Bulgarian Journal of Veterinary Medicine, 2021, **24**, No 1, 159−163
ISSN 1311-1477; DOI: 10.15547/bjvm.2019-0054

Short communication

SIMILARITIES BETWEEN SKIN CULTURABLE BACTERIAL SPECIES OF POOL FROGS (*PELOPHYLAX LESSONAE*) AND THEIR HABITAT

F. P. NOCERA, A. DE FILIPPIS, N. PISCOPO, L. ESPOSITO & L. DE MARTINO

Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Naples, Italy

Summary

Nocera, F. P., A. De Filippis, N. Piscopo, L. Esposito & L. De Martino, 2021. Similarities between skin culturable bacterial species of pool frogs (*Pelophylax lessonae*) and their habitat. *Bulg. J. Vet. Med.*, **24**, No 1, 159−163.

The aim of the present study was to investigate the culturable microflora of pool frogs (*Pelophylax lessonae*) and their belonging aquatic environment. A total of 60 samples (56 frog cutaneous swabs, 4 water samples) were inoculated onto different selective and differential agar plates to isolate Gram-positive and Gram-negative bacteria or yeasts. Microbial investigation of the water hosting frogs was also performed. Isolates were identified by API system and their antibiotic resistance profiles were evaluated by disk diffusion method on Mueller Hinton agar plates. *Aeromonas hydrophila* and *Enterococcus durans* were detected in almost all collected samples. Many of the bacterial isolates showed multidrug-resistant profiles. Importantly, this study highlights that skin frog microbiota is correlated to the belonging environment, and, moreover, some isolated bacterial strains resulted to be of interest in animal and public health, since the park was frequented by visitors of all ages.

Key words: antibiotic resistance, frog, skin microbiota, water environment

Skin is the first line of defense against pathogenic microorganisms and the intimate contact between the epidermis and microbes has been already studied. Commonly, the skin flora is nonpathogenic, and either commensal (not harmful to the host) or mutualistic (offers a benefit). The skin microbiota can protect from transient pathogenic organism's colonisation either by competing for nutrients, secreting chemicals against them, or stimulating the skin's immune system (Cogen *et al*., 2008).

It has been reported that, in a host species-specific manner, amphibian skin may select for microbes that are generally in low abundance in the environment (Walke *et al*., 2014). Researchers in USA have recently identified symbiotic bacteria living on amphibians' skins that protect them from the deadly fungal diseases (Muletz-Wolz *et al*., 2017). The cutaneous microbiota of amphibians can be defined as a biological component of protection, since it can be composed of bacteria that produce antimicrobial compounds (de Assis *et al*., 2017). The rich microbial community harboured on amphibian skin, thus, represents a complex ecosystem which constantly interacts with the environment and host factors that influence colonisation (Rollins-Smith *et al*., 2011).

Here, we investigated, by bacterial growth on selective and differential media agar plates, the skin microbiota of pool frogs correlated to their aquatic environments located in Calabria Region, Italy.

The pool frog (*Pelophylax lessonae*) is a European frog protected by the Biodiversity Action Plan (BAP) which is an internationally recognised programme addressing threatened species and habitats, and it is designed to protect and restore biological systems (Glowka *et al*., 1994). Typically, the pool frog resides in small water pools and prefers places surrounded by woodlands. This research aims to build a basic ecological foundation to better understand the role of culturable microbial communities in frog and their specific habitats. Frog specimens, precisely swabs were collected from back, abdomen, gullet and throat in April 2015 from frogs living in two different habitats, a natural bath and a pool, near the Angitola lake in the Regional Natural Park of Serre (Vibo Valentia, Calabria, Italy). The capture was carried out during the project Angitola FISH2O (EFF Calabria Cod. 02/BA/12 – authorised June 28, 2013 prot. n. 756). All procedures were carried out before the actual Italian law (DgLs 4 marzo 2014, n. 26) but in total accordance with the guidelines of the current European Directive (2010/63/EU) related to care of animals used for scientific purposes. For sampling, the capture and handling of *Pelophylax lessonae* were conducted by hand using fresh latex gloves for each frog. Additionally, all animals were in good health at clinical examinations. Sterile cotton-tipped swabs were drawn across the selected anatomical sites of each frog and immediately transferred to Amies transport medium (Oxoid Ltd, UK), then maintained at 4 °C (not longer than 24 h) until processing.

A total of 60 samples (56 frog cutaneous swabs and 4 water samples) were processed at the Microbiology Laboratory of the Department of Veterinary Medicine and Animal Production, University of Naples Federico II (Italy). All samples were plated on blood agar base supplemented with 5% sheep blood, a selective medium used for the isolation of Gram-positive microorganisms, on mannitol-salt agar, a selective medium to identify staphylococci, and on MacConkey agar, a selective and differential medium for the growth of Gram-negative bacteria, which were all incubated aerobically at 37 °C for 24–48 h. Furthermore, the presence of yeasts on skin was also investigated by incubation in the dark at 30 °C for 3 days. The used agar plates were all produced by Oxoid Ltd, UK.

