PREVALENCE OF RANAVIRUSES IN FRESH FISH AND EDIBLE FROGS AND THE KNOWLEDGE, PRACTICES OF FISHERMEN AND FROG HARVESTERS IN ZARIA, KADUNA STATE, NIGERIA

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BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE

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JUNE, 2018

DECLARATION

I declare that the work in this dissertation titled "**Prevalence of Ranaviruses in Fresh Fish and Edible Frogs and the Knowledge and Practices of Fishermen and Frog Harvesters in Zaria, Kaduna State, Nigeria**" has been performed by me in the Department of Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, Zaria, Nigeria under the supervision of Dr. G.S.N Kia and Professor A.K.B Sackey. The information derived from the literature has been duly acknowledged in the list of references provided. No part of this dissertation was previously presented for another degree in this or another institution.

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CERTIFICATION

This dissertation entitled "**Prevalence of Ranaviruses in Fresh Fish and Edible Frogs and the Knowledge, Practices of Fishermen and Frog Harvesters in Zaria, Kaduna State, Nigeria**" by Inikpi Hannah AMEH for the award of the degree of Masters of Science in Veterinary Epidemiology in the Department of Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literature presentation.

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DEDICATION

This research work is dedicated to the Almighty God, my parents and siblings and people involved in research all over the world

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ABSTRACT

Ranaviruses are globally emerging pathogens which cause systemic infection in fishes, amphibians, and reptiles and have been associated with numerous disease outbreaks in natural and cultured populations.Mortality and morbidity due to ranaviruses have been shown to range from 0% to 100% of infected animals. The aim of this study was to detect ranaviruses in fish and edible frogs from dams and ponds in Zaria, Kaduna State, Nigeria. A total of 415 samples, 215 frogs (Rana spp.) and 200 fishes (*Clarias gariepinus, Labeo senegalensis, Lates niloticus, Marcusenius senegalensis, Oreochromis niloticus, Schilbemystus* and *Tilapia zilli*) were collected based on availability. Liver, kidney and spleen from each sample were pooled, homogenised and screened for ranavirus using the Enzyme linked Immunosorbent assay (ELISA). A total of 37 questionnaires were administered to fishermen and 21 to frog harvesters to ascertain occurrence of mass mortality of fish and frogs and assess their practices and knowledge on frog and fish health. An overall prevalence of 47.2% (196/415) was obtained from the study. Frogs had a higher prevalence of 51.2% (110/215) than fish (43% (86/20). There was no statistically significant relationship between prevalence in fish and that of frogs ($P > 0.05$). Among the fish species; *Marcusenius Senegalensis* had the highest prevalence of 75% (9/12), this was statistically significant ($P < 0.05$). The prevalence in wild fish (51.3%) compared to farmed fish (18%) was statistically significant ($P < 0.05$). Occasional mass mortality of fishes and frogs were observed by fishermen (99.1%) and frog harvesters (76.2%). This study establishes the presence of detectable ranavirus antigen infish and edible frogs in Zaria, Kaduna State, Nigeria. This is the first report on the presence of ranavirus in fishes and edible frogs in Nigeria.

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LIST OF ABBREVIATIONS

ATV	-	Ambystoma tigrinum virus
BIV	-	Bohle iridovirus
FV3	-	Frog virus 3
ECV	-	European catfish virus
EHNV	-	Epizootic haematopoietic necrosis virus
ELISA	-	Enzyme Linked Immunosorbent Assay
OIE	-	World Organisation for Animal Health (Office International des Epizooties)
MCP	-	Major capsid protein
PCR	-	Polymerase chain reaction
Qpcr	-	Quantitative polymerase chain reaction
REA	-	Restriction enzyme analysis
SCRV	-	Santee-Cooper ranavirus
DNA	-	Deoxyribonucleic acid
dsDNA	-	Double stranded DNA
ESV	-	European sheatfih virus
ICTV	-	International Committee on the Taxonomy of Viruses
nm	-	Nanometer
kbp	-	Kilobase pairs
TSGM	-	Tris saline glycerol merthiolate
NaCl	-	Sodium chloride
ABTS	-	2,2'-azino-bis(3- ethylbenzothiazoline-6- sulphonic acid
BF-2	-	Bluegill fry
FHM	-	Fathead minnow
EPC	-	Epithelioma papulosum cyprini
CHSE	-	Chinook salmon embryo cell line
SPF	-	Specific pathogen-free

CPE - Cytopathic effect

H&E - Haematoxylin and eosin

WHO - World health organization

FME - Federal Ministry of Environment

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Ranaviruses are globally emerging pathogens which cause systemic infection in fishes, amphibians, and reptiles, capable of infecting multiple species at a site (Duffus *et al*., 2008), and can be transmitted between taxonomic classes of ectothermic vertebrates (Brenes *et al*., 2014; Brunner *et al*., 2015). Ranaviruses are known to infect at least 175 species across 52 families of cold–blooded vertebrates, and are found on all continents but Antarctica (Duffus *et al*., 2015). Disease outbreaks associated with ranaviruses have been reported worldwide, and their harmful impact on aquaculture and natural populations of host animals has drawn attention to this group of viruses (Holopainen, 2012).

Ranaviruses belong to one of the five genera of the family *Iridoviridae* (Jancovich *et al*., 2011).They are large, icosohedral, DNA viruses that can be found in an enveloped or non-enveloped form.There are six recognized species in the genus *Ranavirus* made up of: frog virus 3 (FV3), Ambystoma tigrinum virus (ATV), Bohle iridovirus (BIV), Epizootic hematopoietic necrosis virus (EHNV), European catfish virus (ECV), and Santee-Cooper ranavirus (ICTV, 2011).Diseases caused by these viruses'presents a growing concern in aquaculture, as well as free-ranging fish, amphibian and reptile populations(Chinchar, 2002). These viruses are found worldwide and cause significant morbidity and mortality in affected species (Williams *et al*., 2005). Ranaviral disease has been placed on the World Organization for Animal Health list of reportable diseases for fishes and amphibians (OIE, 2012).

Ranaviruses have been associated with numerous disease outbreaks in natural and cultured populations of fish, amphibians and reptiles, and have become pathogens of both economic and

ecological importance (Chinchar, 2002; Chinchar *et al*., 2009). The World Organisation for Animal Health (OIE) recognizes one of the ranaviruses, epizootic haematopoietic necrosis virus (EHNV), as a notifiable pathogen for fish, whereas for amphibians all ranavirus infections are listed (OIE, 2011).

Fish is one of the most important sources of protein for humans and other animals in the tropics. According to FAO (1989), fish accounts for more than 40% of the protein diet of two – thirds of the global population. Fish does not only provide food for immediate consumption, but people rely on fishing for economic gains and job provision. A well processed fish product from the tropics has a ready market in developed countries and is therefore a good foreign exchange earner (Imam and Dewu, 2010).

There has been a tremendous increase in global demand for marine and freshwater fish to meet the protein needs of our expanding human population. However, due to the limited capacity of the wild-capture sector and a levelling of production from capture fisheries, the practice of farming aquatic animals has expanded rapidly to become a major global industry (Sahoo and Goodwin, 2012). Aquaculture, particularly freshwater aquaculture is now integral to the economies of many countries. A large number of aquatic animal species are farmed in high density in freshwater, brackish and marine systems, where they are exposed to new environments and potentially new diseases (Bondad-Reantaso *et al*., 2005). Furthermore, environmental stress factors, the use of manufacturedfeeds and prolific global trade has led to the emergence and spread of new diseases (Sahoo and Godwin, 2012). Viral pathogens, established for decades or newly emerging as disease threats, are particularly challenging since there are few efficacious treatments (Sahoo and Goodwin, 2012).

In some regions of India, frogs are called “jumping chickens,” as their taste is similar to chicken. Infact, their palatability to humans is why billions of frogs are consumed annually (Altherr *et al*., 2011). In many countries in Asia, Africa, and Latin America, frogs are collected for subsistence or local consumption. Some of these same countries are engaged in the commercial trade of frogs and frog products— including frogs’ legs, supplying markets in the European Union (EU) and the United States of America (USA) (Altherr *et al*., 2011).Commercial trade and consumption of frogs is becoming popular in some African countries as frogs caught from wild waters are fast becoming a delicacy in these African countries such as Burkina Faso, Benin and Nigeria (Mohneke *et al*., 2009). In Nigeria, frog meat is fast gaining popularity with frog markets springing up nationwide in various states like Ogun, Oyo, Lagos, Kano, Kaduna and Kebbi (Mohneke *et.al.*, 2009;Kia*et al*., 2017).

Trade in live frogs is a potential vector for ranaviruses (Gratwicke *et al*., 2009; Schloegel *et al*., 2010). Ranavirus and chytrid fungus represent the most serious threat to wild frog populations in some American bullfrogs farmed in Brazil (Mazzoni *et al*., 2009). Due to the mounting evidence that the chytrid fungus and ranaviruses are distributed through frogs traded live, in 2009 the World Organization for Animal Health (OIE), specified conditions for regions(for example, health certificates and risk mitigation measuresin its Aquatic Animal Health Code).These pathogens can cause mortality rate of up to 90% in a single pond (Daszak *et al*., 2007;Altherr *et al*., 2011). Die-offs have been reported in the Americas, Europe and Asia (Gray *et al*., 2009). Ranaviruses were found in northern leopard frogs farmed in China for human consumption (Schloegel *et al*., 2009) and led to mass mortality in frogs (Altherr *et al*., 2011).

1.2 Statement of Research Problem

Ranaviruses have the potential to cause population declines and extinctions in fishes, amphibians and reptiles although outcome of infection varies depending on host and strains (Teacher *et al*., 2010;Earl and Gray, 2014; Price *et al*., 2014). Thus, highly susceptible host species that are rare may be at greatest risk (Earl and Gray, 2014). However, common species also can be affected. For example, populations of the common frog (*Rana temporaria*) have declined on average of 80% in the UK where ranavirus die-offs have reoccurred (Teacher *et al*., 2010).

Epizootic haematopoietic necrosis virus (EHNV) is extremely resistant to drying and can survive in water for months (Langdon, 1989). It can persist in frozen fish tissues for more than 2 years and frozen fish carcasses for at least a year (Langdon, 1989). For these reasons, it is presumed that EHNV would persist for months to years on a fish farm in water and sediment, as well as on plants and equipment and so the chances of the spread and transmissions of EHNV are greatly increased (OIE, 2012).

Infections with Epizootic haematopoietic necrosis virus (EHNV) infections are recognized by epizootic mortality in fish of any age and affecting a very large proportion of the population with dramatic population decline (Whittington *et al*., 1996). Typically, fingerling and juvenile fish are affected in endemic areas, but in newly infected areas adults are also affected (Whittington *et al*., 1996). When the disease is first recognized in an area there is a dramatic population decline (Whittington *et al*., 1996).

The cause of the sudden appearance of a global plague from this pathogen is uncertain, but possibilities include trade in food and ornamental fish, reptiles, amphibians, and/or its

emergence from unknown reservoir hosts resulting from changes in the environment (Lee, 2013).

Ranaviruses have not been previously reported in Nigeria, and there are no evidence of surveillance in the country to the best of our knowledge.

1.3 Justification of the Study

Most of what is known about the epidemiology of ranaviruses comes from investigations of obvious die-offs, sporadic surveillance efforts in small numbers of populations at one or two time points, and a few larger-scale surveillance efforts focused on a handful of species of economic importance or conservation interest (Gray *et al*., 2009; Gray *et al*., 2015). Gross signs of ranavirus infection may not be displayed, therefore many host species are cryptic and difficult to detect. As a result of the lack of awareness of ranaviruses as significant pathogens, which are occasionally misdiagnosed, the known geographic distribution and host range of ranaviruses are likely underestimated (Duffus *et al*., 2015).

Ranaviruses are classified as emerging pathogens, because their geographic distribution and host range appear to be expanding (Daszak *et al*., 1999). It is becoming evident that ranaviruses are frequently moved in the regional and international trade of animals. For example, barred tiger salamander (*Ambystoma mavortium*) larvae are sold as fishing bait in the southwestern USA, and as many as 100 % have been shown to be infected with the ranavirus, *Ambystoma tigrinum* virus (Brunner *et al*., 2015).

Frog farming is recently no longer a rarity in Africa and northern Europe with the potential for ranavirus infection in amphibians to have commercial impact. However, the effect of ranavirus on wild populations of amphibians and fishes is potentially devastating. If the virus spreads in

wild populations of amphibians or fishes, it may have severe consequences on the infected populations of amphibians, fishes and other susceptible species and their predators However, there are records of ranaviruses causing disease outbreaks in frogs on all continents except for Africa and Antarctica where there are no literature recording disease outbreak (Williams *et al*., 2005); therefore, the presence of ranavirus in the environment will not be an unusual observation (Green *et al*., 2002).

The use of amphibians as a food source, typically known as frogs' legs is gradually gaining popularity (Jensen and Camp, 2003). The consumption of frogs' legs occurs all over the world (Mohneke *et al*., *2009*) and the trade in this commodity is significant at both local and international levels (Warkentin *et al*., 2009; Emma, 2014).Amphibian ranaviruses have been found in animals that are traded over international borders for a variety of reasons, including human consumption and the pet trade (Schloegel *et al*., 2009; Kolby *et al*.,2014). Schloegel *et al*. (2009) found that 8.5 % of amphibians imported into the USA at three major port cities were infected with ranavirus. Similarly, Kolby *et al*. (2014) found over 50 % of amphibians exported via Hong Kong International Airport wasinfected with ranavirus. Reptiles infected with ranaviruses also have been discovered in internationally traded animals (Stöhr *et al*. 2013, 2015). Finally, internationally traded ornamental fishes have been shown to be infected with ranaviruses (Hedrick and McDowell, 1995). High mortality epizootics were reported among young of the pallid sturgeon in 2001, 2009 and 2013 at the Blind Pony Hatchery in Sweet Springs, Missouri (Zhou *et al*., 2013). Likewise, high mortality epizootics have been reported among Chinese giant Salamanders of all ages and classes on multiple farms in 2010 and 2011 (Zhou *et al*., 2013).Epizootic haematopoietic necrosis is considered to be systemic clinical or subclinical infection of finfish by epizootic haematopoietic necrosis virus (EHNV) (OIE, 2012).

