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Microbiological and histological studies of farmed-bullfrog (*Rana catesbeiana*) tissues displaying red-leg syndrome

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Abstract

Rana catesbeiana is one of the delicacies of international gastronomy. Farming operations often lead to an increased risk of diseases and mortality. The present work addresses microbiological and histological studies in *R. catesbeiana* with red-leg syndrome (RLS), infection that causes significant economic losses in hatcheries. Partial phenotypical identification demonstrated that the microbial populations isolated from the skin of fattening phase animals and freshwater samples during the autumn (June) are grouped into the following taxa: *Lactobacillus* spp, *Pediococcus* spp, *Micrococcus* spp, *Enterococcus faecalis*, *Ent. faecium* and Enterobacteriaceae (*Enterobacter* spp and *Proteus vulgaris*). Microbial infection on target organs (liver/spleen) and blood showed the presence of *Pr. vulgaris*, *Staphylococcus aureus* and *Enterococcus* strains. Histological studies of skin ulcerations showed epithelial necrosis, diskeratosis, apoptosis and espongiosis. No sporangia associated with chitridiomycosis were observed. The dermis presented oedema, dilated vascular light, fibrin-leucocytic exudates and distortion of serous and granular glands. The liver showed centrolobullar necrosis and a decrease in melanin containing cells. The spleen presented wide areas of septic infarct.

This paper reports the presence of lactic acid bacteria and other genera in the skin and freshwater from farmed *R. catesbeiana* during the autumn and a correlation between microbial infection and structural changes in tissues of bullfrogs with RLS. The severity of the structural changes is related to the level of microbial infection in the target organs and could be sustained by the isolation of *Pr. vulgaris* and other pathogens.

Keywords: *Rana catesbeiana*; Skin microbial population; Red-leg syndrome; Histological evaluations

1. Introduction

Aquaculture is one of the fastest-growing sectors of agriculture, having grown at an annual rate of almost 10% from 1984 to 1995; compared with 3% for

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livestock and 1.6% for capture fisheries production (Rana, 1997).

Rana catesbeiana meat is one of the delicacies of international gastronomy that has increased worldwide consumption. Moreover, other products such as the liver, gut and skin are required for other industries. Although Thailand and Taiwan are the main countries involved in *R. catesbeiana* production and export, other countries such as France, Belgium and Holland are also major importers. In Latin America, Brazil is the most important producer and its rana's crop is mainly exported to Chile and Argentina, countries that have available only 10% of their national requirements (Texeira et al., 2002).

The increased meat consumption and the use of its by-products require an intensive *R. catesbeiana* production process where the animals are more susceptible to infectious diseases such as red-leg syndrome (RLS). This disease causes high mortality in bullfrog hatcheries and significant economic losses (Glorioso et al., 1974; Bülher et al., 2000). The etiological agents of RLS are members of the Enterobacteriaceae, such as *Proteus vulgaris*, *Pr. mirabilis*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and by some strains of *Staphylococcus epidermidis* (Glorioso et al., 1974). Recently, the incidence of *Streptococcus iniae*, *Chryseobacterium meningosepticum*, *Chr. indolgenes*, *Citrobacter freundii* and *Edwardsiella tarda* was also reported (Mauel et al., 2002). Clinical signs of RLS include abnormal posture, lethargy, loss of appetite and weight, torticolis, stupor and indifference to stimuli. The initial disease process affects legs with inflammatory signs. Gross lesions consist of subcutaneous haemorrhage and epidermal ulcerations. RLS affects *R. catesbeiana* at different stages of growth (tadpoles, fattening phase animals and reproducers), with tadpoles being more susceptible (Glorioso et al., 1974; Mauel et al., 2002). Other pathogens were related to the declination of the frog species. The cause of mass mortality of *R. temporaria* was studied, and the authors hypothesize that primary iridovirus infection with or without secondary infection with opportunistic pathogens, such as *A. hydrophila*, may cause natural outbreaks of red leg, a disease considered previously to be due to bacterial infection only (Cunningham et al., 1996). Cutaneous chitridiomycosis is a key example of an emerging infectious disease (Daszak et al., 2000) and one of the most important causes of the amphibian wildlife decline in Australia, Europe, Central and North America, in addition to iridoviruses (Berger et al., 1998; Daszak et al., 1999; Rollins-Smith et al., 2002). Chitridiomycosis is caused by a zoosporic fungi, *Batrochochytrium dendrobatidis*, which develop solely in keratinized tissues (Longcore et al., 1999). It was associated with one episode of mass mortality in *R. catesbeiana* from hatchery (Mazzoni et al., 2003). The clinical signs observed in frogs are similar to those of RLS, but the gross lesions are usually not apparent (Berger et al., 2000; Paré, 2003).