Bacteria were identified by macroscopic observation of the colonies, Gram staining, standard laboratory methodologies (catalase, staphylocoagulase tube test, aesculin), and miniaturised biochemical tests API system (bioMérieux SA, Marcy L'Etoile, France). The species identification by miniaturised biochemical tests was accepted when probability was > 88%.

The isolates were tested for susceptibility to 16 antibiotics by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar incubated at 37 °C, as recommended by the principles described in the Clinical and Laboratory Standards Institute standard method for veterinary pathogens (CLSI, 2015). Discs of amoxicillin/clavulanic acid (AUG 30 µg), ampicillin (AMP 10 µg), cefoxitin (FOX 30 µg), ceftiofur (EFT 30 µg), ceftriaxone (CRO 30 µg), ceftazidime (CAZ 30 µg), doxycycline (DO 30 µg), erythromycin (E 15 µg), gentamicin (CN 10 µg), kanamycin (K 30 µg), tetracycline (TE 30 µg), penicillin G (P 10 UI), enrofloxacin (ENR 5 µg), streptomycin (S 10 µg), trimethoprim/sulfamethoxazole (SXT 25 µg) and vancomycin (VA 30 µg) were tested.

By means of the culturing methods, the skin frog microbiota from tub frogs was the same independently from the different sampling sites such as back, abdomen, gullet and throat; precisely, few colonies of *Pantoea* spp., *Acinetobacter baumannii*, *Aeromonas hydrophila* and *Enterococcus durans* were isolated and identified from the different skin districts with no important change in the concentration of each bacterial population. Instead, *Aeromonas hydrophila*, *Enterococcus durans* and *Citrobacter freudii* were detected from the different sampling skin sites of frogs living in pool. The half of skin frog swabs was positive for *Penicillium* spp. yeasts with a higher and significant presence in the tub frogs than the pool frogs.

The different aquatic habitat influenced the composition of the cutaneous microbiota of the frogs, in fact dominant bacterial population on tub frog skin including, *Aeromonas hydrophila* and *Enterococcus durans*, were detected in all the collected samples including tub water samples. The most frequent bacterial isolates from water tub samples, in duplicates of 4 samples, were *Aeromonas hydrophila* (51%; 95% confidence interval [CI]: 40.86–61.06%) and *Acinetobacter baumannii* (26%; 95% CI: 17.97–35.90%). No bacterial and fungal growth was revealed from pool water.

Only from an aquatic environment, both from skin and water samples, the opportunistic pathogen *A. baumannii* was isolated. This last bacterial isolate deserves special attention, since this bacterium has been often isolated from human clinical samples. This bacterial species is generally responsible of nosocomial infections, that are difficult to treat for its documented increased resistance to antibiotics (Howard *et al.,* 2012). Indeed, recent reports indicate that *A. baumannii* has also evolved into a veterinary nosocomial pathogen and, however, it cannot be excluded that animals may occasionally play a role as reservoir for *A. baumannii* (van der Kolk *et al*., 2019).

A. hydrophila, present in all the samples examined, is an aquatic microorganism that is able to produce cytotoxins and enterotoxins associated with acute gastroenteritis and wound infections in human, and haemorrhagic septicaemia in fish, reptiles and amphibians (Fernández *et al.,* 2000). Experiments on pathogenicity of *A. hydrophila* demonstrated that frogs exposed to these bacteria showed no morbidity or mortality (Schadich & Cole, 2010).

Furthermore, both *A. hydrophila* and *A. baumannii* were present in skin samples of frogs from tub, as well as *E. durans*, a commensal member of the normal intestinal flora of humans and animals used also as indicator of hygienic quality of water (Ashbolt *et al*., 2001), and revealed in low percentage, herein, also in tub water samples.

Additionally, *A. viridans*, was only isolated from the tub water samples, while it was not found in the skin samples of frogs living in the tub. *A. viridans* has been associated with different human infections and different animal clinical specimens, such as crustaceans, sea turtles, pigs and cow's milk (Martin *et al.,* 2007).

Penicillium spp., the only yeast identified in our samples, represents the most common fungus observed worldwide that only occasionally is agent of human and animal mycoses (Guevara-Suarez *et al*., 2016).

It was also noticed that the resident bacteria on frogs' skin were the same present in their own aquatic environment, except for the pool water sample containing chlorine able to inhibit the bacterial growth. Chlorine is by far the most commonly used antimicrobial agent and it is known that, even in very low levels, can show high antimicrobial activity.

The present work revealed that a large proportion (80–100%) of the isolates from skin of the tub and pool frogs was resistant to amoxicillin/clavulanic acid, ampicillin, gentamicin and tetracycline, while all the isolates showed high susceptibility (100%) against trimethoprim/sulfamethoxazole and vancomycin. The isolates from tub water showed higher percentages of resistance to amoxicillin/clavulanic acid, ampicillin, cefoxitin, doxycycline and gentamicin.

