pneumonia,* and the organism was reisolated from the hearts, spleens, and livers of affected animals.

Bacterial dermatosepticemia is a fatal disease of frogs. Its epizootic forms are associated with mass mortalities in wild populations[1,15,19] and significant losses in captive populations.[11,17,20,31] Various opportunistic gram-negative bacterial species have been isolated from diseased frogs including *Aeromonas hydrophila*, *Chryseobacterium indologenes*, *Chryseobacterium meningosepticum*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa,* and *Serratia liquefaciens*.[9,11,17,26] These bacterial species can be found among the microbiota of skin and intestine of healthy frogs but are also known to cause disease.[12,14,21] Disease development depends on the virulence of the organism, environmental factors, stress, dietary factors, health, and immune status of the animal.[12,21,22,24]

The pathogenicity of bacterial pathogens typically is evaluated by using pathogenicity models, which aim to reproduce disease in vivo by challenging the selected host with bacterial isolates. These models enable disease to be monitored and are essential in the comparison of pathogenicity of different bacterial isolates.[21] In addition, they can be used to evaluate efficacy of prophylactic and chemotherapeutic treatments,[16] investigate the relationship between environmental factors and bacteria in the etiology of disease,[16] and assess the function of immune defences.[36]

Two modes of bacterial challenge are used in frog pathogenicity models: injection and bath challenge. Injection of pathogens is used widely for analyses of pathogenicity of human and animal bacterial isolates, but its main drawback is that bacteria are introduced in a manner that does not reflect the natural mode of infection.[21,34] Feeding frogs with various bacterial pathogens might resemble a more natural mode of infection but does not lead to disease.[9] Under experimental conditions, immersion of frogs in a bacterial pathogen suspension results in disease and appears to more closely simulate the natural mode of infection because none of the natural immune defense barriers are bypassed.[21]

The requirements for proof of pathogenicity as stated by Koch (1883) are somewhat rigid, and the influences of environmental factors are not considered, although they are important in the development of bacterial disease of frogs. Despite such limitation, Koch postulates are still a standard by which association of bacteria with disease can be established.[32]

Three bacteria were isolated from the hearts of brown tree frogs (*Litoria ewingii*) during a severe mortality outbreak in Oxford Forest, Canterbury, New Zealand. The organisms were identified as *A. hydrophila*, *K. pneumoniae*, and *P. mirabilis*.[26] The aim of the present study was to assess the pathogenicity of isolates of *A. hydrophila*, *K. pneumoniae*, and *P. mirabilis* collected from wild *L. ewingii* frogs.

Materials and Methods

Frogs. Thirty adult *L. ewingii* (2.0 to 2.5 g) were collected by net scoops from Oxford Forest, Canterbury, New Zealand in January 2008; 20 frogs were used in the described experiments, whereas the remaining 10 frogs were used in the captive breeding program. Frogs were housed individually in tanks (560 × 340 × 230 mm, high-density polyethylene) that were placed in a vivarium having a thermostatically controlled temperature of 23 °C and a 12:12-h light:dark period. The lids of these tanks had the holes to allow water to drip constantly and outlets at the bottom of the tanks, allowing water to flow. Aged, nonchlorinated, filtered municipal water from underground aquifers (19.8 °C, ± 0.2 °C) was used. The frogs' diet included fruit flies (*Drosophila melanogaster*) and house flies (*Musca domestca*). One month before experimentation, they were transferred to 7-L polypropylene box tanks with a

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School of Biological Sciences, University of Canterbury, Christchurch, New Zealand
**Corresponding author. Email: ermins@gmail.com*

snap lid (350 × 200 × 100 mm; Sistema, Penrose, Auckland, New Zealand). The lids of the containers had drilled ventilation holes. Each box contained 2 dry Hygenex paper towels (SCA Hygiene Australia, Auckalnd New Zealand) and 2 that were moistened with aged water; all paper towels were changed weekly. In addition, a small glass (height, 55 mm; diameter, 95 mm) containing water was put into each box to provide an additional source of water to maintain humidity at approximately 80%. Approval for animal procedures (AEC 2007/45R) was granted by the University of Canterbury Animal Ethics Committee.