It is therefore important to understand the geographic extent, host range, and phylogenetic relationships of these emerging pathogens (Jancovich *et al*., 2015). Occasional massive economic losses in fishery industry due to deaths of unknown origin prompted this study so as to generate useful data that would guide policy makers and relevant stakeholders concerning possible disease threat to aquaculture in Nigeria.

At the moment, knowledge on the status of ranavirus disease in Nigeria is generally lacking even though it is an OIE reportable disease and fisheries and aquaculture is becoming an important source of livelihood to Nigerians (OIE, 2009). This calls for a baseline data generation for ranavirus in Nigeria.

1.4 Aim of the Study

The aim of this study was to detect the presence of ranavirus in fishes and edible frogs and the practices and knowledge of fishermen and frog harvesters in Zaria, Kaduna State, Nigeria.

1.5 Objectives of the Study

The objectives of the study were to:

I determine the prevalence ofranavirus antigens in fishes and edible frogs from dams and ponds in Zaria, Kaduna State, Nigeria;

II determine the association between source, management system, species of fish and Infection with Ranavirus in Zaria, Kaduna State, Nigeria;

III determine the associations ofthe physiochemical parameters of the environment(water) withRanavirus of some of the sampling areas in Zaria, Kaduna State, Nigeria are within

acceptable limits set by WHO and FME, Nigeria;

IV obtain information on the practices of fishermen and frog harvesters and their knowledgeabout fish and edible frog diseases in Zaria, Kaduna State, Nigeria.

1.6 Research Questions

I Are fishes and edible frogs in Zaria, Kaduna State, Nigeria infected with ranaviruses?

II Are there any associations between source, management system, species of fish and the presence of ranavirus in fishes and edible frogs in Zaria, Kaduna State, Nigeria?

III Are the physiochemical parameters of water of sampled areas in Zaria, Kaduna State, Nigeriawithin acceptable limits set by WHO and FME, Nigeria?

IV What are the practices of fishermen and edible frog harvesters and their knowledge about fish and edible frog diseases?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background Information

Anthropogenic activities such as habitat destruction, pollution and biologic resource exploitation have undoubtedly intensified the impacts of disease on ecosystems worldwide (Aguirre and Tabor, 2008). Amphibian and fish populations worldwide have suffered severe declines, partly because of the spread of diseases, including those caused by ranavirus infection (Daszak *et al*., 1999). Ranaviruses of the family, Iridoviridae can infect a variety of species of amphibians, fish, and reptiles, but there is some host-dependent susceptibility (Forza´n and Wood, 2013).Several ranaviruses may present a significant threat to host species that are geographically isolated or exist at low abundance (Earl and Gray, 2014; Price *et al*. 2014).

Infection with ranavirus is generally characterised by systemic disease involving multiple internal organs such as liver, spleen, kidneys and gut, and typically results in mortality (Chinchar, 2002). However, ranaviruses have also been isolated from apparently asymptomatic animals (Tapiovaara *et al*., 1998) and can also cause low-level mortality (Whittington *et al*., 1994). It has been suggested that these animals could act as reservoirs of the virus (Gray *et al*., 2009).This questions the role of ranavirus as a primary pathogen in edible frogs. It may be an opportunistic pathogen which coexists at low levels within the host except when the host is weakened and the environmental conditions are conducive to viral replication. Under such conditions the virus may turn pathogenic and cause disease in the host (Ariel *et al*., 2009).

The recent listing of *Batrachochytrium dendrobatidis* (Bd) and ranavirus infection in amphibians by the World Organisation for Animal Health (OIE) reflects a global concern for the health of farmed and declines of wild populations of amphibians (OIE 2008). Several reports on ranavirus disease in amphibians abound in literatures (Speare and Smith 1992; Zupanovic *et al*., 1998; Zhang *et al*., 2001; Green *et al*., 2002; Weng *et al*., 2002; Greer *et al*., 2005; Fox *et al*., 2006) with several publications indicating that ranaviruses are part of the cause of amphibian declines across the world (Cunningham *et al*., 1996; Chinchar 2002; Green *et al*., 2002; Docherty *et al*., 2003; Pearman *et al*., 2004; Greer *et al*., 2005; Jancovich *et al*., 2005;Ariel *et al*.,2009).

Despite the severity of amphibian declines due to infectious diseases, it is difficult to envisage how control and containment of ranavirus in wild frogs can be achieved (Mendelson *et al*., 2006; Gascon *et al*., 2007). The nature of wild animals makes it very difficult to ensure non-destructive containment of animals in an infected area. When frogs migrate from a pond in the autumn they may be in contact with other frog populations and contaminate them. Viruses could also spread by vectors such as birds, fish or downstream via the water. The risk of ranavirus outbreaks is not limited to amphibians. Evidence for transmission of ranavirus between frogs and fish has been recorded (Moody and Owens 1994; Ariel and Owens 1997; Mao *et al*., 1999) and one host species may act as reservoir for the other (Mao *et al*., 1999; Duffus *et al*., 2008). EHNV, ECV and European sheatfish virus (ESV) are listed by the OIE for fish and they are closely related to this new strain. All the listed viruses were involved in natural outbreaks in wild populations of perch, catfish and sheatfish (Bigarré *et al*., 2008) and several other species of fish and frogs are experimentally susceptible to ranavirus infection (Langdon 1989; Moody and Owens 1994; Ariel and Owens 1997; Cullen and Owens 2002; Bang-Jensen *et al*., 2009).

Potentially, this new *Pelophylax esculentus* viral isolate could therefore pose a risk to both amphibian and piscine fauna. To balance these apocalyptic statements there are also many reports of ranavirus isolations from animals with no clinical symptoms, where the virus apparently is not causing the host any distress (Tapiovaara *et al*., 1998; Harp and Petranka 2006; Duffus *et al*., 2008).

Natural epizootics of amphibian ranaviruses appear to be similar for piscine ranaviruses (e.g. EHNV) (OIE, 2012). Epizootics appear to be seasonal and can be related to poor husbandry (captive populations) and overcrowding (wild and captive) (OIE, 2012). It has been assumed that for some amphibians such as salamanders,the disease is related to the annual appearance of large numbers of non-immune young animals and their subsequent exposure to the virus in shallow waters (Jancovich *et al*., 2001;Green *et al*., 2002;Brunner *et al*., 2004; Rojas *et al*., 2005).

2.2 History of Ranavirus

Ranaviruses are believed to have evolved in fish and only later began to infect amphibians and reptiles (Jancovich *et al*., 2010). The type species of the ranavirus genus, frog virus 3 (FV3) was the first virus to be isolated and for the first time in 1965 (Granoff *et al*., 1965; Rafferty, 1965). In Europe, ranaviruses were isolated from moribund edible frogs *Pelophylax kl. esculentus* (formerly Rana kl. esculenta) in Croatia (Fijan *et al*., 1991) and Italy (G. Bovo pers. comm.) and from wild populations of the common frog *Rana temporaria* in the UK (Drury *et al*., 1995). The origin of these viruses is unknown, as is the influence of ranaviral disease on the wild populations of amphibians in Europe (Ariel *et al*., 2009).

Granoff, *et al*.(1965) first reported ranavirus from amphibians in the 1960's in a population of northern leopard frogs, *Lithobates pipiens*. Yet the impact of widespread virus related die-offs was not recognized until the 1990's. Since the mid-1990's, Ranaviruses have been documented as being responsible for amphibian, reptile and fish die-offs, ranging from a few individuals to thousands, and some of themmassive events occurring in different states and countries (Lee, 2013).

2.3 Structure and Classification of Ranavirus

Ranaviruses are large icosahedral DNA viruses measuring approximately 120 – 200 nm in diameter with a large single linear dsDNA genome of roughly 105 kbp (Williams *et al*., 2005) which codes for around 100 gene products (Chinchar,2002) The main structural component of the proteincapsid is the major capsid protein (MCP). (Figure 2.1). The core of the virion consists of a nucleoprotein filament surrounded by a lipid membrane containing transmembrane proteins and a capsid composed of identical capsomers (Jancovich *etal*., 2011).

Ranaviruses are members of the family *Iridoviridae*. There are six recognized species and numerous strains with the six species being Ambystoma tigrinum virus (ATV), Bohle iridovirus (BIV), Epizootic haematopoietic necrosis virus (EHNV), European catfish virus (ECV), Frog virus 3 (FV3) and Santee-Cooper ranavirus (SCRV)(Lee, 2013).

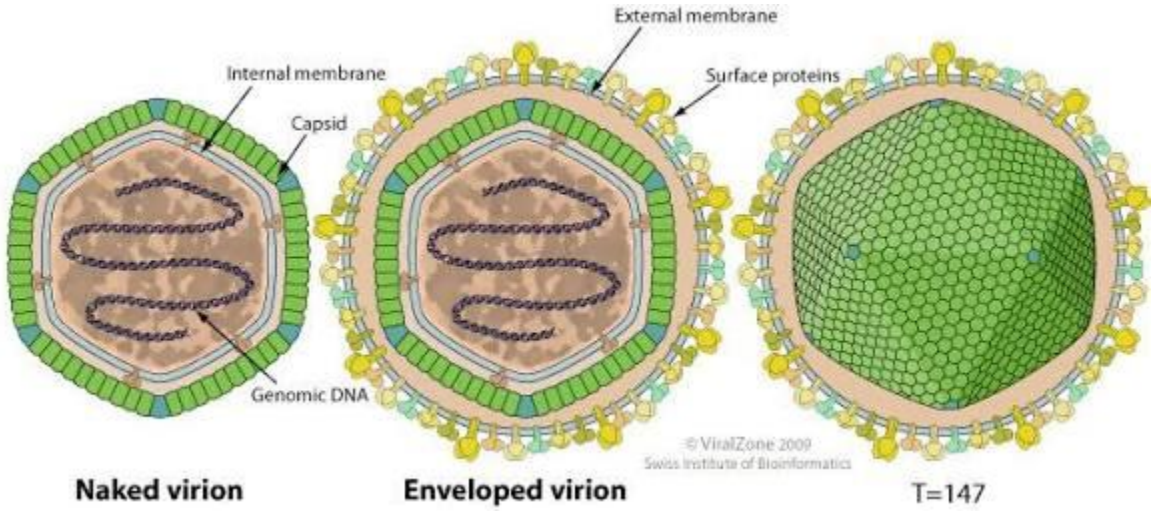


Figure 2.1: Structure of Iridoviruses (ViralZone,2009).

2.4 Epidemiology

Ranavirus infections have been reported in five continentssuch as Asia, North and South America, Europe, Australia (Gray *et al*., 2009) and itsprevalence, based on intensive widespread serosurveillance and antigen detection, is not known (OIE,2012).

Ranaviruses have been isolated from healthy or diseased frogs, salamanders and reptiles in America, Europe and Australia (Chinchar, 2002; Drury *et al*., 2002;Hyatt *et al*., 2002;Whittington *et al*., 2010). Ranavirus-associated mortality and morbidity has been reported among both farmed and wild fish populations in several countries around the world (Holopainen, 2012).

Worldwide, amphibian populations are declining, with approximately a third of all known amphibian species threatened with extinction (Stuart *et al*., 2004). Factors associated with this global amphibian decline phenomenon include habitat loss, climate change, infectious disease, overexploitation, pollution and introduced species (Beebee and Griffiths, 2005). Also numerous amphibian population declines have been attributed to increased mortality rates following infection with the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* Bd,(Lips *et al*., 2006; Doherty-Bone *et al*., 2013).

Mortalities due to infection with ranavirus, another emerging infectious disease of amphibians, have also been shown to cause amphibian population declines (Teacher *et al*., 2010). When a die-off of an amphibian population is encountered for the first time, it might automatically be assumed that a decline is taking place, but further, often long-term, investigation is needed to determine whether this is the case. The interpretation of investigations into amphibian declines is dependent upon species specific information and requires the teasing apart of causative factors

(e.g. distinguishing natural fluctuations from anthropogenic impacts)(Pechmann and Wilbur,1994). Such investigations can be challenging, especially in Africa where many amphibian faunas have been less studied and background data of amphibian population dynamics, habitat requirements and background pathogen presence are lacking (Doherty-Bone *et al*., 2013).

2.5 Transmission of Ranavirus

Dissemination of ranavirus infection has been enhanced by introduction of foreign amphibian species into naıve populations (Harp and Petranka, 2006). Ranavirus infections can occur from animal-to-animal contact, ingestion of infected, dying and dead individuals (Cullen and Owens, 2002; Picco and Collins, 2008). Viruses can also be spread between widely separated river systems and impoundments. Transmission is understood to occur by means other than water, e.g. translocation of live fish or bait by recreational fishers (Pico *et al*., 2007; OIE,2012)

When infected frogs migrate from a pond in the autumn they may be in contact with other frog populations and contaminate them. Viruses could also spread by vectors such as birds, fish or downstream via the water (Ariel *et al*., 2009).It can be readily transmitted from one location to another on nets, fishing lines, boats and other equipment (Whittington *et al*., 1999). The risk of ranavirus outbreaks is not limited to amphibians. Evidence of transmission of ranavirus between frogs and fish has been recorded by Mao *et al*.(1999). One host species may act as ranavirus reservoir for the other (Mao *et al*., 1999; Duffus *et al*., 2008). EHNV, ECV and European sheatfish virus (ESV) are listed by the OIE for fish and they are closely related to this new strain. All the listed viruses were involved in natural outbreaks in wild populations of perch,

catfish and sheatfish (Bigarré *et al*., 2008) and several other species of fish and frogs are experimentally susceptible to ranavirus infection (Bang - Jensen *et a*l., 2009).