Since RLS is the most common disease associated with *R. catesbeiana* hatcheries, the purpose of this work was to perform microbiological investigations in organs of healthy *R. catesbeiana*, animals with RLS and freshwater samples during the autumn and also to carry out histological studies in order to report the structural changes produced in the affected organs.

2. Materials and methods

2.1. Animals

Ten specimens of healthy and eight of non-healthy fattening phase *R. catesbeiana* (12 months age; weight between 200 and 250 g) were provided from a hatchery located at the Northwest of Argentina during the autumn (June 2003) and kept at 20 °C until utilization.

Groups of healthy and non-healthy animals were taken from separate areas of the hatchery.

2.2. Isolation and partial identification of bacterial populations from R. catesbeiana hatchery

Microbiological samples were taken in aseptic conditions from freshwater and the skin of living healthy and non-healthy animals. The skin samples were obtained by gently scraping the surface by using sterile cotton swabs. The samples were plated on LBS (Lactobacillus Selection Media) for *Lactobacillus*, MSA (Mannitol Salt Agar) for *Staphylococcus*, MC (MacConkey) for *Enterobacteriaceae*, CATCA (Citrate Azide Tween Carbonate Agar) for *Enterococcus faecalis* and *Ent. faecium* (Reuter, 1992), PCA (Plate Count Agar) for total aerobic heterotrophic

micro-organisms and SAB (Sabouraud Agar) for mycelial fungi and yeast.

Plates were incubated aerobically for 48–72 h at 30 °C, except those from SAB which were incubated for 15 days. Identification of the isolated micro-organisms was performed by morphologic and phenotypic characteristics, using the following biochemical assays: Gram staining, catalase reaction, nitrate reduction, indole production, citrate and urea utilization, mobility, coagulase, arginine and hipurate hydrolysis, Voges-Proskauer reaction, test of haemolysis, growth in 6.5% (w/v) NaCl, growth at different pH and temperatures and fermentation patterns of some sugars (Holt et al., 1994).

The strains were stored at −20 °C in LAPTg medium (Raibaud et al., 1963) supplemented with 20% (v/v) glycerol. All culture media were obtained from Britania (Argentina).

2.3. Cell counts in tissue homogenates and blood to evaluate microbial infection

Healthy and non-healthy animals were euthanized by applying standard procedure for pithing (Petrino et al., 1984). Tissues and blood samples were aseptically removed. Livers and spleens were weighed, rinsed and homogenized in 5 ml peptone–water (meat peptone 0.1% (w/v) Britania, Argentina) with a Teflon pestle. Blood was obtained by cardiac puncture and collected in 1 mg ml^{-1} EDTA (ethylendiaminetetraacetic acid). Quantification of the micro-organisms was performed by serial dilutions (up to 10^{-8}) using 0.1% (w/v) peptone–water. 100 μl dilutions were plated in triplicate onto the different culture media cited above. Plate counts were determined for each tissue and expressed as CFU g^{-1} of organ or ml^{-1} of blood.

2.4. Histological preparations

Hepatic lobes (middle fractions), spleen, and skin from both healthy and non-healthy animals were aseptically removed, fixed with 10% (v/v) formaldehyde for 24 h at room temperature, dehydrated and embedded in paraffin for 24 h according to standardized method for light microscopy (Martoja and Martoja-Pierson, 1970). Four- to six-micrometer-serial paraffin sections of skin, livers and spleens were stained with haematoxylin and eosin (Grignaschi et al., 1983).

