PREVALENCE AND CHARACTERISATION OF *FASCIOLA* SPECIES FROM SNAILS, SLAUGHTERED CATTLE AND SHEEP FROM MAIDUGURI, GOMBE AND JOS ABATTOIRS, NIGERIA

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DECLARATION

I declare that the work in this thesis entitled **"Prevalence and Characterisation of *Fasciola* species from Snails, Slaughtered Cattle and Sheep from Maiduguri, Gombe and Jos Abattoirs, Nigeria"** has been performed by me in the Department of Veterinary Parasitology and Entomology. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or other institution.

Joshua LUKA _____ _____

Signature Date

CERTIFICATION

This thesis entitled, "**PREVALENCE AND CHARACTERISATION OF *FASCIOLA* SPECIES FROMSNAILS, SLAUGHTERED CATTLE AND SHEEP FROM MAIDUGURI, GOMBE AND JOS ABATTOIRS, NIGERIA**" by JOSHUA LUKA meets the regulation governing the award of the degree of Doctor of Philosophy of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

The prevalence, morphometry and molecular characterisation of *Fasciola* species from slaughtered cattle and sheep, and snails from Maiduguri, Gombe and Jos were investigated. Prevalence of *Fasciola* spp. was studied by determination of eggs of the parasite in both faeces and bile, while morphometric description was done using standard keys and descriptions. For molecular characterisation, the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA (rDNA), 28S rDNA and NADH dehydrogenase subunit 4 (NAD4) respectively were amplified from individual *Fasciola* isolated from bile duct by polymerase chain reaction (PCR). All collected snails were subjected to morphological identification using standard keys. The DNA of *Fasciola* spp. was similarly characterised in *Lymnaea natalensis* by the use of 28S rDNA, while the *Lymnaea (Radix) natalensis* was characterised by the use of 18S rDNA. Representative amplicons of both *Fasciola* spp and *Lymnaea natalensis* were sequenced, and NCBI databases were used for sequence homology analysis using BLAST and ClustalW programs, while phylogenetic analysis was done in ApE and Molecular Evolutionary Genetics Analysis (MEGA). Combined location prevalence rate was 27.52% for cattle and 11.97% for sheep. For cattle, sex, age or breed had no significant ($p \geq 0.05$) impact on prevalence rate, while for sheep, only age had an impact, as more adult than young sheep were infected (12.62% compared to 2.63%). Jos, had a significantly ($p<0.05$) higher prevalence of 35.43% followed by Gombe (26.99%), while Maiduguri had the lowest (19.63%). In cattle, there was a negative association between the number of positive animals and egg per gram of faeces and bile (EPG), with Maiduguri having a mean EPG of 65.85±13.2 followed by Gombe 45.48± 10.8 and Jos the least (14.4± 1.34). For sheep, Jos had significantly ($p<0.0.010$) higher prevalence rate (24.35%)

than both Maiduguri (6.16%) and Gombe (5.52%). Actual mean EPGs were 19.71, 36.34 and 14.47 for the respective locations. For both cattle and sheep, mean EPG were significantly ($p<0.05$) higher by the bile sedimentation method than by faecal analysis. For cattle, values were 41.12±5.8 versus 15.72± 3.8, while for sheep values were 50.88±15 versus 8.36±1.9 for bile and faeces respectively. Month of sampling had a significant ($p\leq0.05$) influence on infection rate with most animals infected in January - Febuary, being the months with highest infections. Morphological differences were observed in linear measurements and ratios. Three useful morphological parameters (BL, CL, CW) and one ratio (BW/BL) showed significant ($p<0.05$) variations among samples from the locations, and may therefore be relevant for phenotypic differentiation of species. The molecular identification using ITS-1, 28S rDNA and NAD4 and the sequencing revealed the presence of both *F.hepatica* and *F.gigantica* in the study areas. Analaysis of the overall genetic sequence data showed that 64.7% of the sequences of *Fasciola* isolates were *F. gigantica,* while 35.3% were *F. hepatica*. Of the *F.hepatica* isolates, 66.6% were from Jos. The phylogenetic tree constructed based upon the ITS-1 sequences revealed a close relationship (95-98%) with isolates of *F. gigantica* from Bukina Faso and South Africa, while the *F. hepatica* isolate had 84% identity with that from Iran. The 18S rRNA of *Lymnaea natalensis* was identified molecularly at 450bp. The sequences had between 99-100% similarities among themselves and 98-100% with other deposited reference *Lymnaea natalensis* sequences. All our *Lymnaea (Radix) natalensis* sequences formed a clade different from the clade formed by other reference sequences. *Lymnaea* (*Radix) natalensis* sequences from Nigeria seem to clade separately from deposited sequences from GenBank. Conclusively, the prevalence of *Fasciola* spp indicates a high morbidity in the sampled animals. The study had shown that, a well defined relationship exists between egg counts in bile and faeces of cattle, but not sheep in this study.