2.6 Clinical Features of Ranavirus Infection in Affected Species

Infection with *Ranavirus* can cause two disease syndromes; ulcerative skin syndrome, characterised by dermal ulceration and haemorrhagic syndrome, characterised by systemic haemorrhaging within the skeletal muscles and visceral organs (Cunningham*et al.*, 1996). In most cases, the first sign of an outbreak of ranavirus infection is the sudden onset of illness and death in large numbers of amphibians and/or reptiles over a 1-5 day period. At necropsy, there may be fluid accumulation in the body cavity and hemorrhages on the surfaces of the heart, stomach, and liver. Occasionally, the liver or spleen is affected and ulcers in the skin and palate may be observed.

Mass mortality events may last from days to months (Converse *et al*., 2005). However, later deaths may be due to individuals succumbing to secondary invaders (e.g., bacterial, fungal infections) rather than ranavirus (Warne *et al*., 2011). Mortality is often the only clinical finding reported in cases of ranaviral disease; however, weak or erratic swimming, buoyancy problems, gasping for air, lethargy, and anorexia frequently occur (Converse *et al*., 2005). Miller *et al*. 2011 reports that in fatal cases, gross findings may include swelling of the legs and body, erythema (redness) of the legs and ventrum, ecchymosis (red blotches) near the vent and/or urostyle; petechiation (pinpoint hemorrhages) or ecchymosis of the skin and internal organs (especially the mesonephros (kidneys and liver); and irregular patches of discoloration on the skin. In metamorphs and adults, the gastrointestinal tract may be empty or contain minimal ingesta and the gall bladder may be enlarged, both of which are consistent with fasting. Although hemorrhages and swellings are the most common gross lesions noted in the larvae,

cutaneous erosions and ulcerations are more frequently seen in adult anurans in Europe and adult caudates in North America (Bollinger *et al.,* 1999). There have been additional gross lesions reported in caudates, including hemorrhages on the plantar surfaces of the feet and tail and edema in the gular region(Converse *et al*., 2005). Jancovich *et al*.(1997) further described lesions spanning 4 stages of ranaviral disease in experimentally challenged tiger salamanders (*Ambystoma tigrinum*). In Stage 1 white polypoid lesions covered approximately 10% of the body, followed by Stage 2 wherein they covered 50% of the body. By Stage 3, the polyps covered nearly 90% of the body and epidermal hemorrhages were occasionally noted. By Stage 4, hemorrhages were common and were present in internal organs, as well as in the epidermis, in addition to lethargy, anorexia, buoyancy problems, and bloody discharge. Interestingly, the polypoid lesions observed by Jancovich *et al*.(1997) in the experimentally-challenged salamanders were not observed in field collected specimens (Miller *et al*., 2011).

2.7 Replication and Pathogenecity of Ranaviruses

Virions enter cells by one of two possible routes and initial events in virus replication (early viral transcription and the synthesis of unit length genomes) take place within the nucleus. Viral genomes are subsequently transported into the cytoplasm where they are methylated and serve as templates for concatemer formation. Viral assembly sites contain viral DNA and a number of virus-encoded proteins and serve as the loci of virion formation. Newly synthesized virions are found free within the cytoplasm or within paracrystalline arrays, and, a minority, at least in vitro, bud from the plasma membrane and in the process acquire an envelope (Jankovich *et al*.,2015).

Ranaviruses replicate at temperatures between 12 and 32 C, because of this, birds and mammals are not suitable hosts (Chinchar, 2002). *Ranavirus* isolates from the UK grow *in vitro* between 8-30°C, with slower replication below 15°C and the fastest replication at 30°C (Cunningham, 2001). Likely reflecting this temperature dependence, deaths caused by *Ranavirus* at all developmental stages (larval, metamorph and adults) occur predominantly in the summer months (Chinchar, 2002). Findings from North American ranaviruses in other host species have, however, shown that lower temperatures can cause increased mortality, perhaps due to immunosuppression at low temperatures (Rojas*et al.*, 2005).

Ranaviral replication (Figure 2.2) is well-studied using the type species for the genus, frog virus 3 (FV3) (Chinchar *et al*., 2005). Ranaviruses enter the host cell by receptor-mediated endocytosis (Eaton *et al*., 2010).Viral particles are uncoated and subsequently move into the cell nucleus, where viral DNA replication begins via a virally encoded DNA polymerase (Goorha,1982). Viral DNA then abandons the cell nucleus and begins the second stage of DNA replication in the cytoplasm, ultimately forming DNA concatemers (Goorha, 1982). The viral DNA is then packaged via a headful mechanism into infectious virions (Chinchar *et al*., 2005).The *ranavirus* genome, like other iridoviral genomes is circularly permuted and exhibits terminally redundant DNA (Goorha, 1982).

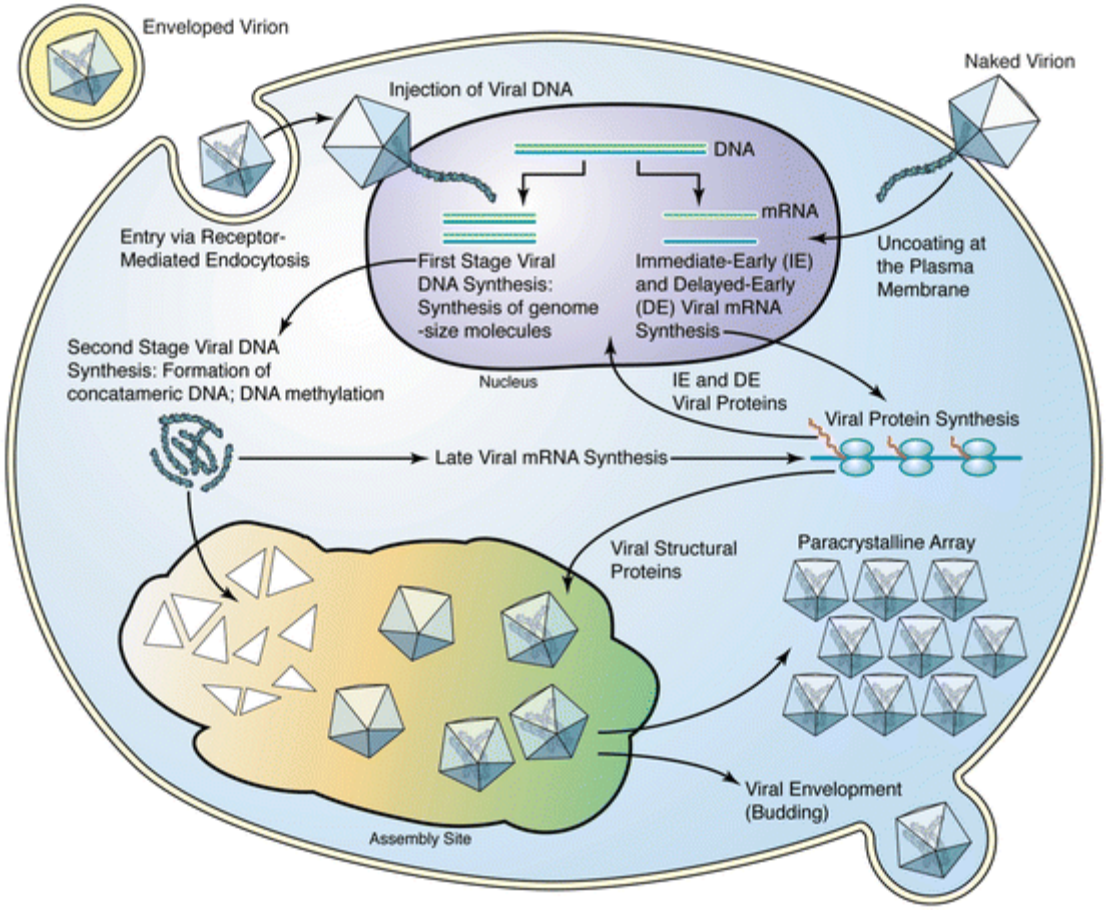


Figure 2.2: Schematic diagram of ranavirus replication (Jankovich *et al*., 2015).

2.7.1 Species Susceptibility

Susceptibility to ranaviruses varies among populations within species and across phylogenetic lineages (Schock *et al*., 2010). Among populations, the amount of genetic variability appears to be correlated with ranavirus susceptibility (Miller *et al*., 2011).

The susceptibility to ranaviruses differs among amphibian developmental stages, even in the same species (Gray *et al*.,2009). The adult stage tends to be the least susceptible, probably owing to more competent immune function compared to pre-metamorphic stages (Robert *et al*.,2005). However, die-offs of adult frog and newt species in the wild have been reported in Europe (Ariel *et* al., 2009). In captivity, adult mortality from ranaviral disease is common (Miller *et al*., 2007), and may be a consequence of increased transmission associated with high host density, elevated viral titers, or stress induced from the unnatural environment. Lastly, environmental conditions such as ambient temperature or pesticides have been associated with increased prevalence of ranavirus infection and disease (Gray *et al*., 2007; Miller *et al*., 2011)

2.8 Diagnosis

Detection of infected individuals, particularly carriers, is necessary to prevent and control outbreaks. (Forza´n and Wood, 2013). Prevention of the spread of disease, in the wild or in captivity, requires accurate diagnosis of the infection status of representative individuals in a population. Detection of ranavirus infection in frogs can be achieved by PCR testing on liver tissue (from adults and tadploes) (Forza´n and Wood, 2013).

2.8.1 Field diagnostic methods

Field diagnostic methods involve using clinical signs and behavioural changes to diagnose infection.

2.8.1.1 Clinical signs

There are two syndromes in frogs, fish and reptiles associated with ranavirus infection: a chronic ulcerative syndrome and an acute haemorrhagic syndrome (Cunningham *et al*., 1996). Salamanders infected with *Ambystoma tigrinum* virus develop ulcerative dermatitis and enteritis. Affected larvae have small multifocal haemorrhages affecting subcutaneous tissue on the plantar surface of feet, the inguinal area, and the vent area, with ventral oedema and the skin may contain pale foci (Bollinger *et al*., 1999; Docherty *et al*., 2003).

2.8.1.2 Behavioural changes

Field and behaviour changes differ between species of fish and frogs, life stage and severity of disease. Changes include lordosis, erratic swimming, lethargy and loss of equilibrium (Gray *et al*., 2009).

2.8.2 Clinical diagnostic methods

Clinical diagnostic methodsinvolve the use of gross pathology and histopathology for diagnosis.

2.8.2.1 Gross pathology

There may be no gross lesions or non-specific lesions. There are two syndromes in frogs associated with ranavirus infection: ulcerative syndrome and haemorrhagic syndrome(Cunningham *et al*., 1996). In salamanders infected with Ambystoma tigrinum virus there may be ulcerative dermatitis, pale foci in the skin, small multifocal haemorrhages affecting subcutaneous tissue on the plantar surface of feet, the inguinal area, the vent, the subserosal surface of the intestine, and the liver may be pale and swollen; there may be ventral oedema (Bollinger *et al*., 1999; Docherty *et al*., 2003).

2.8.2.2 Histopathology

The BIV and FV3 cause multifocal multi-organ haemorrhage and necrosis (Cullen and Owens, 2002; Robert *et al*., 2005). Salamanders infected with *Ambystoma tigrinum* virus develop necrosis in many tissues including spleen, liver, renal tubular epithelial cells, and lymphoid and haematopoietic tissues (Bollinger *et al*., 1999). Amphophilic intracytoplasmic inclusion bodies may be present in cells in many organs together with single cell or variable sized areas of focal necrosis (Bollinger *et al*., 1999, Docherty *et al*., 2003). In skin there may be foci of spongiosis and ballooning degeneration, erosion and ulceration and hyperplasia of epidermal epithelial cells which may have intracytoplasmic inclusion bodies (Bollinger *et al*., 1999). In electron microscopy/cytopathology, affected tissues (e.g. kidney liver and spleen) contain cells exhibiting necrosis. Cells contain conspicuous cytoplasmic inclusions that are rarefied areas of the cytoplasm in which the viruses are assembled. Within the cytoplasm, aggregates (paracrystalline arrays) of large (ranaviruses can vary greatly in size ranging from approximately 150 nm to -180 nm) non-enveloped icosahedral viruses are apparent; single viruses are also present. Complete viruses (containing electron-dense cores) bud/egress from the infected cells through

the plasma membrane. The nuclei of infected cells are frequently located peripherally and are distorted in shape (OIE, 2012).

Figure 2.3 shows the clinical manifestation of Ranavirus in infected American bullfrog and wood frog tadpole. Plate 1) Showing marked swelling of the body in a ranavirus infected American bullfrog. (II) Showing hemorrhages in a ranavirus infected wood frog tadpole. (III) Phytomicrograph of mesonephros (kidney) from a ranavirus infected tadpole showing necrosis of the hematopoietic tissue and degeneration and necrosis of a glomerulus. (IV) Phytomicrograph of mesonephros (kidney) from an uninfected tadpole (Miller *et al*., 2011).