3. Results

3.1. Isolation and phenotypic identification of bacterial populations from R. catesbeiana skin and freshwater samples

The identification of the micro-organisms isolated from the skin of fattening phase animals evidenced the presence of the following bacterial genera and species: *Lactobacillus* spp., *Pediococcus* spp., *Micrococcus* spp., *Ent. faecalis*, *Ent. faecium* and members of the Enterobacteriaceae. In both healthy and non-healthy animals, all of these genera and species were isolated. The prevalence of the genera *Pediococcus* and *Lactobacillus* was detected in healthy animals, while in non-healthy a predominance of Enterobacteriaceae was determined. In all the cases, the members of the Enterobacteriaceae were identified as *Enterobacter* spp. and *Pr. vulgaris* (responsible of RLS).

Freshwater samples from both healthy and non-healthy frogs indicate that all the genera cited above were present, but no *Micrococcus* spp. strains were isolated under our experimental conditions. Samples from healthy animals demonstrated a similar proportion of all the genera, while in non-healthy animals a higher number of *Lactobacillus* and Enterobacteriaceae, represented by *Pr. vulgaris*, were isolated.

3.2. Microbial infection in R. catesbeiana organs

Since the non-healthy animals studied differed in their clinical signs related with RLS (such as indifference to stimuli and torticolis), a total of eight representative live frogs were euthanized for evaluating the microbial organs infection. Only two specimens were selected to present the results of the numbers of micro-organisms isolated from tissues according with their clinical signs. The histological studies were performed in a higher number of animals. The diseased animals were highly affected showing abdominal skin ulceration, subcutaneous bloody accumulation, and blood-filled blisters over the eyes.

The microbiological results from the tissues of non-healthy animals showed that the microbial populations varied with organ/tissue processed and the stage of RLS, as summarized in Table 1. The range of each group of micro-organisms is very wide, as for example the total bacterial counts from liver varied

Table 1
Microbial infection in organs of *R. catesbeiana* with red-leg syndrome

Organ/tissue	Micro-organisms					
	Plate counts[a]					
	Enterobacteriaceae	Staphylococcus	Enterococci	Total aerobic	Lactobacilli	Yeast
Healthy liver	0	0	0	0	0	0
Liver[1]	1×10^{2}	0	5.4×10^{5}	4×10^{5}	0	0
Liver[2]	9.5×10^{2}	0.8×10^{1}	4×10^{1}	4×10^{1}	0	1.5×10^{2}
Healthy spleen	0	0	0	0	0	0
Spleen[1]	2.6×10^{4}	2.8×10^{1}	4.7×10^{5}	2.4×10^{6}	0	0
Spleen[2]	3.6×10^{4}	0	0	9×10^{1}	0	0
Healthy blood	0	0	0	0	0	0
Blood[1]	4.7×10^{6}	1×10^{5}	3×10^{5}	9.5×10^{6}	0	4×10^{2}
Blood[2]	0	0	0	0	0	0

The results represent the means of values from triplicates plates.
[a] Plate counts are expressed as CFU g^{-1} of organ or CFU ml^{-1} of blood. The method and culture media used are described in Materials and methods. Organs from [1]animal with skin ulcerations and [2]animal without skin ulcerations.

from 4×10^{1} to 4×10^{5} CFU g^{-1}. The spleen also showed results with a difference of 5 log units (9×10^{1} to 2.4×10^{6} CFU g^{-1}), while in blood the populations varied from 0 to 9.5×10^{6} CFU ml^{-1}. The organs and tissues from 10 representative healthy animals did not present bacterial or fungal infection.

The micro-organisms identified from the tissues of diseased animals were *Pr. vulgaris, Ent. faecalis*, *Ent. faecium*, *S. aureus* and yeasts. No *Lactobacillus* strains or other lactic acid bacteria (LAB) were isolated from the liver, spleen and blood under our experimental conditions.

