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A NON-HEMOLYTIC, GROUP B *STREPTOCOCCUS* INFECTION OF CULTURED BULLFROGS, *RANA CATESBEIANA*, IN BRAZIL

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ABSTRACT: A systemic streptococcal infection in cultured bullfrogs in Brazil was characterized by necrotizing splenitis and hepatitis with hepatic and renal hemorrhage. A non-hemolytic Group B *Streptococcus* appeared to be the cause of the lesions, and the stimulus for the splenic reticuloendothelial hyperplasia observed in the animals. Stress may have been a factor in the development of the pathological condition.

INTRODUCTION

Streptococcosis in an aquatic poikilotherm was first characterized in rainbow trout, *Salmo gairdneri*, by Hoshina et al. (1958). Episodes of this disease have since been reported in a variety of both freshwater and saltwater fish (Bullock, 1981). Infections caused by alpha hemolytic, beta hemolytic, and non-hemolytic streptococci have been described (Plumb et al., 1974; Cook and Lofton, 1975; Boomker et al., 1979; Bullock, 1981). This report characterizes an epizootic of streptococcosis among cultured bullfrogs.

MATERIALS AND METHODS

Bullfrogs were maintained in a commercial facility located near the mouth of the Amazon River approximately 30 miles from the city of Belem, Brazil. Separate outdoor breeding and production pens provided all animals with free access to both flowing water and dry land. Spawns were collected daily, transferred to a hatchery and used either as a food along with insect larvae for the adults or to stock production pens. The system had functioned without major problems for approximately 1 yr. During February and March of 1982 equatorial rainfall, greater than that normally experienced, resulted in flooding of the outdoor pens, and with a reduction in daily sunlight hindered proper sanitation. Some pens were more suitable than others and movement of frogs to these pens resulted in overcrowding. Frogs, demonstrating a pronounced difficulty in resubmerging, started to appear at the surface of the water during March. Over the next 2 mo mortalities reduced the population by 80%. The pens originally contained a total of approximately 100,000 frogs.

Frogs were killed by pithing and examined for gross external and internal lesions. Tissues from six clinically diseased frogs and three non-diseased frogs were collected in neutral buffered 10% formalin for histological examination. The tissues were processed 3 days after collection. Paraffin embedded tissues were sectioned at 5–6 μm and stained with hematoxylin and eosin. Selected tissues were stained by a Gram method (Brown and Hopps, 1973).

Blood was collected by cardiac puncture into citrated frog Ringer's solution, and solid tissues were aseptically removed from 10 each of moribund and apparently healthy frogs for microbiological studies. Samples of water, soil, tadpoles, and insect larvae were processed to determine the presence of bacteria and viruses according to techniques previously described (Glorioso et al., 1974). Identification of the bacterial isolates was carried out with a combination of microscopy and biochemical profiles according to Glorioso et al. (1974). Additional biochemical tests and selective-enrichment techniques developed for streptococci (Facklam, 1980; Waitkin, 1982) and serological grouping with the Phadebact *Streptococcus* Test System (Pharmacia Diagnostics, Piscataway, New Jersey 08854, USA) were performed as needed.

The spleens of diseased frogs were two times the size of the non-diseased frog spleens. There was an increase in reticuloendothelial cells (splenic macrophages) and acute necrosis (Fig. 1). There was depletion of small lymphoid cells which were present in the non-diseased frogs in multifocal accumulations. Gram positive bacterial cocci were very numerous in the splenic tissue of some diseased frogs. Microscopic changes in the liver of diseased frogs varied from moderate diffuse hyperplasia of melanocytes to moderate acute multifocal coagulation necrosis and focal hemorrhage. Atrophy of hepatocytes and disorganization of hepatic cords was a diffuse hepatic alteration (Fig. 2). There was hyperplasia of hepatic reticuloendothelial cells, and these contained slight to large numbers of gram positive bacterial cocci. The livers of non-diseased frogs had moderate diffuse vacuolation of hepatocytes. One non-diseased frog had slight hepatic congestion with moderate hyperplasia of melanocytes. The kidneys of one dis-