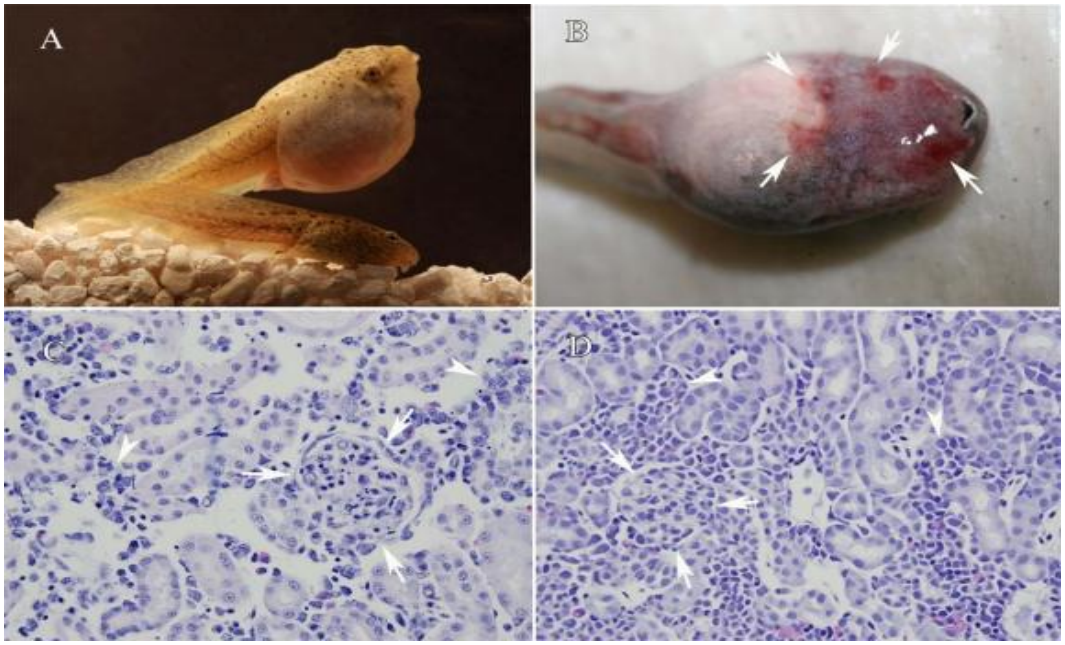


Figure 2.3 (Plates I – IV): Images of observed pathologies for ranavirus infection in bullfrogs and tadpoles (Miller *et al*., 2011).

2.8.3 Agent detection and identification methods

Ranavirus are detected using direct detection methods (microscopy), agent isolation and identification, immunoelectron microscopy, PCR and other molecular techniques.

2.8.3.1 Direct detection methods

Direct detection methods detect Ranaviruses using microscopic methods.

2.8.3.1.1 Microscopic methods

Includes light microscopy, electron microscopy, negative contrast electron microscopy.

2.8.3.1.1.1 Light microscopy

Routine microscopy methods can be used for tissue fixation in 10% buffered neutral formalin, paraffin embedding, preparation of 10 µm sections and staining with H&E to demonstrate tissue necrosis and basophilic intracytoplasmic inclusion bodies. These inclusion bodies are indicative but not confirmatory for ranavirus. Formalin-fixed paraffin-embedded sections can also be stained using an immunoperoxidase method (Hyatt, 1991)

2.8.3.1.1.2 Electron microscopy

Ultrathin routine sectioning methods can be used for preparation of tissues and cell cultures (Eaton *et al*., 1991) to demonstrate tissue necrosis, presence of viruses and virus inclusion

bodies. Tissues and cells fixed with an alternative fixation and embedding regime can be used for antigen detection (Hyatt, 1991).

2.8.3.1.1.3 Negative contrast electron microscopy

Supernatants from dounce homogenised tissues (10% [w/v]) and cell cultures can be used to detect viruses. Ranaviruses have a definitive appearance. They vary in diameter (150–180 nm) and have a limiting cell-derived (plasma membrane) envelope that surrounds a capsid of skewed symmetry. Underlying the capsid is a de novo membrane that itself surrounds a core containing the double-stranded (ds) DNA and minor proteins. These preparations can also be used to confirm ranavirus antigenicity (Eaton *et al*., 1991).

2.8.3.2 Agent isolation and identification

Cell culture is the gold-standard test but is costly and time consuming. Ranaviruses grow well in many fish cell lines including BF-2 (bluegill fry ATCC CCL 91), FHM (fathead minnow; ATCC CCL 42), EPC (epithelioma papulosum cyprini [Fijan *et al*., 1983]), and CHSE-214 (Chinook salmon embryo cell line; ATCC CRL 1681) at temperatures ranging from 15 to 22°C (Crane *et al*., 2005).

The identity of viruses in cell culture is determined by Immunostaining, ELISA, Immuno-electron microscopy, Polymerase chain reaction (PCR) and other methods (OIE,2012)

2.8.3.2.1 Detection of ranaviruses using immunoperoxidase staining of infected cell cultures

Principle of the test: ranaviruses replicate within cultured cells. The addition of a mild detergent permeabilises the cells allowing an affinity purified rabbit antibody to bind to intracellular viral

proteins. Ranavirus is detected by a biotinylated anti-species antibody and a streptavidin–peroxidase conjugate. The addition of a substrate results in 'brick-red' staining in areas labelled with antibodies. The staining is conspicuous and specific. However, the test has not been validated with respect to sensitivity or reproducibility (OIE,2012)

2.8.3.2.2 Detection of ranavirus using antigen-capture ELISA

ELISA has been validated to detect EHNV in cell cultures and directly in fish tissue homogenates. The same assay can be applied to amphibian tissues. The analytical sensitivity is 103 to 104 TCID50ml–1. Specificity approaches 100% and sensitivity for direct detection in fish tissues is 60% relative to the gold standard of virus isolation in BF-2 cells (Drury *et al*., 1995; Marsh *et al*., 2002). ELISA is useful for both diagnosis and certification (OIE,2012). This ELISA test is based on the principle that EHNV particles are captured from the sample by an affinity purified rabbit antibody that is coated to the plate. EHNV is detected by a second antibody and a peroxidaselabelled conjugate using the chromogen ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid). The enzyme is inactivated after 20 minutes and the resulting optical density (OD) is compared with standards (OIE,2012).

2.8.3.2.3 Immunoelectron microscopy

Gold-labelling of sections containing tissues or cell cultures

Principle of the test: cell cultures, tissues and/or tissue homogenates can be used for examination by electron microscopy. Conventional electron microscopy (examination of ultra-thin sections) will generate data on virus structure and morphogenesis. Negative contrast electron microscopy will produce images that can be used to examine the particulate structure of the virus. The use of ranavirus-specific antibodies and conjugated gold with these preparations permits both

ultrastructure and antigenicity to be examined (Hyatt, 1991). These collective data enable classification to the genus Ranavirus. Viruses within the cytoplasm of infected cells will be specifically gold-labelled. Viruses will be located singularly, within assembly bodies (inclusion bodies) and within paracrystalline arrays.

2.8.3.2.4 PCR and other molecular techniques

Identification of ranavirus at genus and species level is also possible using two PCR methods based on the MCP gene. In the first method, two PCR assays using MCP primers are used with restriction analysis to detect and rapidly differentiate fish ranaviruses (EHNV, ECV) from amphibian ranaviruses (FV3, BIV) (Harp and Petranka, 2006). This can be completed in less than 24 hours at relatively low cost. In the second method, a single MCP PCR assay is used to generate a 580 bp product, which is then sequenced to identify the type of ranavirus (OIE, 2012).

2.8.3.2.4.1 PCR and restriction endonuclease analysis

Amplified product from PCR assay MCP-1 is digested with PflM I and enables differentiation of Australian iridoviruses (EHNV and BIV) from non-Australian iridoviruses (FV3, Americas; and ECV, Europe). Amplified product from PCR assay MCP-2 digested with Hinc II, Acc I and Fnu4H I (individually) enables differentiation of EHNV and BIV (Australia) from each other and from FV3 (Americas) and ECV (Europe) (OIE, 2012).

2.8.3.2.4.2 Alternative PCR and sequencing for viral identification

In this assay two primers, a reverse primer (5′-AAA-GAC-CCG-TTT-TGC-AGC-AAA-C-3') and a forward primer (5′-CGC-AGT-CAA-GGC-CTT-GAT-GT-3'), are used for amplification

of the target MCP sequence (580 base pairs [bp]) of EHNV DNA by PCR (OIE, 2012). This PCR procedure can be used for the specific detection of ranaviruses from redfin perch, rainbow trout, sheatfish, catfish, guppy fish (*Poecilia reticulata*), doctor fish (*Labroides dimidatus*) and a range of amphibian ranaviruses (Eaton *et al*., 1991). Nucleic acid (1 µl) is added to Taq polymerase(Promega) buffer containing 0.1 µM of each primer, 2.5 U Taq polymerase (Promega) and 2.5 mM MgCl2. The mixture is incubated in an automatic thermal cycler programmed for 35 cycles at 95°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, and finally held at 72°C for 15 minutes. Amplified DNA (580 bp) is analysed by agarose gel electrophoresis, excised and sequenced using a range of standard technologies). Each viral species is identified by its unique DNA sequence available from GenBank. Samples can be submitted to the OIE reference laboratory for specific identification.

The definition of confirmed case of ranavirus are demonstrated by the Characteristic CPE in cell culture in which cell culture is positive for ranavirus in immunoperoxidase test or antigen-capture ELISA or PCR, or tissues positive in antigen-capture ELISA or immunoperoxidase stain or immunoelectron microscopy or PCR. Sequences consistent with ranavirus are demonstrated by PCR-REA or PCR-sequencing (OIE,2012).

2.9 Prevention and Control

There are no known methods of prevention or control. Since there is no treatment for ranavirus infection, management is focused on quarantine and sterilization to prevent the spread of the virus to other animals and new environments. Any infected animals should be quarantined to prevent infection of other animals. Water from captive facilities and home aquariums should be disinfected prior to disposal to prevent the virus from spreading into new water sources. All

equipment, surfaces, and storage tanks in captive facilities should be disinfected after use (CWHC, 2017).

The use of specific pathogen-free (SPF) stocks under biosecure conditions is the recommended method for prevention of ranavirus disease in amphibian or fish farms. Infected amphibians, fish or reptiles should not be transported into areas known to be free of ranavirus. A 4% concentration of sodium hypochlorite can be used to disinfect premises and fomites (Miocevic *et al*., 1993).

By maintaining good general husbandry practices, disease control at the farm level relies on reducing the impact of infection by maintaining low stocking rates and adequate water quality. The mechanism of protection may be through maintenance of healthy integument (OIE, 2012).

2.10 Treatment

There is currently no treatment or vaccine agent for ranavirus infection (OIE,2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was carried out in Zaria (Figure 3.1), Kaduna State; an ancient city popularly known as the centre of learning, leading to the influx of people to the town and making it an increasingly developing town in the last few decades (Agbogu *et al.*, 2006).Zaria lies 2, 800 feet above sea level and is located between Latitude 11^0-04'N and 7^0-42'E longitude, with a total area of about 300km^2 and a population of 408,198 (NPC, 2006). It has an annual rainfall of 1092.8mm and monthly mean temperature ranging from 13.8°C to 36.7°C (Agbogu *et al.*, 2006).

Zaria is divided into Zaria and Sabon Gari Local Government Areas administratively (Ministry of Economic Development, 1996). Approximately 40 – 70% of its working population derive their livelihood from agriculture with a substantial number engaging in fishing/fish farming (Agbogu *et al.*, 2006).

3.2 Study Design

A cross sectionaldesign method was employed forthis study, where fresh fish and edible frogs were randomly collected and used for the study.

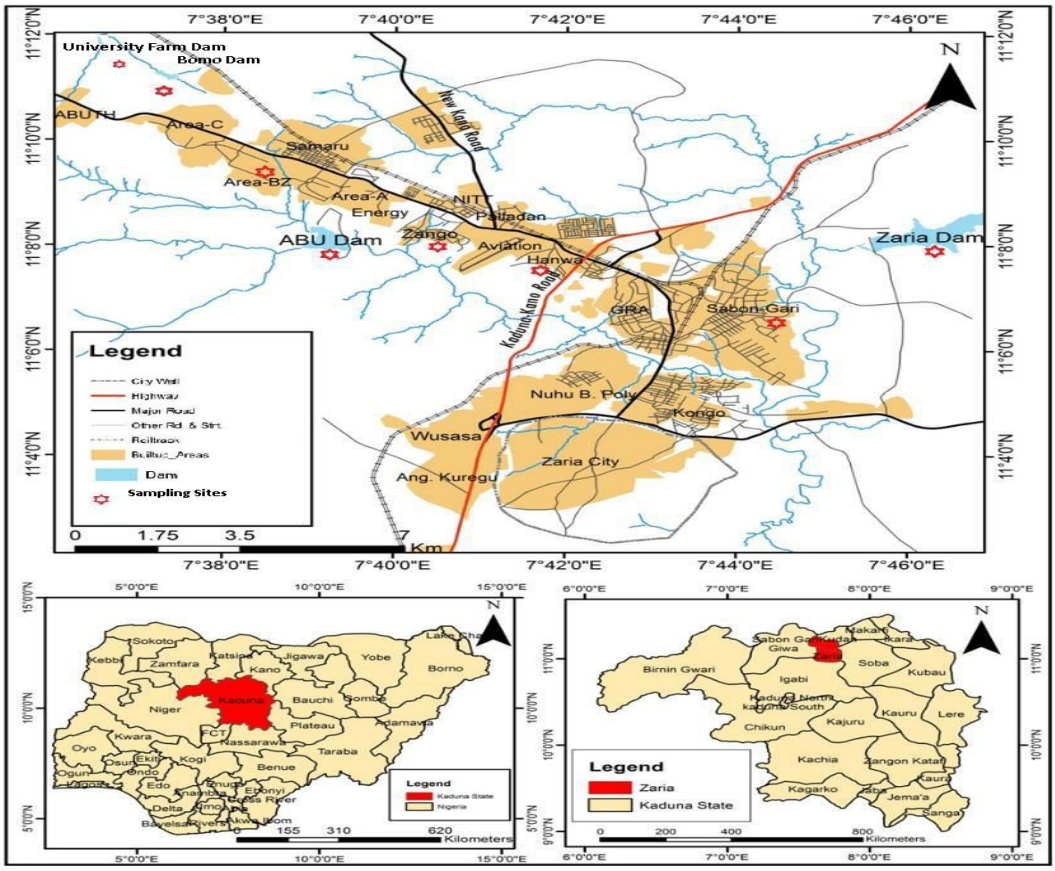


Figure 3.1: Map of Zaria showing Zaria and its environs andthe sampling areas

Source: Adopted and modified from satellite imagery (2013).