3.3. Histological studies in organs of R. catesbeiana

On the basis of the microbiological results obtained from different tissues, histological studies were carried out in *R. catesbeiana* skin, spleen and liver, in order to determine if some structural changes were produced by the microbial infection. Clinically, the frogs with RLS demonstrated torticolis, stupor and indifference to stimuli. In some cases, gross lesions were present. Cutaneous hyperemia was noted especially on the extremities and the dorsal and ventral skin. The legs were often swollen with marked subcutaneous oedema and focal areas of haemorrhage within the skeletal muscle. The skin presented areas with ulceration mainly in the legs (Fig. 1) and abdomen. The liver and spleen were enlarged. Both organs were discoloured with multiple pale foci.

Histologically, the ventral abdominal skin was studied in areas closed to the ulcerations (Fig. 2A). The epidermis exhibited ortokeratosis and parakeratosis, apoptosis, espongiosis, vacuolisation of the cells

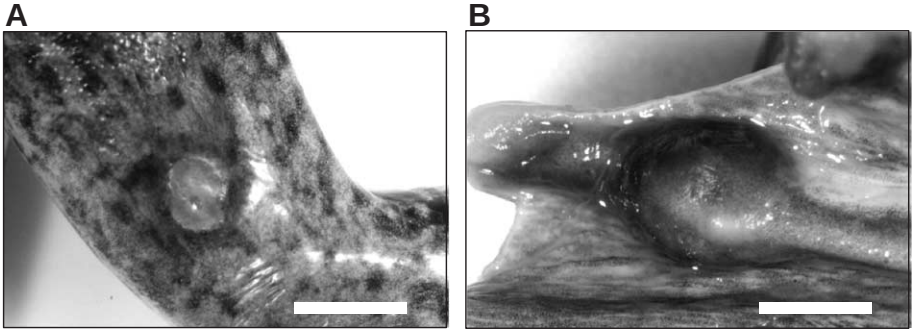

Fig. 1. *R. catesbeiana* with red-leg syndrome. Skin ulcerations with inflammatory signs in (A) leg (Bar=8.43 mm) and (B) finger (Bar=2.82 mm).

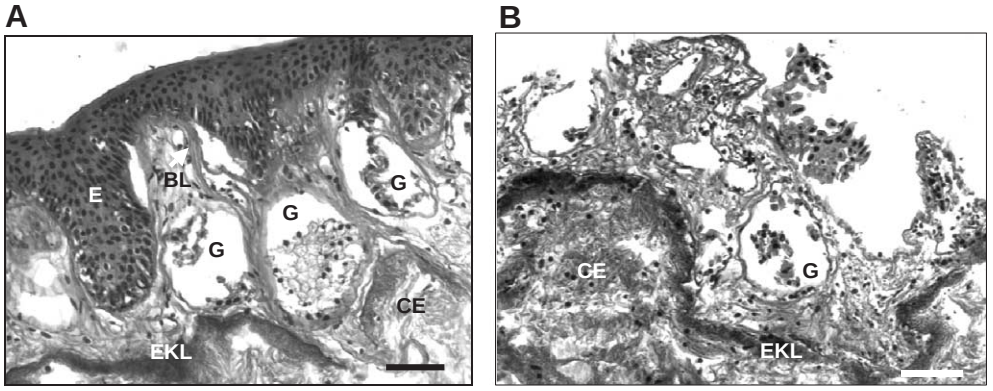

Fig. 2. Light microscopy photograph of histological sections of *R. catesbeiana* skin. (A) Epithelium (E) and dermis alterations in areas close to ulceration: prominent basal lamina (BL, white arrow), spread infiltrate of lymphomonocytic and polymorphonuclear cells, glandular and connective tissue oedema (CE), Eberth-Kastschenko layer (EKL). Bar=2000 μm. (B) Skin ulceration showing epithelial necrosis and disjoined epithelial cells. Alterations in papillar dermis with necrosis of glands (G), connective tissue oedema (CE) and fibrin-leucocytic exudates. Bar=2000 μm.

from the basal layer, prominent and discontinuous basal lamina. The dermis showed glandular oedema, diffuse infiltration of lymphomonocytic and polymorphonuclear cells, dilated vascular light, and connective tissue oedema.