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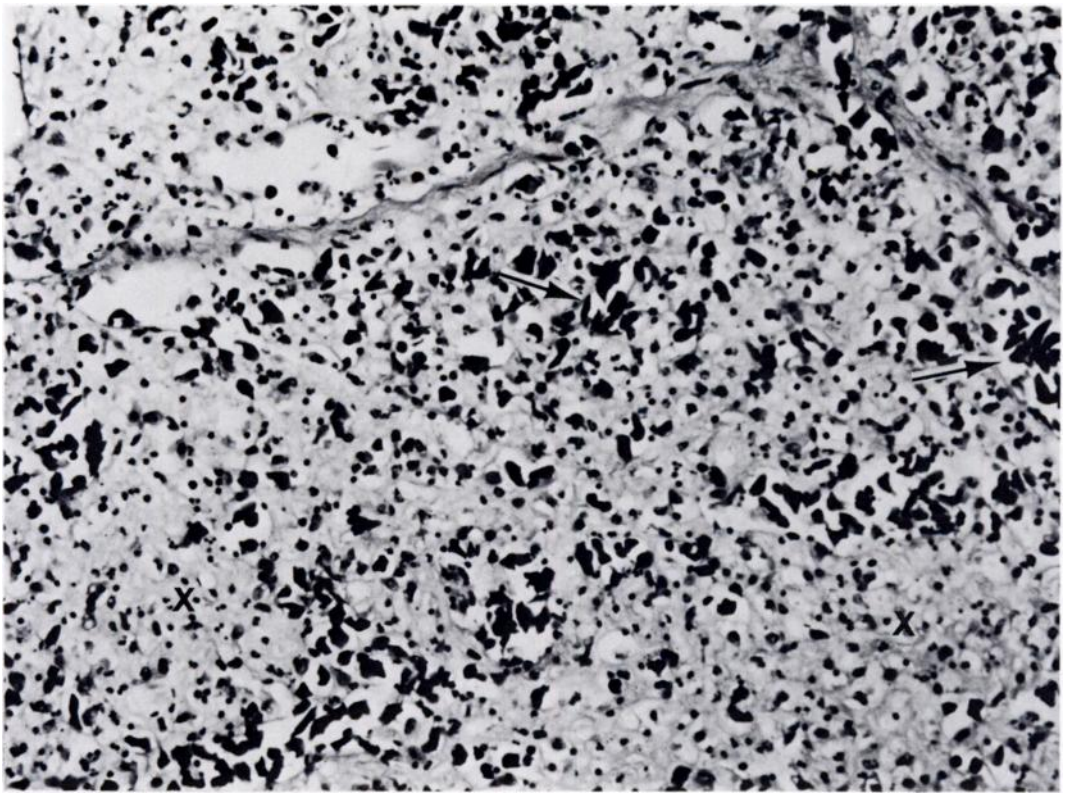