Bacterial cultures. Heart isolates of *A. hydrophila*, *K. pneumoniae*, and *P. mirabilis* were collected from septicemic *L. ewingii* frogs taken from a site in Oxford Forest in December 2005 at the time of a severe mortality outbreak.[26] The isolates were stored on cryobeads at –80 °C. Prior to experimentation, they were grown aerobically in 10 mL tryptone soya broth at 30 °C overnight and then 20 µL broth culture was spread on tryptone soya agar plates and incubated as the broth cultures.

Calibration curve for bacterial cultures. A colony from overnight growth on tryptone soya agar was used to inoculate 10 mL tryptone soya broth. Bacterial cells were harvested by centrifugation at 4000 × *g* at 4 °C for 15 min and resuspended in sterile saline (0.85% NaCl). The optical density (at 610 nm) of bacterial suspension was adjusted with sterile saline to yield suspensions with optical densities of 1.2, 1.0, 0.8, 0.6, 0.4, 0.2, and 0.1. A sample was taken from each suspension to estimate the number of colony-forming units by overnight incubation on tryptone soya agar. The number of colony-forming units was plotted against optical density to generate a calibration curve. The regression equation obtained from the calibration curve was used to estimate the number of bacteria in the broth cultures.

Preparation of bacterial challenge inocula. Cells from an overnight bacterial culture were harvested by centrifugation at 4000 × *g* at 4 °C for 15 min and resuspended in sterile saline. The optical density of bacterial suspensions was measured and the number of colony-forming units per1 mL of suspension was estimated by using the calibration curves. Bacterial suspensions were diluted with sterile saline to give a final concentration of 2×10^8 cfu/mL; this suspension was used as the inoculum.

Bacterial baths. We used 3 experimental groups of 5 frogs each to test 3 different pathogens: *A. hydrophila*, *K. pneumoniae*, and *P. mirabilis*. Another group of 5 animals was used as controls. Two successive short baths were performed instead of an overnight bath, which could disturb osmoregulation in arboreal species like *L. ewingii*. Frogs were put into individual 500-mL tissue culture jars (the lids had drilled ventilation holes) containing 300 mL autoclaved artificial pond water, 10% Holtfreter solution (60 mM NaCl, 0.67 mM KCl, 0.90 mM $CaCl_2$, 2.40 mM $NaHCO_3$), with bacteria at a concentration of 2×10^7 cfu/ mL and were monitored during a 40-min bath. This bacterial concentration does not exceed that found in ponds during disease outbreaks.[13,19] To check that the challenge dose was at the correct concentration, 1 mL of the resulting pond water was taken from each container for a viable cell count on tryptone soya broth agar. Frogs were allowed to rest for 30 min in empty 7-L tanks before undergoing a second 40-min bath exposure to bacteria. Control animals were exposed individually to pond water only. After bacterial exposure, the animals were returned to their living containers and their health was monitored for 3 wk.

Euthanasia of animals. Diseased animals were euthanized by using 3% chloral hydrate.[33] Each animal was placed in a container with chloral hydrate solution covering its floor to a depth of 5 mm. This method causes death of small poikilothermic animals like frogs in a few minutes, and the animal dies in a relaxed state.[33] Small pieces of heart, spleen, and liver tissue (approximately 0.5 mg) were taken and cultured in tryptone soya broth (Oxoid, Basingstoke, UK). All cultures were incubated aerobically for 24 h at 30 °C. At the end of experimentation, all animals were euthanized and samples of internal organs taken for bacterial culturing.

Identification of bacteria. Bacteria from internal organs were identified by using a commercial system (API 20E, Biomérieux, Marcy-L'etoile, France) containing 23 conventional biochemical tests and 4 supplementary tests: analysis of motility, growth on MacConkey agar, oxidation of glucose, and fermentation of glucose.

Results

None of the control frogs or those exposed to *A. hydrophila* or *P. mirabilis* ($n = 5$ per group) showed any signs of morbidity or mortality during the 3 wk after bath exposure to the bacteria. Among the 5 frogs challenged with *K. pneumonia*, 2 became fully symptomatic for disease 7 d after exposure, and the final morbidity and mortality was 40%. Both frogs had all 3 gross clinical signs of disease including hemorrhages, ulcers, and redness of the ventral skin area. *K. pneumonia* was isolated as a pure culture from the hearts, spleens, and livers of both affected animals. The remaining animals in the *K. pneumoniae* group remained healthy until the end of the study. Samples from the internal organs of all healthy frogs were negative for bacterial growth.