Our results about microbial resistance, that is of crucial significance to public health, have important implications and deserve considerable attention for the park environment where samplings were made. It is important to continuously monitor antibiotic-resistant bacteria because resistance may be spread among bacteria presenting on aquatic animals and in the water that hosts them.

Taken together, these results suggest that frog skin microbiota is susceptible to the belonging environment, the adopted chlorine treatment works well to sanitise water pool and several isolated bacterial strains resulted to be of interest in animal and public health. The percentage of multidrug resistant strains was high, and more studies are needed to understand this dissemination. However, in order to better characterise the interaction between animal and habitat, future research is required. The implications of this research are stimulating and they suggest that monitoring the skin-associated bacteria may provide a useful way to define the health of these animals and inferences with their habitat, also in relation to health of humans as visitors and insiders.

REFERENCES

Ashbolt, N. J., W. O. K. Grabow & M. Snozzi, 2001. Indicators of microbial water quality. In: *Water Quality: Guidelines, Standards and Health,* eds L. Fewtrell & J. Bartram, IWA Publishing: London, UK.

Clinical and Laboratory Standards Institute (CLSI), 2015. Performance Standard for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, 3rd edition CLSI VET01S (Wayne, Pennsylvannia, Clinical Laboratory and Standards Institute).

Cogen, A. L., V. Nizet & R. L. Gallo, 2008. Skin microbiota: A source of disease or defence? *British Journal of Dermatology*, **158**, 442–455.

de Assis, A. B., C. C. Barreto & C. A. Navas, 2017. Skin microbiota in frogs from the Brazilian Atlantic Forest: Species, forest type, and potential against pathogens. *PLoS One,* **12**, e0179628.

Fernández, M. C., B. N. Giampaolo, S. B. Ibañez, M. V. Guagliardo, M. M. Esnaola, L. Conca, P. Valdivia, S. M. Stagnaro, C. Chiale & H. Frade, 2000. *Aeromonas hydrophila* and its relation with drinking water indicators of microbiological quality in Argentine. *Genetica*, **108**, 35–40.

Glowka, L., F. Burhenne-Guilmin, H. Synge, J. A. McNeely & L. Gündling, 1994. Guide to the Convention on Biodiversity. IUCN

Guevara-Suarez, M., D. A. Sutton, J. F. Cano-Lira, D. García, A. Martin-Vicente, N. Wiederhold, J. Guarro & J. Gené, 2016. Identification and antifungal susceptibility of penicillium-like fungi from clinical samples in the United States. *Journal of Clinical Microbiology*, **54**, 2155–2161.

Howard, A., M. O'Donoghue, A. Feeney & R. D. Sleator, 2012. *Acinetobacter baumannii*: An emerging opportunistic pathogen. *Virulence*, **3**, 243–250.

Martin, V., A. I. Vela, M. Gilbert, J. Cebolla, J. Goyache, L. Dominguez & J. F. Fernández Garayzabal, 2007. Characterization of *Aerococcus viridans* isolates from swine clinical specimens. *Journal of Clinical Microbiology*, **45**, 3053–3057.

Muletz-Wolz, C. R., G. V. DiRenzo, S. A. Yarwood, E. H. Campbell Grant, R. C. Fleischer & K. R. Lips, 2017. Antifungal bacteria on woodland salamander skin exhibit high taxonomic diversity and geographic variability. *Applied and Environmental Microbiology*, **83**, 1–13.

Rollins-Smith, L. A., J. P. Ramsey, J. D. Pask, L. K. Reinert & D. C. Woodhams, 2011. Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. *Integrative and Comparative Biology*, **51**, 552–562.

Schadich E. & A. L. Cole, 2010. Pathogenicity of *Aeromonas hydrophila*, *Klebsiella pneumoniae* and *Proteus mirabilis* to brown tree frogs (*Litoria ewingii*). *Comparative Medicine*, **60**, 114–117.

van der Kolk, J. H., A. Endimiani, C. Graubner, V. Gerber & V. Perreten, 2019. Acinetobacter in veterinary medicine with emphasis on *A. baumannii*. *Journal of Global Antimicrobial Resistance*, **16**, 59–71.

Walke, J. B., M. H. Becker, S. C. Loftus, L. L. House, G. Cormier, R. V. Jensen & L. K. Belden, 2014. Amphibian skin may select for rare environmental microbes. *The ISME Journal*, **8**, 2207–2217.

Paper received 15.04.2019; accepted for publication 29.06.2019

Correspondence:

Francesca Paola Nocera
Section of Infectious Diseases
Department of Veterinary Medicine and Animal Production
Via F. Delpino, 1
I – 80137 Naples, Italy
e-mail: francescapaola.nocera@unina.it