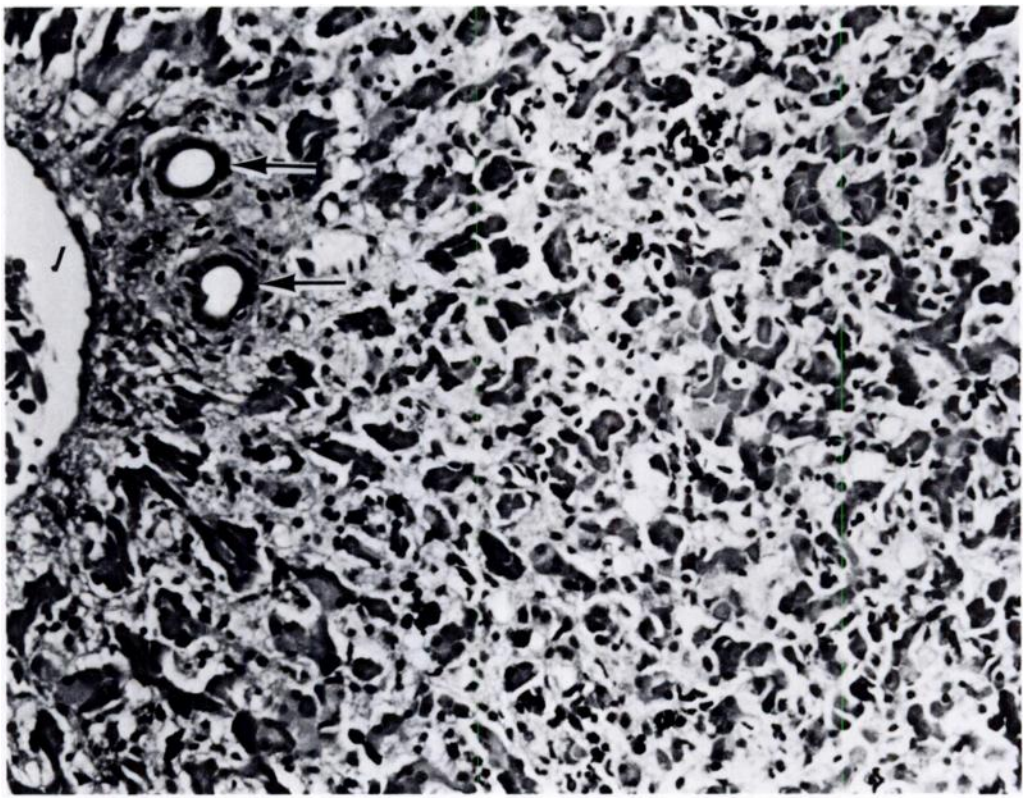
FIGURE 1. Diseased bullfrog spleen with acute necrosis and loss of small lymphoid cells (X). Darkly stained cells are erythrocytes (arrows). H&E. ×200.

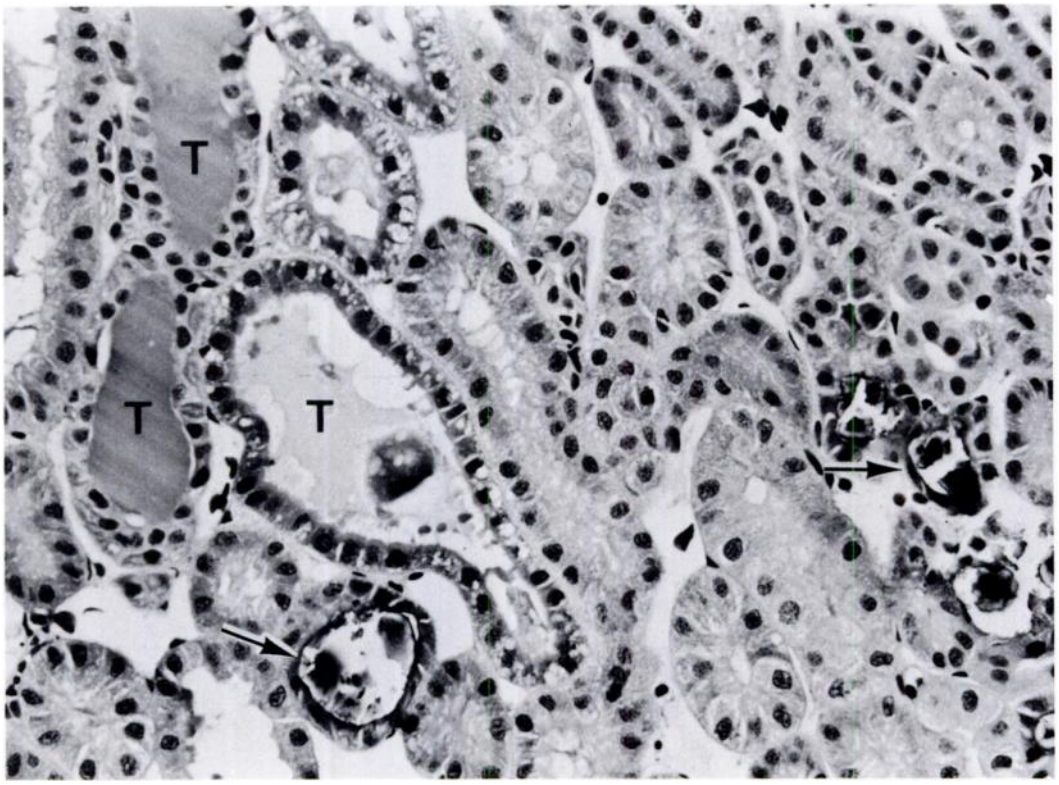
eased frog had marked interstitial hemorrhage, another had segmental necrosis of the glomerular tufts, and one had numerous bacterial cocci with minimal tissue alteration. In diseased and non-diseased frogs there was moderate diffuse multifocal mineralization of the tubule epithelium often with occlusion of the lumen (Fig. 3). Also present were slight to moderate widely dilated tubules containing proteinaceous material. The lung, stomach, testes, and pancreas of diseased frogs were essentially normal. However, there were gram positive bacterial cocci in blood vessel lumens and in endothelial cells of the stomach (Fig. 4) and other tissues.