3.2.1 Questionnaire adminstration

A structured questionnaire was administered to 37 fishermen (Appendix I), 21 to edible frog catchers (Appendix II) 9 to frog sellers (Appendix III). This was to obtain information on, management practices, observed pathologies commonly seen in fish and edible frogs and information on mortality of fish and edible frogs from the water sources.

3.2.2 Fresh fish and edible frog sampling

Fresh fish and edible frogs were sampled from the different locations as shown in Figure 3.2 and Figure 3.3

3.2.2.1 Sampling technique

A purposive sampling technique was used and fishes were sampled based on their availability from Fish farms; ABU Dam; ABU University farm; Zaria Dam. Edible frogs were also sampled based on availability from ABU Dam; ABU University farm; Zaria Dam, Bomo Dam and the edible frog market monthly from February to June, 2016.

3.3 Sample Size Determination

Using an expected prevalence of 50%,(as there is no available baseline data on Ranavirus in Nigeria), a precision (Maximum acceptable error) of ±5% and a confidence level of 95%; the number of samples to be collected was determined.

Using the formula, by Thrusfield (2005).

$$N = \frac{Z^2pq}{d^2}$$

N = Sample Size

Z = Standard Normal deviation for 95% confidence interval (1.96)

P = Prevalence (50%)

d = Desired Precision (0.05)

q = 1 – P

$$N = \frac{(1.96)^2 \times 0.50 \times (1-0.50)}{(0.05)^2} = 384$$

=384 of fish and edible frogs

However, 8% was added to increase precision and so 415 samples was arrived at. Thus 215 edible frog samples and 200 fish samples was collected.

3.4 Sample Collection

Two hundred fish and 215 edible frog samples were bought from farms, dams and the Hanwa frog market and used for this study.

3.4.1 Fish samples collection

Fish were purchased from the collection sites i.e farms/dams (Figure 3.2) and labelled according to the location of collection. The fish samples (Appendix V) were then transported in a cold box to the Viral Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria for processing**.**

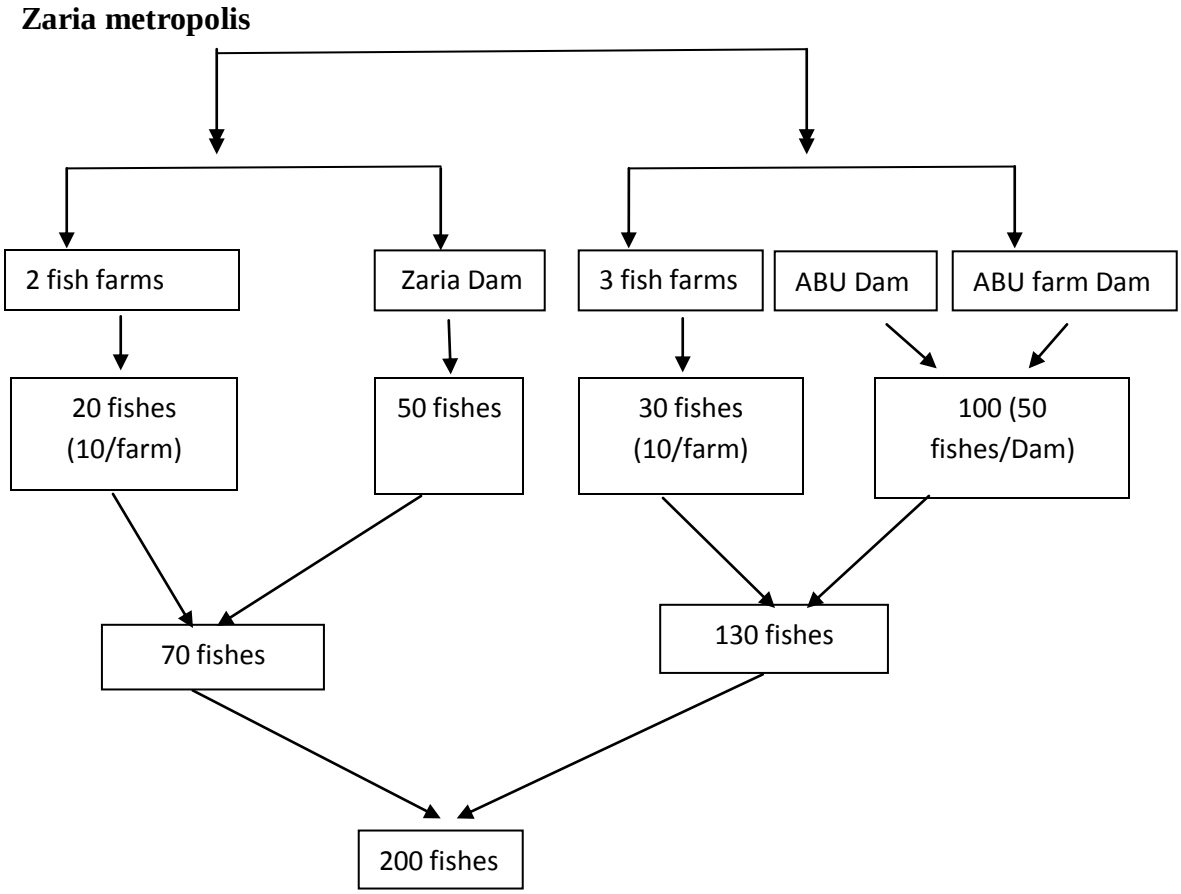


Figure 3.2: Schematic representation of sample collection sites for fish in Zaria, Kaduna State, Nigeria.

3.4.2 Edible frog samples collection

Frogs were purchased from the collection sites as shown in Figure 3.3 and labelled according to the location of collection. The edible frog samples (Appendix VI) were then transported in a cold box to the Viral Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria for processing**.**

3.5 Laboratory Procedures

This includes the measurements of weight and length of fishes and weight of frogs; processing and preparation of fish and frog samples for ELISA; preparation of buffers and reagents and ELISA analysis.

3.5.1 Measurement of fish and frogs

The standard length and total length, of each fish was measured using a measuring tape, and the weight evaluated using a weighing scale. Each frog was placed in a plastic bag with sealed ends to prevent jumping off, and then placed on a weighing scale and the weight taken.

3.5.2 Fish species identification

Fish species sampled were taken to the Department of Biological Sciences, Ahmadu Bello University, Zaria for identification.

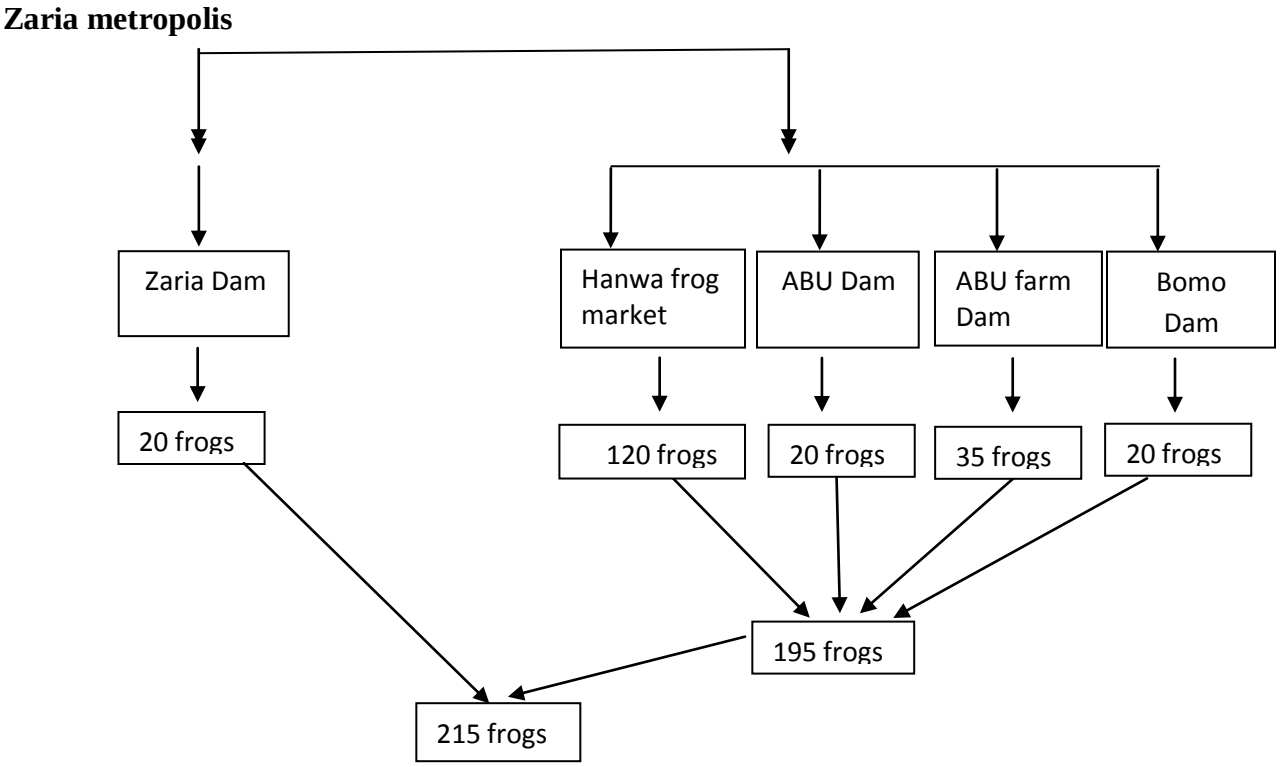


Figure 3.3: Schematic representation of sample collection sites for edible frogs in Zaria, Kaduna State, Nigeria.

3.5.3 Processing of fish samples

Large fishes(>60 mm fork length) was bathed with 70% ethanol for 30 seconds and fingerlings for 5 seconds and then rinsed in deionised water and later dissected. From each large fish, 0.1 g each of liver, kidney, spleen was removed and placed in sterile 1.5 ml tubes suitable for use with pestles for grinding tissues. For medium sized fish (30-60 mm fork length):all viscera were scraped into a sterile tube. For the small fishes (<30 mm fork length): the head and tail were removed, and the rest of fish placed into the sterile tube. All the tubes containing tissues were frozen until used.

3.5.4 Processing of frog samples

Frogs were bathed in 70% ethanol and then rinsed in deionised water before dissection. From each large frog, the liver, kidney, spleen were removed and placed in sterile 1.5 ml tubes suitable for use with pestles for grinding tissues.All the tubes containing tissues were frozen until utilised.

3.5.5 Preparationof fishand frog tissues for ELISA

Half a ml of homogenising medium (minimal essential medium [MEM] Eagle, with Earle's salts and glutamine], 200 International Units [IU] ml^{-1} penicillin, 200 µg ml^{-1} streptomycin and 4 µg ml^{-1} amphotericin B) was added to each tube and the tissue ground to a fine mulch using a sterile glass rod. Another0.5 ml of homogenising medium was added to each tube and mixed using the glass rod.

Threesterile glass beads each of 3 mm diameter wereadded to each tube and the lid of the tubeclosed. Thesuspension was vortexed vigorously for 20–30 seconds and placed in refrigerator at 4°C for 2 hours. Thesuspension was again vortexed as described above and centrifuged for 10 minutes at 2500 g in a benchtopmicrocentrifuge.

Thesupernatant, now called "clarified tissue homogenate", was then transferred to a fresh sterile tube and frozen between –20°C and –80°C until required for ELISA. (Whittington and Steiner, 1993).

3.6 Laboratory Analysis

All samples of fish and frog tissue homogenates were screened for the presence of antigens to Ranavirus using enzyme linked Immunosorbent assay (ELISA).

3.6.1 Preparation of buffers and reagents- see Appendix IV

3.6.2 Test principle

The EHNV particles were captured from the sample by an affinity purified rabbit antibody that is coated to the plate. The EHNV is detected by a second antibody and a peroxidise labelled conjugate using the chromogen ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid). The enzyme is inactivated after 20 minutes and the resulting optical density (OD) is compared with the OIE standards (OIE, 2012). There is cross-reactivity of antibodies to all ranaviruses (Miller *et al*., 2015), So EHNV antigen and antibody was used.

Reagents: Affinity purified-anti-EHNV anitibody, sheep-anti-EHNV antibody and the EHNV control antigenswere provided by the OIE Reference Laboratory, Faculty of Veterinary Science, University of Sydney, Australia. The conjugate, Peroxidase conjugate was purchased from Sigma Aldrich®.