This study also confirmed the presence of *F.gigantica* and strongly suggests the existence of *F.hepatica* for the first time, to the best of our knowledge, using molecular tools. The findings of the two species of *Fasciola (F. hepatica and F.gigantica)* may have implications for livestock and human infections and vaccine types to be developed in the control of fasciolosis in the study locations and Nigeria.The findings also confirmed the existence of *Lymnaea (Radix) natalensis* and its role as intermediate host of *Fasciola* spp in the study areas. In addition, experimental infection of different breeds of cattle and sheep with *Fasciola* spp, complete sequence of the ITS-1, 28S rDNA, NAD4 of *Fasciola* spp and 18S rDNA of *Lymnaea (Radix) natalensis* and investigation of snails such as *Melanoides tuberculata* and *Biomphalaria pfeifferei* as potential intermediate hosts of *Fasciola* spp are recommended for further studies.

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LIST OF ABBREVIATIONS

AGDT	Agar gel diffusion test
ALT	Alanine amino transferase
ASL	Aspartate transaminase
BCG	Bacille-Calmete Guierin
BL	Maximum body length
BW	Maximum body width
CatL1	Cathepsin L1
CDC	Centre for Disease Control
CE	Crude extract
CL	Cone length
COI	Cytochrome C oxidase gene
CRT	Coproantigen test
US$	Dollar(s)
DBIL	Direct bilirubin
DCPH	Di-nitro-o-cyclohexyl-phenol
ddH_2O	Double distilled water
dNTPs	Dinucleotide triphospahtes
DNA	Deoxyribonucleic acid
EC	Egg count(s)
EHA	Egg hatching assay
ELISA	Enzyme linked immunosorbemt assay
ES	Excretory-secretory antigen

ESPs	Excretory-secretory products
FABP	Fatty acid binding protein
FEC	Faecal egg count
FECRT	Faecal egg count reduction test
FER	Faecal egg reduction
FERT	Faecal egg reduction test
FEA	SD-Formo ether acetate sedimentation
Fg-ELISA	*Fasciola gigantica* soluble ELISA antigen
FgGST	*Fasciola gigantica* glutathione-S-transferase
Fh-ELISA	*Fasciola hepatica* soluble ELISA antigen
FIA	Freund Incomplete Adjuvant
G1	Haplotype 1
G2	Haplotype 2
GGT	Gamma glutamyl transferase
GIS	Geographic Information System
GST	Glutathione-S-transferase
H_2O	Water
ICR	Imprinting control region
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHAT	Indirect haemagglutination test
IL-4	Interleukin 4
IL-5	Interleukin 5

INF-γ	Interferon gamma
ITS	Internal transcribed spacers 1and 2
ITT	Indonesian thin tail
kDa	Kilodalton
$MgCl_2$	Magnesium chloride
mg/ml	milligram per milliliter
mg/kg	milligram per kilogram
MT04	1,2,4,5-tetraoxane
NADH	Nicotinamide adenine dehydrogenase
NEJ	Newly excysted juveniles
OZ78	1, 2, 4-trioxolane
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-Restriction fragment length polymorphism
qPCR	Quantitative PCR
RAPD-PCR	Random amplified polymorphic DNA-PCR
rFABP	Recombinant fatty acid binding protein
rproFgCatL1	Recombinant pro-*F.gigantica* cathepsin L1
rmatFgCatL1	Recombinant mature *F.gigantica* cathepsin L1
(rFhTGR)	Recombinant *Fasciola hepatica* thioredoxin glutathione reductase
rRNA	ribosomal Ribonucleic acid
rDNA	ribosomal Deoxyribonucleic acid
SCITT	Single comparative intradermal tuberculin test
SEM	Scanning electron microscopy