Gram stained blood smears demonstrated the presence of encapsulated, gram positive diplococci with occasional short chains of cocci in the diseased samples. A similar gram positive coccus was subsequently isolated in almost pure culture from the blood samples at 10^3 to 10^7 colony forming units/ml, and at 10^2 to 10^6 colony forming units/gram in samples of spleen, liver and kidney. *Proteus vulgaris* and an unidentified yeast were isolated from the liver of one diseased frog. *Aeromonas hydrophila* was isolated from a kidney of a second diseased frog. No other bacteria were isolated from the samples, and obligate anaerobes could not be demonstrated. Attempts to

TABLE 1. Phenotypic properties of the gram positive coccus isolated from diseased bullfrogs.

Test or substrate	Response
Hemolysis	
Human blood agar	–
Frog blood agar	–
Susceptibility to	
Sulfamethoxyzole and trimethoprim	–
Optochin	–
Bile solubility	–
Bile-esculin	–
Hippurate hydrolysis	+
Growth	
10 C	–
45 C	–
6.5% NaCl	–
Lancefield serological grouping	
A	–
B	+
C	–
G	–





demonstrate viruses on monolayer cultures of fat head minnow and bullfrog tongue cells were negative.

The gram positive coccus could not be demonstrated among the apparently healthy frogs in the pens at the primary isolation stage. However, the coccus was recovered after 5 days of enrichment culture at 25 C in 0.02% azide-Brain Heart Infusion broth from four of 10 spleen samples removed from pen reared, apparently healthy frogs. Other tissue samples were negative. Even after using enrichment culture, the coccus was not isolated from laboratory-raised bullfrogs.

Biochemical and serological responses of the isolate (Table 1) identified the coccus as a non-hemolytic Group B *Streptococcus*. Growth of the isolate in pure culture was accompanied by extreme viscosity which made it difficult to remove the growth from the surfaces of solid media. The pure culture of the isolate did not produce pigment when grown either aerobically or anaerobically. Attempts to isolate the organism from environmental samples, tadpoles, and insect larvae were not successful. With the available selective media, species of staphylococci, non-Group B streptococci, and bacilli were the predominant isolates from these samples under both aerobic and anaerobic conditions.