3.6.3 Protocol for analysis

A 96-well ELISA plate (100µl/well) was coated with affinity purified rabbit-anti-EHNV (an antibody) diluted 1/12,800in borate coating buffer and incubated overnight at 4°C. The plate was washed five times with wash buffer (Milli-Q (MQ) purified water plus 0.05% Tween 20).

Blocking solution was prepared (1% [w/v] gelatin inphosphate buffered saline + Tween + gelatin PBSTG diluent [PBS, 0.05% [v/v] Tween 20, 0.1% [w/v] gelatin]) by warming the solutions in a microwave oven or water bath to dissolvethe gelatin, and then cooled to room temperature. The remaining binding sites were then blocked using blocking solutionPBSTG (100µl/well) and then incubated at 37ºC for 30 minutes. The plate was then washed 5 times with wash buffer.

The control antigen was diluted inphosphate bovine serum + Tween + gelatin (PBSTG). The tissue homogenate samples and diluted control antigens was added at 100 µl/wellto the lower right-hand corner of the plate. All test samples and controls wereadded to duplicate wells and incubated for 90 minutes at 37ºC. The wells were then aspirated using a multichannel pipette and the plate washed five times using the wash buffer.

The second antibody, sheep-anti-EHNV diluted 1/32,000 in PBSTG was added at 100 µl/well and Incubated for 90 minutes at 37ºC. The plate was then washed five times in the plate washer.

The conjugate, Peroxidase conjugate (Sigma Aldrich®) was diluted 1/1500 in PBSTG and added at 100 µl/well and incubated for 90 minutes at 37ºC. The plate was then washed five times using wash buffer.

The 2,2'-azino-bis(3- ethylbenzothiazoline-6- sulphonic acid) substrate (ABTS substrate) (22 ml ABTS + 10 µl H_2O_2) was added(100 µl/well) and the plate shaken and incubated for 20 minutes (this step was timed from the moment substrate was added to the first wells of plate 1).

ABTS stop solution (50µl/well) was immediately added and the plate shaken briefly and optical density (OD) read at 405 nm using an ELISA plate reader. The mean ELISA OD of duplicate wells was calculated.

Prevalence was calculated with the formula

$$\text{Prevalence} = \frac{\text{Total no. of positive samples}}{\text{Total no. of samples}} \times 100$$

3.7 Analysis of Physiochemical Parameters of Water

Water samples were collected from the sampling locations using plastic containers sterilized in 10% bleach water andrinsed with boiled water and allowed to dry. They were then used to collect water samples for analysis.

Physiochemical parameters of water namely water temperature, pH, dissolved oxygen and turbidity from the locations from which fish and frogs are sampled from were measured.

3.7.1 Temperature and pH of water at some sampling locations

The hydrogen ion concentration (pH) and temperature of each sample were measured using a HACH digital pH/temperature meter. This was carried out by the insertion of the electrode probe into a glass beaker containing 20 ml of the water sample and the result was read from the screen and recorded. The pH meter was calibrated before and after each reading using freshly prepared pH buffers (7.00), (4.00) and (9.00) (Ologbosere*et al.*,2016).

3.7.2 Dissolved oxygen content of water at some sampling locations

The modified Winkler-azide method (Lind, 1979; APHA, 1985)) was used to determine the dissolved oxygencontent of water.The water sample was collected into a 250ml bottle carefully without allowing air bubbles. Manganous sulphate (1ml) and 1ml of alkali iodide – azide reagent was added. A brown precipitate of basic manganic oxide formed was allowed to settle. 1ml of concentrated sulphuric acid was added and mixed well until the precipitate dissolved. About 100ml of the solution was taken and titrated against sodium thiosulphate until a straw yellow colour appeared. Few drops of starch indicator was added and titrated again until the blue colour disappeared (Lind, 1979;APHA, 1985)

3.7.3 Turbidity of water of some sampling locations

Turbidity was measured using the turbidity tube method which is based on the visual interpretation of the turbidity of water. The visual appearance of black cross mark at the bottom of the tube, through the open end was used for turbidity measurement. PROCEDURE: A well-mixed sample of water collected was poured into the cleaned turbidity tube placed above a white sheet placed on the floor. The open end of the tube was observed to visualize the black markings from the distance of 7 to 10cm. The level of water at which the black mark was seen was noted (Lind, 1979; APHA, 1985).

3.8 Data Analysis

Data generated, were subjected to descriptive statistics using Statistical Package for Social Sciences (IBM SPSS) version 20.0 and results were presented in tables and expressed as frequencies and percentages. Chi square, Fisher's exact test was used to test for associations between variables. P values <0.05 were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of Ranavirus Infection in Fishesand Edible Frogs in Zaria, Kaduna State.

A total of 415 samples, made up of 215 frogs and 200 fishes were collected. Of the 215 frogs sampled, 110(51.2%) were Ranavirus positive by Indirect ELISA test while 43% (86/200) were positive for fishes, giving an overall prevalence rate of 47.2% (196/415) (Table 4.1). There was no statistically significant difference between prevalence in frogs and fish($\chi^2 = 2.7770$, df = 4, p = 0.00) (Table 4.1).

4.2. Prevalence of Ranavirus in Edible Frogs and Fishes Based on Location of Capture

Results obtained for frogs based on location of capture showed Zaria Dam to have the highest prevalence of positive ranavirus antigen in frog samples collected at 65% (13/20) and A.B.U. Dam, the lowest with 20% (Table 4.2).A statistically significant difference was observed between the different locations of frog capture ($\chi^2 = 43.165$, df = 4, p = 0.00)

Fish samples from Zaria Dam also had the highest prevalence of ranavirus infection of 70% (35/50).Sabon Gari pond B was the lowest with 10%, (1/10) while Sabon Gari pond C fish samples were not positive (Table 4.3). There was a statistically significant difference between prevalence of ranavirus and sampling location of fish *(*p = 0.001, fisher's exact test).

Table 4.1: Prevalence of Ranavirus in fishes and edible frogs in Zaria, Kaduna Sate, Nigeria.

	Number Sampled	Number Positive	Percentage Positive (Prevalence)	Chi Square	P-Value
Frogs	215	110	51.2	2.7770	0.096
Fish	200	86	43		
Total	**415**	**196**	**47.2**		

Table 4.2: Prevalence of Ranavirus in frogs based on location of capture in Zaria, Kaduna Sate, Nigeria.

Location	Number Sampled	Number Positive	Percentage Positive (Prevalence)	Chi Square	P-Value
ABU Dam	20	4	20	43.165	0.00
UF Dam	35	18	51.4		
Zaria Dam	20	13	65		
Bomo	20	10	50		
Hanwa frog market	120	65	54.1		
Total	**215**	**110**	**52.1**		

Key:

UF Dam – University Farm Dam

ABU Dam – Ahmadu Bello University Dam

Table 4.3: Prevalence of ranavirus infection in fishes in Zaria, Kaduna State, Nigeria based on sampling location.

Variable	Number Sampled	Number Positive	Percentage Positive (Prevalence)	Fisher's Exact TestP-Value
Sabon Gari A	10	1	10	0.001
Sabon Gari B	10	1	10	
Sabon Gari C	10	0	0	
Samaru A	10	6	60	
Samaru B	10	1	10	
UF Dam	50	16	32	
Zaria Dam	50	35	70	
ABU Dam	50	26	52	
Total	**200**	**86**	**43.0**	

Key:

UF Dam – University Farm Dam

ABU Dam – Ahmadu Bello University Dam

4.3 Prevalence of Ranavirus in Frogs and Fishes Based on Month of Sample Collection

The collection period was from the month of February to the month of June, 2017. The month of June had the highest prevalence rate of 57.1% (16/28) and February had the lowest prevalence of 20% (1/5) (Table 4.4).A non- statistically significant difference was observed between the different months of sample collection and positivity (p = 0.534, fisher's exact test).

For fishes, the prevalence of Ranavirus was found to be highest in the month February with 42.5% (18/40). May, had the lowest with 34% (17/50). However, the differences between the months were not statistically significant (χ^2 = 2.608, df = 3, p = 0.456,) from the other months (Table 4.5).

4.4 Prevalence of Ranavirus Infection in Fresh Fish and Edible Frogs Based on Sex in Zaria, Kaduna State, Nigeria.

Female frogs had a higher percentage positivity of 52.3% (58/111) than male frogs with 50% 52/104), though the difference was not statistically significant (χ^2 = 0.109, df = 1, p = 0.741) (Table 4.6).

Male fishes had a prevalence of 39.8% (41/103) while female fishes had a prevalence of 48.5% (45/97) (Table 4.7). Though there was no significant statistical difference between sex and prevalence of ranavirus in fish(χ^2 = 0.300, df = 1, p = 0.254).

Table 4.4: Prevalence of ranavirus in frogs in Zaria, Kaduna State, Nigeria based on month of sample collection.

Month	Number Sampled	Number Positive	Percentage Positive (Prevalence)	Fisher's Exact Test P-Value
February	5	1	20	0.534
March	43	20	46.5	
April	39	22	56.4	
May	100	51	51	
June	28	16	57.1	
Total	**21511051.2**			

Table 4.5: Prevalence of ranavirus infection in fishes in Zaria, Kaduna State, Nigeria based on month of sampling.

Month of Sampling	Number Sampled	Number Positive	Percentage Positive	Chi Square	P-Value
February	40	17	42.5	2.608	0.456
March	70	34	48.6		
April	40	18	45.0		
May	50	17	34.0		
Total200	**86**				

Table 4.6: Prevalence of ranavirus infection in edible frogs in Zaria, Kaduna State, Nigeria based on sex of frogs

Sex	Number Sampled	Number Positive	Percentage Positive (Prevalence)	Chi Square	P-Value
Male	104	52	50	0.109	0.741
Female	111	58	52.3		
Total	**215**	**11051.2**			

Table 4.7: Prevalence of ranavirus infection in fishes in Zaria, Kaduna State, Nigeria based on sex of fish

Variable	Number Sampled	Number Positive	Percentage Positive	Chi Square	P-Value
Male	103	41	39.8	0.300	0.254
Female	97	45	48.5		
Total	**200**	**86(43%)**			

4.5 Prevalence of Ranavirus in Fish in Zaria, Kaduna State, Nigeria Based on Species of Fish

*Marcusenius senegalensis*species of fish had the highest prevalence of 75% (9/12). *Clarias gariepinus* hadthe lowest prevalence of 26% (25/96) (Table4.8). There was a statistically significant difference between positive samples and species of fish *(*p = 0.005, fisher's exact test).

4.6 Prevalence of Ranavirus Infection in Wild and Farmed Fishes in Zaria, Kaduna State, Nigeria

The wild fishes had a prevalence of 51.3% (71/150) for ranavirus infection while that of the farmed fishes was 18% (9/50) with the difference between them being statistically significant *(*χ^2 = 17.000, df = 1, p = 0.000) (Table 4.9).

4.7 Mean Physiochemical Parameters of Some of the SamplingLocation for RanavirusInfection in Fishes and Edible Frogs in Zaria, Kaduna State, Nigeria

Sabon Gari fish pond C had the highest temperature of 27.4°C and ABU Dam with the lowest temperature (25.2°C). The ABU University farm dam and Samaru fish ponds A and B had a pH of 7.3 with Sabon Gari fish pond A having the lowest pH (6.8). Samaru fish pond B had the highest amount of dissolved oxygen (2.8mg/l) and Samaru fish pond A had the lowest (1.9mg/l). Samaru fish pond B had the highest turbidity with 59.9 ppm and Sabon Gari fish pond B had the lowest with 44.4ppm. Samaru fish pond B had the highest PH, dissolved oxygen and turbidity.

Table 4.8: Prevalence of ranavirus infection in fishes in Zaria, Kaduna State, Nigeria based on species of fish

Fish specie	Number Sampled	Number Positive	Percentage Positive	Fisher's Exact Test	P-Value
Clarias gariepinus	96	25	26	28.427	0.000
Labeo senegalensis	5	2	40		
Lates niloticus	12	7	58.3		
Marcusenius senegalensis	12	9	75		
Oreochromis niloticus	27	18	66.7		
Schilbe mystus	21	14	66.7		
Tilapia zilli	27	11	40.7		
Total	**200**	**86**			

Table 4.9: Prevalence of ranavirus infection in wild and farmed fish in Zaria, Kaduna State, Nigeria

Variable	Number Sampled	Number Positive	Percentage Positive (Prevalence %)	Chi Square	P-Value
Farmed fish	50	9	18	17.000	0.000
Wild fish	150	77	51.3		
Total	**200**	**86(43%)**			

Table 4.10: Mean physiochemical parameters of some of the sampling locations for ranavirus infection in fishes and edible frogs in Zaria, Kaduna State, Nigeria

Sampling location	Temperature (°C)	pH	Dissolved oxygen (mg/l)	Turbidity (ppm)
ABU Dam	25.2	7.2	2.7	49.1
Zaria Dam	26.2	7.1	2.3	45.9
University Farm Dam	26	7.3	2.6	48.7
Samaru A	25.7	7.3	1.9	45.6
Samaru B	25.2	7.3	2.8	59.9
Sabon Gari A	26.1	6.8	2.0	46.3
Sabon Gari B	25.3	7.2	2.3	44.4
Sabon Gari C	27.4	6.9	2.3	52
WHO standards	30	6.5-8.5	3.0	15
FME limits	20-33	6.0-9.0	7.5	15

Keys:
WHO – World Health Organization
FME - Federal Ministry of Environment, Nigeria

4.8Demographic Profile of Fishermen, Frog Harvesters and Frog Sellers in Zaria, Kaduna State, Nigeria.