SPSS	Statistical package for social sciences
TAE	Tris acetate EDTA buffer
Taq	*Thermus aquaticus*
TBIL	Total bilirubin
TCBZ	Triclabendazole
TIC	Trypsin inhibitor complex
Th1	T helper cell 1
Th2	T helper cell 2
TrisNaOH	Tris base NaOH
US	United States
Vit-P	Distance between the union of vitelline gland and the posterior end of the body
VS-P	Distance between the ventral sucker and the posterior end of the body
WAAVP	World Association for the advancement of veterinary parasitology
WHO	World Health Organization
µm	Micrometer

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

The disease, fasciolosis is a parasitic disease of domestic ruminants caused by two liver fluke species: *Fasciola hepatica* and *Fasciola gigantica* (Trematoda). *Fasciola hepatica* has a cosmopolitan distribution, mainly in temperate zones, while *Fasciola gigantica* is found in tropical regions of Africa and Asia (Mulugeta *et al*., 2011). Infection with *Fasciola* is of veterinary and medical importance, particularly in areas of high-density cattle and sheep production (Taha *et al.*, 2014). *Fasciola* spp. occur commonly in the bile duct and liver of sheep, goat, cattle and buffalo, mule, pig and may rarely be found in unusual hosts such as man and horse (Bhatia *et al.*, 2006).

The economic importance of fasciolosis is mostly due to mortality and high losses from reduced feed efficiency, weight gains, milk production, reproductive performance, carcass quality and work output in draught animals, and condemnation of livers at slaughter (Vassilev and Jooste, 1991). An estimate of more than US$200 million dollars globally, is lost every year in livestock products due to the effects of fasciolosis (Moazeni *et al.*, 2005).

Fasciolosis also has the widest geographic spread of any emerging vector-borne zoonotic disease.World Health Organisation (WHO) estimated that at least 2.4 million people are infected in more than 70 countries worldwide, with several million at risk. Although, the infection in humans was described as accidental, and characterised by jaundice caused by obstruction of the biliary tree (Moghadami and Mardani, 2008), there have been increased reports of the occurrence

of the infection in man of recent. No continent is free from fascioliasis, and it is likely that where animal cases are reported, human cases also exist (WHO, 2016).

Varying prevalence of the infection has been reported in animals and man (Mulugeta *et al.*, 2011; Biu *et al.*, 2013; Jean-Richard *et al.*, 2014). In animals, the infection is more important in cattle where high prevalence rates have been reported in comparison to other domestic animals (Jean-Richard *et al.*, 2014).

In Nigeria, several investigators have reported the disease in cattle and small ruminants; for example Ardo and Aliyara (2014) reported 0.32% and 0.23% in sheep and goats respectively in Yola, while Magaji *et al*., (2014) reported 27.68% prevalence among slaughtered cattle at Sokoto central abattoir. Similarly, previous reports from Zaria, Nigeria, ranked fasciolosis among top pathological conditions encountered in slaughtered animals at abattoirs (Raji *et al.*, 2010; Alawa *et al.*, 2011).