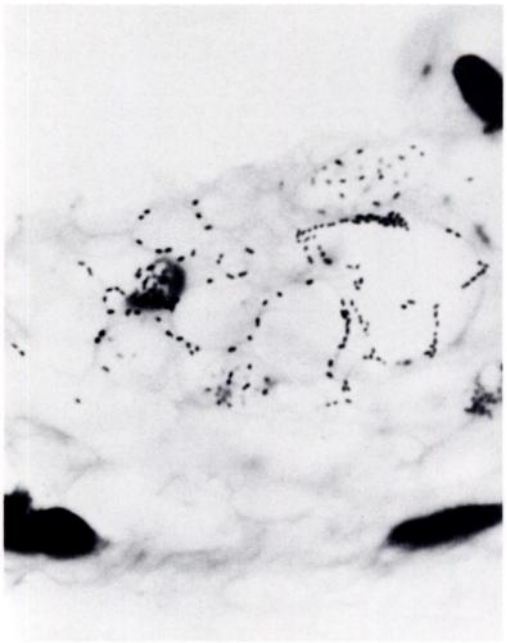


FIGURE 4. Submucosal vein in diseased bullfrog stomach with bacterial cocci in endothelial cells. Gram. ×1,000.

DISCUSSION

There have been relatively few reports on streptococcosis in aquatic poikilotherms (Bullock, 1981), with only two of these studies identifying non-hemolytic Group B streptococci in diseased fish (Plumb et al., 1974; Cook and Lofton, 1975). Histopathological studies were not carried out in the fish studies.

Bullfrog streptococcosis was characterized by septicemia, necrotizing splenitis, and hepatitis with hepatic and renal hemorrhage. A non-hemolytic Group B *Streptococcus* appeared to be the cause of the lesions, and the stimulus for the splenic reticuloendothelial hyperplasia observed in the natural infection.

A major feature of the splenic lesions was a loss of small lymphoid cells. This depletion, loss, or necrosis of small lymphoid cells suggested either severe systemic stress or a direct effect of the bacteria. As most bacteria were isolated in reticuloendothelial cells (macrophages) rather than the parenchymal cells, it is most likely that stress factors produced the depletion of lymphoid cells. The effects of the stress and the bacterial disease appear comingled. The effect of pre-existing stress was probably contributory to the development of the streptococcosis in the frog population. Renal changes including mineralization and tubular dilation were present in both diseased and non-diseased frogs. This mineralization may reflect a relatively normal situation or a problem in mineral metabolism of dietary or metabolic origin.

The exact nature of the stressor(s) has not been determined. Any one or combination of effects associated with either the flooding, overcrowding, unavoidable deterioration in sanitation or suggested problem in mineral metabolism may have contributed to the enhancement of the disease. Other stressors, which do not appear to be applicable to the present situation,

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FIGURE 2. Diseased bullfrog liver with atrophy and disorganization of hepatic cords. Portal area with bile ducts (arrows) and vein (V). H&E. ×200.

FIGURE 3. Non-diseased bullfrog kidney with multifocal tubular mineralization (arrows) and slightly dilated tubules (T). H&E. ×300.

have been suggested to contribute to streptococcosis (Plumb et al., 1974; Boomker et al., 1979).

Available records on mortalities within the pens indicated that moribund frogs with inflated lungs and splenomegaly were occasionally observed 8 mo prior to the epizootic. This suggests that the *Streptococcus* may have been in the system for some time. However, no microbiological studies were performed on these animals. Given the fact that Group B streptococci appear to be part of the bacterial flora indigenous to humans (Wilkinson, 1978), the exact source of the organism may never be known.

As reported with other non-hemolytic Group B streptococci (Waitkin, 1982), the frog isolate did not produce the orange pigment characteristic of hemolytic Group B streptococci when grown under anerobic conditions.

Our results demonstrated that naturally occurring episodes of streptococcosis among poikilotherms are not limited to fish. As in other epizootics of this disease, stress may have been a factor in the development of the pathological condition.

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