A total of 37 questionnaires were administered to fishermen, 21 to frog harvesters and 9 to frog sellers. Most of the people interviewed were between ages 21 – 40. Majority have had some form of education and were very involved with farming (Table 4.11 – frog harvesters, Table 4.12 – fishermen, Table 4.13 – frog sellers).

Majority fished or harvested frogs for income, with 81% of the frog harvesters doing so mainly for income while 19% did so for income and personal use. Also 64.9% of fishermen interviewed fished for income, 3% fished for personal use and 24.3% fished both for income and personal use (Table 4.14).

4.9 Practices of Fishermen and Frog Harvesters in Zaria, Kaduna State, Nigeria.

Regarding the management ofequipment's used to fish or catch frogs after each catch, 2.7% of fishermen indicated washing of their fishing nets with soap, 35.1% only rinsed their nets,56.8% just hanged the nets to dry after fishing while 5.4 did nothing. For the frog harvesters, 61.9% did nothing to their nets, while 38.1% left theirs out to dry after frog catching (Table 4.15).

On the issue of what is done to dead fishes found in water, 37.8% of fishermen left the dead fishes in water, 2.7% harvest for sale, 40.5% normally harvest and throw away and 13.5% harvest to feed animals (Table 4.15).

Table 4.11: Demographic profile of frog harvesters in Zaria, Kaduna State, Nigeria

Variables	Frequency	Percent (%)
Age		
<20	2	9.5
21-40	11	52.4
41-60	8	38.1
Total	**21**	**100**
Occupation		
Farming	7	33.3
Fishing	7	33.3
Civil Servant	1	4.8
Trading	4	19
Student	2	9.5
Total	**21**	**100**
Education		
Primary	3	14.3
Secondary	7	33.3
Tertiary	2	9.5
Islamic school	3	14.3
None	6	28.6
Total	**21**	**100**

Table 4.12: Demographic profile of fishermen in Zaria, Kaduna State, Nigeria

Variables	Frequency	Percent(%)
Age		
<20	1	2.7
21-40	20	54.1
41-60	15	40.5
>60	1	2.7
Total	**37**	**100**
Occupation		
Farming, Fishing	23	62.2
Farming, Fishing, Trading	13	35.1
Civil Servant	1	2.7
Total	**37**	**100**
Education		
Primary	11	29.7
Secondary	2	5.4
Tertiary	1	2.7
Islamic School	12	32.4
None	11	29.7
Total	**37**	**100**

Table 4.13: Demographic profile of frog sellers in Zaria, Kaduna State, Nigeria

Variables	Frequency	Percent(%)
Sex		
Male	6	66.7
Female	3	33.3
Total	**9**	**100**
Age		
21-40	3	33.3
41-60	6	66.7
Total	**9**	**100**
Occupation		
Farming	2	22.2
Farming, Trading	1	11.1
Civil Servant	1	11.1
Trading	5	55.6
Total	**9**	**100**
Education		
Secondary	6	66.7
Tertiary	1	11.1
Islamic school	1	11.1
None	1	11.1
Total	**9**	**100**

Table 4.14: Reason for fishing and frog harvesting by fishermen and frog harvesters in Zaria, Kaduna State, Nigeria.

Variables	Fishermen		Frog harvesters	
	Frequency	Percent (%)	Frequency	Percent (%)
Reason				
Income generation	24	64.9	17	81.0
Family Use	3	8.1	0	0
Family use and Income generation	10	27.0	4	19.0
Total37 **100**	**21**	**100**		

Table 4.15: Practices of fishermen and frog harvesters in Zaria, Kaduna State, Nigeria.

Variables	Fishermen		Frog harvesters	
	Frequency	Percent (%)	Frequency	Percent (%)
Action after catch (management of nets)				
Wash with soap	1	2.7	0	0
Rinse and hang to dry	13	35.1	0	0
Hang to dry	21	56.8	8	38.1
Nothing	2	5.4	13	61.9
Total	**37**	**100**	**21**	**100**
Action on dead fishes/frogs				
Leave in water	15	40.5	15	71.4
Harvest and Sell	2	5.4	0	0
Harvest and throw away	15	40.5	6	28.6
Harvest and feed to dogs	5	13.5	0	0
Total	**37**	**100**	**21**	**100**

4.10 Respondents Observed Frog and Fish Mortality, Mortality Occurrence, Season of Occurrence and Appearance of Dead Frogs and Fish

A high percentage of the fishermen observed cases of massive mortality of fish with 91.9%of fishermen having observed massive mortality and 8.1% had never. Also 55.9% had observed it more than once, 38.2% just once and 5.9% said fish death was observed yearly. 73.5% of fishermen respondents said the rainy season was the period mass mortality was observed. 20.6% said the hot dry season and 5.9% the cold dry season.Similarly,44.1% observed bloated fishes, 17.7% observed blood blotches on the skin and 38.2% observed the fishes being both bloated and had haemorrhages (Table 4.16).

For frog harvesters, 76.2% had observed cases of massive mortality and 23.8% had never observed such. Majority of the frog catchers said they have observed such mortality occurring during the rainy season with a frequency percentage of 68.8% and the hot dry season with 31.3%. Also 62.5% observed bloated frogs, 12.5% blood blotches on the skin while 25.0% observed bloated frogs with haemorrhages (Table 4.16).

4.11 Respondents Observed Frequencies of Suspected Cause of Death of Fishes and Frogs by Fishermen and Frog Catchers in Zaria, Kaduna State, Nigeria

Death of fishes and frogs were believed to be caused by disease and chemicals with 45.9% of the fishermen saying that a combination of disease and chemicals caused death, 32.4% said death was caused by exposure to chemicals, while 10.8% said death was caused by disease but 10.8% had no opinion of the cause of death in fish. Also 81% of the frog harvesters attributed death to disease and chemicals, while 19% believed chemicals to be the main cause of death (Table 4.17).

Table 4.16: Respondents Observed frog and fish mass mortality, observed mortality occurrence, season of occurrence and appearance of dead frogs and fish

Variables	Fishermen		Frog harvesters	
	Frequency	Percent (%)	Frequency	Percent (%)
Observed mass mortality				
Yes	34	91.9	16	76.2
No	3	8.1	5	23.8
Total	**37**	**100**	**21**	**100**
Observed Mortality Occurrence				
Once	13	38.2	10	62.5
More than Once	19	55.9	6	37.5
Yearly	2	5.9	0	0
Total	**34**	**100**	**16**	**100**
Season of observed Mortality				
Rainy season	25	73.5	11	68.8
Cold dry season	2	5.9	0	0
Hot dry season	7	20.6	5	31.2
Total	**34**	**100**	**16**	**100**
Appearance of dead fishes/frogs				
Bloated	15	44.1	10	62.5
Blood blotches	6	17.7	2	12.5
Bloated and blood blotches	13	38.2	4	25.0
Total	**34**	**100**	**16**	**100**

Table4.17: Respondents observed frequencies of suspected cause of death of fishes and frogs by fishermen and frog catchers in Zaria, Kaduna State, Nigeria

Variables	Fishermen		Frog harvesters	
	Frequency	Percent (%)	Frequency	Percent (%)
Cause of death				
Disease	4	10.8	0	0
Chemicals	12	32.4	4	19
Disease and Chemicals	17	45.9	17	81
No idea	4	10.8	0	0
Total	**37**	**100**	**21**	**100**

CHAPTER FIVE

5.0 DISCUSSION

The study showed a total prevalence rate of ranavirus infection in fishes and frogs in Zaria, Kaduna State to be 47.2% with a prevalence of 51.2% in frogs and 43.0% in fishes. The prevalence rates obtained in this study are similar to those reported by other works: Gray *et al*.(2007) who found that 34% of American bullfrogs (*L. catesbeianus*) and 30% of green frog (*L. clamitans*) tadpoles inhabiting eight farm ponds were positive for ranavirus, though at the time of collection and necropsy, they did not present any visible symptoms for ranaviral disease. Hoverman *et al*.(2012) obtained a higher prevalence rateof 83%in an expanded study at 40 different sites in Tennessee, USA with only one population experiencing a die-off from ranaviral disease. Brunner *et al*. (2004) reported subclinical, but infected, salamanders returning to breeding sites. These findingsagree with that of this study as there was no observed die off or massive mortality during the study periodthough fish and frogs tested positive to ranavirus infection by ELISA testindicating that ranavirus isolates are not always virulent (Chinchar *et al*., 2005). Subclinical infections have been documented, although their role in ranavirus persistence and emergence remains unclear (Miller *et al*., 2011).

There was no statistically significant relationship between prevalence of ranavirus and month of capture in this study. This could be because the sampling time frame is short. Period of sampling in this study is similar to the period of sampling in a study by Kolby *et al*.(2015) in Hong Kong who detected ranavirus in 5 out of 97 amphibians sampled between February to April. A study by Schloegel *et al*.(2009) on imported frogs in the US reported frogs collected in spring to have the highest probability of ranavirus infection followed by those collected in summer, fall and

winter with significant differences between frogs collected in spring and summer and those collected in winter ($p < 0.05$).

Female frogs and fishes had a higher percentage positive prevalence for ranavirus than male frogs and fishes, although, this was not statistically significant. Similarly, in a study by Doherty-Bone *et al.* (2013), there was no significant difference between the number of infected male or female frogs ($p > 0.05$) suggesting no apparent relationship between the gender of frogs and frog disease.

All species of fish sampled in this study were positive for ranavirus. *Marcusenius senegalensis*had the highest overall prevalence for ranavirus. Several studies report ranavirus diseases in different species of fish; two ranaviruses have been detected in dead catfish collected in France in two lakes during the summer of 2007 by Bigarre *et al*.(2008). Whittington *et al*.(2010) also reported epizootic haemtopoietic necrosis virus (EHNV) to cause epizootic mortality in finfish in Australia. Ariel and Owens(1997) reported epizootic mortalities in Tilapia *Oreochromis mossambicus.* The Ranavirus Bohle Iridovirus has been reported to be the causative agent for the 'Spinning Tilapia Syndrome' in *Oreochromis mossambicus, Oreochromis aureus*, *Oreochromis niloticus* and *sarotherodon galilaeus*species of fish (Fishbase, 2004; Ariel and Owen, 1997).

Wild fishes had the highest prevalence for ranavirus in this study. Most reports of outbreaks of ranaviruses are in the wild than personal owned farmssince people source stock from parent stocks believed to be pathogen free which are usually bred on farms under favourable conditions

Also fish and frogs can transmit this disease when they migrate from one water body to another. Water is also an effective means for transmission of ranavirus (Miller *et al*., 2011).

The physiochemical parameters of water measured were within the normal standards set by WHO and Federal Ministry of Environment. The natural temperatures of water bodies' in this study fall within the range of temperature in which ranaviruses are able to replicate. Chinchar, (2002) gives the optimal temperature for ranavirus replication to be between 15℃ to 32℃.Laboratory studies have suggested that ranavirus virions are less virulent at warmer water temperatures and are more virulent at cooler temperatures (Rojas *et al*., 2005). This could be an explanation for presence of ranavirus infection observed by ELISA test in this study with no visible clinical sign or mortality observed during the study period. It is reasonable to assume other stressors, such as water pH, oxygen concentration and crowding of animals, may act to suppress the immune system and thereby increase susceptibility to ranavirus infection. Though studies, have failed to find significant relationship between pH and disease occurrence (Gahl and Calhoun, 2010).

Fishermen and frog harvesters fished and caught frogs mainly for income generation. Frog farming is slowly gaining popularityaround the world and could be an alternative source of income for Nigerians with frog consumption becoming more popularand socially acceptable, mostly for its medicinal qualities (Mohneke *et al*., 2009)

Fishermen and frog harvesters interviewed in this study carry out only some minimal form of cleaning for nets and other fishing/harvesting gears with majority of the fishermen respondents only hanging their nets to dry and frog harvesters doing nothing. This allows for the transmission of disease agents from one water body or fishing area to anotheras possible vectors include nets, boats and other equipment (OIE, 2012). This is so because water is an effective

transmission medium for ranaviruses, and survival outside the host may be for significant duration, weeks to months (Miller *et al*., 2011).Majority of the fishermen and frog harvesters usually leave dead fishes/ frogs in water or harvest and throw away in bushes, and this could be a means for transmission when other fishes or frogs consume the carcasses.

Some of thefrog harvesters, fishermen and frog sellers who were administered questionnaires had observed some of the clinical symptoms associated with ranaviruses at one point or the other. They had noticed fish and frogs to be either bloated or had red blotches or hemorrhages on skin. These symptoms observed are similar to clinical signs observed for ranavirus disease in reports and studies byJankovich *et al*. (1997), Converse *et al*. (2005), Ariel *et al*. (2009) and Miller *et al*.(2011).About 99.1% of fishermen and 76.2% frog harvesters who respondedto the questionnaire survey in this study had witnessed massive frog and fish mortality. No previous studies have documented massive mortalities in fish and frogs. Mortalities were said to be observed more during the rainy season and hot dry seasons when the temperatures are warmer.This is similar to findings in studies by Brunner *et al*. (2005), Ariel *et al*. (2009) and Bayley *et al*. (2013).

The weather conditions during observed mortalities reported by fishermen and frog catchers in this study was similar to a study of an outbreak of ranavirusinfection in frogs in Denmark by Ariel *et al*.(2009), a very high density of frogs was observed in and near the pond over the summer. In the weeks prior to the outbreak, the weather was unusually hot and the weather was described as a heat wave for Danish climate conditions: the maximum air temperature for that week was registered as 30.7°C for the area.Bayley *et al*.(2013) also reports higher mortality with warmer temperatures comparing experimental exposure at 15°Cand 20°C.