The histological analysis of skin ulcerations (Fig. 2B) showed epithelial necrosis, diskeratosis, apoptosis and espongiosis. Connective tissue presented oedema, dilated vascular light, fibrin-leucocytic exudates, and distortion of serous and granular glands.

Normal skin of *R. catebeiana* is basically composed of epidermis and dermis. The epidermis is formed by a stratified epithelium, which exhibited ortokeratosis, diskeratosis and a prominent basal lamina. The dermis is composed by two layers or strata: the stratum spongiosum and the stratum compactum or papillar and reticular dermis, respectively. The stratum spongiosum is mostly filled with loose connective tissue and contains abundant serous and granular glands, numerous vascular lights and regular cells of the connective tissue. The compactum stratum is composed by dense connective tissue where collagen fibers lie in parallel bundles to the surface of the animal. The calcified Eberth-Kastschenko layer is between the stratum spongiosum and the stratum compactum.

In the histological sections of the skin from both healthy and non-healthy animals, no sporangia associated with chitridiomycosis were observed.

The isolation of micro-organisms from tissues (as pathogenicity marker) leads to the carrying out of histological studies in the liver and spleen of *R. catesbeiana* specimens. The histological sections from *R. catesbeiana* liver with RLS showed centrolobullar necrosis and diminution of the melanin containing cells related to structural alterations. Central vein presented endothelial alteration at the blood vessels level as well. The parenquima showed abnormal distribution of hepatocytes, which presented signs of espongiosis and apoptosis (Fig. 3). However, the liver from healthy animals showed normal distribution of hepatocytes and the presence of pigmented cells.

The spleen of unhealthy animals showed wide areas of septic infarct with lymphomonocytic and

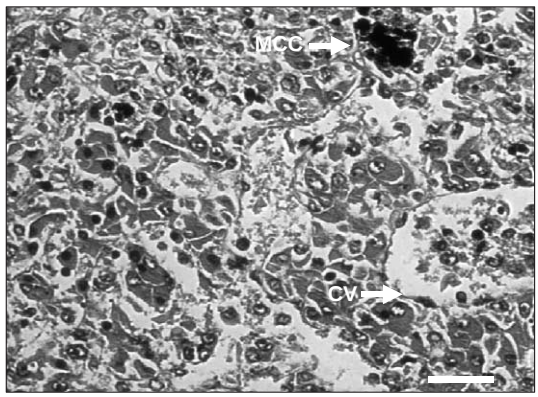

Fig. 3. Light microscopy photograph of histological section of *R. catesbeiana* liver with RLS showing centrolobullar necrosis, diminution of melanin containing cells (MCC, white arrow), alterations in central vein (CV, white arrow) and blood vessels. Bar=1000 μm.

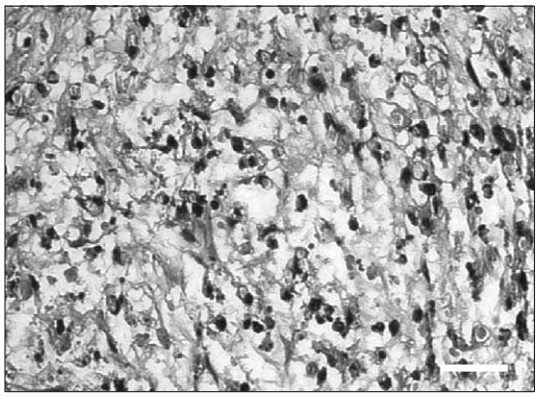

Fig. 4. Light microscopy photograph of histological section of *R. catesbeiana* spleen with RLS showing necrotic area with lymphomonocytic and polymorphonuclear cells infiltration. Bar=1000 μm.

polymorphonuclear cells infiltration (Fig. 4), while the healthy animals presented normal distribution of red and white pulp.