Discussion

Results from this study demonstrated the pathogenicity of *K. pneumoniae* toward *L. ewingii*. *K. pneumoniae* was isolated from diseased *L. ewingii*, grown in pure culture, induced septicemic disease in healthy individuals, and was reisolated, thus fulfilling all required Koch postulates for confirmation of pathogenicity.

The septicemic disease that occurred in infected frogs progressed slowly, becoming fully developed only day 7 after bath challenge. This pattern confirms previous results,[21] in which exposure of *R. rugulosa* frogs to *Aeromonas* spp. through bacterial baths at 10^6 cfu caused slowly progressing disease that was fully developed 6 d after exposure. In contrast, injection of *Aeromonas* caused a rapid septicemic disease and death within 24 h.[21] Slow progression of disease after bath challenge may reflect the extended time necessary for developing skin infections before systematic bacterial dissemination.[21] Prolonged time also is required for disease development in various frog species after bath challenge with the fungal pathogen *Batrachochytrium dendrobatidis*[35] and in European eels exposed to various bacterial pathogens through bath exposure.[8] Through bath exposure, pathogens are introduced in a manner by which none of the immune defenses are bypassed; the multiple and varied contributions from these diverse immunologic responses may synergize to delay progression of disease.

Although pathogenic for frogs, *K. pneumoniae* is not a frog-specific pathogen. This bacterium has been associated with disease outbreaks in humans and other animals including birds, civets, dogs, monkeys, horses, sea lions, and cows[2,5,7,18,25] Mammalian

species have been described to become infected through exposure to contaminated water or soil, and the bacterium can survive for 2 mo in the environment.[5,7,18,25] The ability of mammals to resist infection depends on different mechanisms of innate immunity, because the adaptive immune system cannot provide a sufficiently rapid, efficient response to a high number of invading pathogens.[6,39] The observation that *L. ewingii*, the only introduced *Litoria* species that does not have active skin antimicrobial peptides,[26,27] is susceptible to disease both in the wild[26] and after bath challenge supports a role for the innate immune defenses of other frog species in protection from *K. pneumoniae*. In particular, one study[28] showed that the resistance of *Litoria raniformis* to disease after bath challenges with *K. pneumoniae* correlated with the antimicrobial activity of skin antimicrobial peptides against this bacterium. Furthermore, the antimicrobial activity of skin antimicrobial peptides against *B. dendrobatidis* in various Australian and Central American frog species correlates with resistance to disease caused by this fungus.[35-38] Other effective components of innate immunity against bacterial pathogens are complement and the macrophages of the large lymph sinus under the skin.[12,30]

Pathogenicity of the *A. hydrophila* and *P. mirabilis* isolates that did not cause disease in the present study cannot be ruled out because bath challenge without skin scarification might underestimate pathogenicity. Skin scarification is required for demonstration of pathogenicity of some frog bacterial isolates.[29] We tested pathogenicity at the concentration of 2×10^7 cfu/mL, a level shown by previous studies[9,21] to be appropriate. The morbidity and mortality of *Rana rugulosa* frogs exposed to bacterial pathogens at 1×10^8 cfu/ mL were much higher than those of animals exposed 1×10^6 and 1×10^7 cfu/mL.[21] Similar concentration-dependent pathogenicity was observed in various fish species exposed to bacterial pathogens through bath challenge.[23] Therefore a more sensitive experimental design that included skin scarification of tested frogs and multiple concentrations of challenge bacteria would be required to rule out the possibility that the *A. hydrophila* and *P. mirabilis* isolates we tested are not potentially pathogenic.

Environmental stressors have profound and diverse effects on the immune defenses of frogs. The inhibitory effects of these stressors on resistance to disease have been demonstrated in many different studies.[3,4,10,12] Low temperature was one of the most significant stressors that increased morbidity and mortality of frogs after bath challenges with *A. hydrophila.*[29] In the present study, *L. ewingii* was maintained at 23.5 °C both before and after bacterial bath challenge, and this temperature level corresponded to the environmental temperatures observed during disease outbreaks.[13,26] However, the morbidity and mortality of *Rana tigrina* after bath challenges with *A. hydrophila* was increased significantly by lowering the environmental temperature to below 20 °C.[29] Environmental temperatures lower than 20 °C have a profound inhibitory effect on various immune responses of frogs,[10,35] and this effect could account for increased susceptibility to disease among frogs that undergo bath challenge with pathogens.

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