Fishermen and frog catchers interviewed in this study believe chemicals and disease in combination were the causes of such massive mortalities which corroborated with several other studies that revealedenvironmental conditions such as ambient temperature or pesticides to be associated with increased prevalence of ranavirus infection and disease (Gray *et al*., 2007; Miller *et al*., 2011).

This study is the first survey for ranaviruses in Nigeria and the first record of ranavirus in fish and frogs to our knowledge. Although ranavirus could occur naturally as part of the ecosystem, the finding of ranavirus and the possible association with the observed mass mortalities in frogs demonstrates the possible consequences of ranavirus infection given the right environmental circumstances.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The total prevalence of ranavirusobtained in this study for frogs and fishes was 47.2% with 51.2% in frogs and 43.0% in fish, though there was no statistically significant relationship between prevalence in fishes and frogs. The prevalence of ranavirus infection was higher in wild fishes (51.3%) than in farmed fish (18%) and the difference was statistically significant (P-value – 0.000). *Marcusenius senegalenis* type of fish had the highest prevalence rate of 75%, among the fish species studied. This was statistically significant.The month of June had the highest ranavirus prevalence rate in frogs and March in fishes. Fishermen and frog harvesters had observed mass mortality of frogs and fish at some point in their business though undocumented. This study demonstrated the presence of detectable ranavirus antigen in fresh fish and edible frogs Zaria, Kaduna State, Nigeria.

6.2 RECOMMENDATIONS

i. More surveys and detailed experimental investigations should be carried out todetermine the extent of spreadand the dynamics of rananvirusinfection in Zaria, Kaduna State and other parts of Nigeria and Africa generally.

ii. Molecular studies should be done to ascertain the true picture of this disease and the different strains or species of the ranavirus which may be circulating in Nigeria.

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Appendix I: Questionnaire for Fishermen

DEPARTMENT OFVETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, SAMARU ZARIA.

TOPIC: PREVALENCE OF RANAVIRUS AND DETERMINATION OF RISK FACTORS FOR RANAVIRUS IN FISH AND EDIBLE FROGS IN ZARIA, KADUNA STATE, NIGERIA.

Dear Respondent,

I am Inikpi Hannah Ameh, a masters' student in the Department of Veterinary Public Health and Preventive Medicine of the above named University. I am carrying out a research on the Prevalence of Ranavirus and the Risk Factors for Ranavirus in Fish and Edible Frogs in Zaria, Kaduna State, Nigeria. This questionnaire is intended to obtain information on the fishing practices and knowledge of fish diseases and mortality from fishermen in Zaria, Kaduna State, Nigeria. I kindly request your cooperation to respond to questions in this questionnaire. Your full cooperation will be greatly appreciated. Responses will not be identified by individuals but all responses will be compiled together and analyzed. Your responses will be used for academic purpose only.

Participation in this research is voluntary and if during the course of the interview you feel uncomfortable with any of the questions, you may stop or skip such question.

Do I have your consent to proceed with this interview? If yes, please sign below.

Signature of respondent ……..

Date of interview

1. Location of fishing activities involved in (tick all that apply)

   ABU Dam [ ] Zaria Dam [ ] University Farm Dam [ ] Shika Dam [ ] Others, please specify _____

2. Sex of respondent

   Male [ ] Female [ ]

3. Age of respondent

   < 20 [ ] 21-40 [ ] 41-60 [ ] 61 and above [ ]

4. Occupation_____

5. Level of Education

Primary [ ] Secondary [ ] Tertiary [ ]None [ ] Others, please Specify_____

6. Why do you fish?

   For income [ ] for pleasure [ ] For family use only [ ] Others, please specify _____

7. What kind of fish do you fish?

   mention_____

8. How regularly do you fish?

   Daily [ ] Weekly [ ] Monthly [ ] Others, please specify _____

9. What do you do with your fishing equipment after fishing?

   Rinse in water [ ] Wash [ ] Hang to dry [ ] Nothing [ ]

   Others, please specify _____

10. Have you ever observed cases of massive fish mortality or death?

    Yes [ ] No [ ]

11. How many times have you seen this occur?

    Yearly [ ] More than once in a year [ ] Only Once [ ] More than Once [ ]

12. What season of the year does this occur?

    Rainy season [ ] Cold dry season [ ] Hot dry season [ ]

13. If yes to 10 above, how does the sick or dead fish look like? (refer to picture).

    _____

14. What do you do with sick or dead fish?

    Leave in water [ ] Harvest to sell [ ] Harvest and throw into the bush [ ]

    Others, please specify _____

15. What do you think causes massive fish mortality?

    Disease [ ] Chemicals [ ] Others, please specify _____

16. If you ticked disease or chemicals in 15 above, please name the ones you know

    _____

Appendix II: Questionnaire for Frog Harvesters

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, SAMARU ZARIA

TOPIC: PREVALENCE OF RANAVIRUS AND DETERMINATION OF RISK FACTORS FOR RANAVIRUS IN FISH AND EDIBLE FROGS IN ZARIA, KADUNA STATE, NIGERIA.

Dear Respondent,

I am Inikpi Hannah Ameh, a masters' student in the Department of Veterinary Public Health and Preventive Medicine of the above named University. I am carrying out a research on the Prevalence of Ranavirus and the Risk Factors for Ranavirus in Fish and Edible Frogs in Zaria, Kaduna State, Nigeria. This questionnaire is intended to obtain information on the frog harvester's practices and knowledge of frog diseases and mortality from frog harvesters in Zaria, Kaduna State, Nigeria. I kindly request your cooperation to respond to questions in this questionnaire. Your full cooperation will be greatly appreciated. Responses will not be identified by individuals but all responses will be compiled together and analyzed. Your responses will be used for academic purpose only.

Participation in this research is voluntary and if during the course of the interview you feel uncomfortable with any of the questions, you may stop or skip such question.

Do I have your consent to proceed with this interview? If yes, please sign below

Signature of respondent ……..

Date of interview

1. Location of frog capture _____
2. Sex of respondents
   Male [ ] Female [ ]
3. Age of respondents
   < 20 [ ] 21-40 [ ] 41-60 [ ] 61 and above [ ]
4. Occupation _____
5. Level of Education

Primary [ ] Secondary [ ] Tertiary [ ] None [ ]
Others, please Specify_____

6. Do you harvest only frogs or frogs and fish?
   Frogs [ ] Frogs and Fish [ ]
7. Why do you harvest frogs?
   For income [ ] for pleasure [ ] For family use only [ ] Others (specify) _____
8. What kind of frogs do you harvest? mention_____
9. How regularly do you harvest frogs?

Daily [ ] Weekly [ ] Monthly [ ] Others (specify) _____

10. Do you eat frogs?
    Yes [ ] No [ ]
11. How many frogs do you harvest per catch on the average?
    Per Day [ ] Per Week [ ] Per Month [ ]
12. How do you harvest Frogs? _____
    Attract with torchlight [ ] Hit with Stick [ ] Catch with rope [ ]
    Others (specify) _____
13. What do you do with your equipment after harvesting frogs? (choose all that apply)

Rinse in Water [ ] Wash [ ] Hang to dry [ ] Nothing [ ]
Others, please specify _____

14. Do you slaughter and process the harvested frogs yourself?
    Yes [ ] No [ ]
15. If no, who does the slaughter and processing, and their age range?
    Children <10 [ ] 10 - 14 [ ] 15 – 17 [ ]

    Market Women 18 – 20 [ ] 21-40 [ ] 41- 60 [ ] > 60 [ ]
    Adult Men at market 18 – 20 [ ] 21-40 [ ] 41- 60 [ ] > 60 [ ]
    Others, please specify _____

16. Have you ever observed cases of massive frog mortality or death?
    Yes [ ] No [ ]

17. What season of the year does this happen?
    Rainy season [ ] Cold dry season [ ] Hot dry season [ ]
18. How does the sick or dead frog look like? (refer to picture).
    _____
19. What do you do with sick or dead frogs?
    Leave in water [ ] Harvest to sell [ ] Harvest and throw into the bush [ ]
    Personal consumption []Others, please specify_____
20. What do you think causes massive frog mortality?
    Disease [ ] Chemicals [ ] Others, please specify _____
21. If you ticked disease or chemicals in 20 above, please name the ones you know
    _____

Appendix III: Questionnaire for Frog Sellers

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, SAMARU ZARIA.

TOPIC: PREVALENCE OF RANAVIRUS AND DETERMINATION OF RISK FACTORS FOR RANAVIRUS IN FISH AND EDIBLE FROGS IN ZARIA, KADUNA STATE, NIGERIA.

Dear Respondent,

I am Inikpi Hannah Ameh, a masters' student in the Department of Veterinary Public Health and Preventive Medicine of the above named University. I am carrying out a research on the Prevalence of Ranavirus and the Risk Factors for Ranavirus in Fish and Edible Frogs in Zaria, Kaduna State, Nigeria. This questionnaire is intended to obtain information from frog and fish sellers in Zaria, Kaduna State, Nigeria. I kindly request your cooperation to respond to questions in this questionnaire. Your full cooperation will be greatly appreciated. Responses will not be identified by individuals but all responses will be compiled together and analyzed. Your responses will be used for academic purpose only.

Participation in this research is voluntary and if during the course of the interview you feel uncomfortable with any of the questions, you may stop or skip such question.

Do I have your consent to proceed with this interview? If yes, please sign below.

Signature of respondent ……..

Date of interview

1. Location of Sale/ Stall _____
2. Sex of respondents
   Male [ ] Female [ ]
3. Age of respondents
   < 20 [ ] 21-40 [ ] 41-60 [ ] 61 and above [ ]
4. Occupation_____
5. Level of Education

Primary [ ] Secondary [ ] Tertiary [ ] None [ ] Others, please Specify_____

6. Who are the people that catch and supply frogs for you, and their age range?
   Children <10 [ ] 10 - 14 [ ] 15 – 17 [ ]
   Adults 18 – 20 [ ] 21 - 40 [ ] 41 - 60 [ ] > 60 [ ]
7. How often are the frogs supplied?
   Daily [ ] Weekly [ ] Every two weeks [ ] Monthly [ ] Others, please specify _____
8. Who slaughters and processes the frogs? Yourself [ ] Children [ ] Payed Workers [ ]
9. What is the approximate number of frogs you sell?
   Per Day [ ] Per Week [ ]
10. What month of the year do you sell frogs the most?
    January [ ] February [ ] March [ ] April [ ] May [ ] June [ ] July [ ] August [ ] September [ ] October [ ] November [ ] December [ ]
11. Who do you sell frogs to in Zaria?
    Students [ ] Lecturers [ ] Business Women /Men [ ] Civil Servants [ ] Others, please specify _____
12. Are people from Zaria the only people you sell frogs to or do you send to other places for sale?
    Yes, Only Zaria [ ] No, not only Zaria [ ]

13. If no to 12 above, please mention other places you send your frogs for sale. _____

14. In what form are the frogs sold to you?
    Live [ ] Apparently healthy [ ] Sick [ ] Dead [ ]

15. If sick or dead, how many?
    1 - 10 [ ] 11- 20 [ ] 21 - 30 [ ] 31 – 40 [ ] 41 – 50 [ ] 51 and above [ ]

16. How does the sick or dead frog look like? (refer to picture)
    _____

17. What do you do with sick or dead frogs?
Sell [ ] Throw away [ ] Others, please specify_____

18. What do you think causes massive frog mortality?
    Disease [ ] Chemicals [ ] Others, please specify _____

19. If you ticked disease or chemicals in 18 above, please name the ones you know _____

Appendix IV: Preparation of Buffers and Reagents

- Borate coating buffer

The following reagents: boric acid (6.18g), Disodium tetraborate ($Na_2B_4O_7.10H_2O$) (9.54g) and NaCl (4.38g) were mixed with 1 litre distilled water and autoclaved.

- 10 × phosphate buffered saline

NaCl (80g), KCl (2.00g), Na_2HPO_4 (11.50g), KH_2PO_4 (2.00g) were mixed with 900 ml distilled water and the pH adjusted to 7.2 with NaOH. The solution was then made up to 1 litre with distilled water and autoclaved

- 2,2'-azino-bis (3- ethylbenzothiazoline-6- sulphonic acid (ABTS)

Citrate phosphate buffer

Citric acid (21.00g) was added to 14 g of Na_2HPO_4 and dissolved in 800 ml of distilled water. The pH was adjusted to 4.2 and made up to 1 litre with distilled water.

ABTS (0.55g) was added to 1 litre citrate phosphate buffer and dispensed in 22-ml aliquots and frozen. Immediately prior to use, 10 µl H_2O_2 was added per 22-ml aliquot.

- ABTS stop solution (0.01% NaN_3 in 0.1 M citric acid)

Citric acid weighing 10.5 g was dissolved in 500 ml distilled water and 1 ml of 5% sodium azide solution was added.

- Tris saline glycerol merthiolate cryoprotectant (TSGM cryoprotectant)

Fifty ml of 10 × Tris/saline at pH 7.4, 250 ml Glycerol and 250ml sterile purified water were combined in a conical flask and autoclaved. To this, 1 ml of 10% Merthiolate was added and stored in a dark bottle at 4°C.

- 10 × Tris/saline (250 mM Tris, 1.5 M NaCl)

Tris, (15.14g) and NaCl, (43.83g) were dissolved in 500 ml, sterile purified water and the pH adjusted to 7.

Appendix V: Image of Fish Sampled



Fish with bloody fins



Appendix VI: Images for Frogs Sampled



Fried frogs spread out to dry in Hanwa frog Market



Fried frogs spread out to dry in Hanwa frog Market