4. Discussion

The current study was undertaken to investigate the *R. catesbeiana* hatchery microflora and its relationship to bacterial diseases. Farming operations require frogs to be placed in captivity and this can lead to an increased risk of disease and mortality. Within the aquatic environment, frogs are in intimate contact with a number of potentially pathogenic micro-organisms. Under normal conditions, the animals remain healthy, but when stressed by crowding or unsanitary conditions, bacterial opportunists may overcome weakened immune barriers and cause disease (Glorioso et al., 1974; Mauel et al., 2002).

The genera and species isolated from *R. catesbeiana* hatchery during the autumn were different from those in spring (September) and summer (December), where the microbiota varied significantly. A higher number of LAB, mainly represented by *Lactobacillus*, were isolated (Pasteris et al., 2004). Although LAB are generally considered to be non-pathogenic and classified as GRAS (Generally Regarded as Safe) (Reid et al., 2003), a growing number of diseases that appeared with the worldwide development of fish aquaculture may be assigned to distinct bacteria belonging to the genera *Streptococcus*, *Lactococcus*, *Vagococcus* and *Carnobacterium* (Ringø and Gatesoupe, 1998).

Taking into account the diversity of the microbial population found in frogs and that their different biological cycles require an aquatic environment, the results could indicate that this medium is the main cause of dissemination of diseases. Sometimes, bullfrog farmers use antibiotics to control infectious diseases (Bülher et al., 2000), but this would modify both intestinal and skin microbiota and would contribute to the spread of antibiotic resistance and to pathogens infection. The interactions of the pathogen with mucosal surfaces are the first step in adhesion, colonisation and bacterial translocation (Nikoskelainen et al., 2001). The binding of *Vibrio anguilarum* and *A. salmonicida* (responsible of vibriosis and furunculosis in fish, respectively) to intestinal mucus was studied; *A. salmonicida* adhered better, which may help to explain its virulence (Horne and Baxendale, 1983). Other authors suggest that skin (Svendsen and Bøgwald, 1997), lateral line, gills (Svendsen et al., 1999) and the gastrointestinal tract (Lødemel et al., 2001; Ringø et al., 2003) or a combination of these organs, could be the infectious routes of fish pathogenic bacteria. The port of entry for *R. catesbeiana* pathogens is associated with skin lacerations or the gastrointestinal tract (Glorioso et al., 1974). Our microbiological results correspond with the health status of the animals. Healthy animals did not present microbial infection, while the microbial counts of the organs were different in each one of the non-healthy animals studied. Thus, frogs without apparent skin damages presented lower microbial infection than frogs with skin ulcerations. These observations could indicate that both of the infection routes for pathogens entries proposed by Glorioso et al. (1974) could be involved in microbial infection. Regarding the cutaneous way of entry, the skin glands produce and release peptides that play various roles, either in the regulation of physiological functions of the skin or in defence against predators micro-organism (Simmaco et al., 1994; Goraya et al., 1998; Rollins-Smith et al., 2002). Skin ulcerations revealed gland distortions and this fact could contribute to the pathogens infection of target organs where structural changes are related to the degree of microbial infection.

The micro-organisms isolated from tissues are related to the skin and freshwater bacterial microflora

and with *Pr. vulgaris* isolated from the lesions. Other authors showed different species of potentially pathogenic micro-organisms isolated from *R. catesbeiana* tissues with RLS and propose *S. iniae* as the etiological agent (Mauel et al., 2002).

In this paper, we report the isolation and partial identification of *R. catesbeiana* microflora during the autumn and a correlation between microbial infection and structural changes in tissues of farmed-bullfrog with RLS. Histological studies performed in normal tissues of *R. catesbeiana* were an important tool to establish a correlation between normal and pathological findings produced by microbial infection. The severity of the structural changes observed could be explained by the high level of bacterial infection in the target organs and by the isolation of *Pr. vulgaris* as responsible to RLS and other pathogens species.

Further work is necessary to study the potentially probiotic properties of LAB isolated from farmed-bullfrog to find an effective way of preventing the main infectious diseases, such as RLS, which affect the production costs in *R. catesbeiana* hatcheries.

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