Adult *Fasciola* live in bile ducts producing eggs that are excreted with the faeces. The life cycle of the disease involves hatching of the eggs in 14 days at a temperature of 22-26^{o}C (Bhatia *et al.*, 2006). Hatching occurs in moist conditions only after the first larval stage, the miracidium, has formed and when ambient temperatures rise above 5-6^{o}C (Radostits *et al*., 2006). Miracidia must find and invade the tissues of a suitable snail host within 24-30 hours.Cercariae which emerge from snails about 6-8 weeks after infection, encyst on pasture as metacercariae, the infective form for ruminants and other susceptible hosts. Following ingestion of metacercariae by grazing animals, the parasites (newly excysted juveniles, NEJ) emerge from their cysts in the intestine,

traverse the intestinal wall, which takes just a few hours, before migrating through the liver capsule and into the parenchyma (Molina-Hernández *et al.*, 2015). Here, their feeding and migratory activities cause tissue perforation and haemorrhage, leading to extensive tissue damage. After about 7–8 weeks, the parasites migrate into the bile ducts, mature and produce 20,000–24,000 eggs per fluke per day which are released onto the pasture with the faeces (Urquhart *et al.*, 1987).

Clinically, fasciolosis presents with distended abdomen, ascites, anaemia, stiff gait, loss of appetite, black scouring and bottle jaw, similar to signs seen in amphistosmosis and haemonchosis. Three types of clinical presentations; acute, subacute and chronic forms have been reported (Bhatia *et al.*, 2006). These presentations may however depend on the state of the animal at the time of infection especially cattle. Cattle may overtime develop a partially protective immune response to *F. hepatica* in regions where the infection occurs. The interaction of such factors as age of the host, innate resistance, previous exposure and present level of parasite exposure determine the degree of parasite establishment and the pathologic impact of the infection. Older cattle with previous exposures have a greater resistance to infection than young parasite-naive calves (Kaplan, 2001).

The snails belonging to the genera; *Lymnaea*, *Amphipepla*, *Simlimnaea*, *Galba*, *Fossaria*, *Stagniciola* and *Pseudosuccine*a serve as intermediate hosts, with specific species restricted to different geographical regions of the world (Smyth 1994; Bhatia *et al.*, 2006). In Nigeria, the most important snail is *Lymnaeaa natalensis*. These intermediate hosts are found in shallow

water and are capable of migrating for long distances over mud and wet pasture, thereby increasing the chances of exposure of susceptible animals (Bhatia *et al.,* 2006)

1.2 Statement of Research Problem

Fasciola hepatica infects more than 300 million cattle and 250 million sheep worldwide and together with *F. gigantica,* cause significant economic losses to global agriculture; through lost productivity such as reduction in milk and meat yields (Mas-Coma *et al.,* 2005). Previous report by Talukder *et al.* (2010) estimated a conservative amount of over US$ 3.2 billion per annum, as losses due to fasciolosis in production animals worldwide, which was slightly higher than the US$ 3 billion estimated recently by Elelu and Eisler (2017). According to a World Health Organization (WHO) report in 2007, fasciolosis was limited in the past to specific and typical geographical areas (endemiotopes), but is now widespread throughout the world. Until recently, human cases occurred occasionally but are now increasingly reported from Europe, the Americas and Oceania (where only *F. hepatica* is transmitted) and from Africa and Asia (where the two species overlap)(WHO, 2016).

The epideomiology of fasciolosis is linked to a myriad of factors. The presence of streams, wetlands and pastures on farms were significantly associated with the presence F. *hepatica* infection in cattle herds previously (Olsen *et al*., 2015).

The metacercariae of *Fasciola* spp. take about six days to reach the liver or only about 48 hours in exceptional cases (Bhatia *et al.,* 2006).The acute phase of fasciolosis characterised by severe haemorrhage caused by the migrating juvenile flukes in the hepatic tissue rupturing the blood vessels. The liver parenchyma particularly the ventral lobe associated with gall bladder is

severely damaged, assuming an uneven surface covered with blood clots (Gupta, 2014). Most of the pathologies described in fasciolosis are often due to the migration of the immature stages of the parasite and are manifested as early as 2 weeks after the infection (Guobadia and Fagbemi, 1997).

Fasciola gigantica has been previously characterised from African countries of Burkina Faso, Senegal, Kenya, Zambia and Mali, while *F. hepatica* has been reported from Morocco and Tunisia, and both species have been observed from Ethiopia and Egypt on the basis of morphometric parameters (Amor *et al*., 2011). For sometimes now, the identification of *Fasciola* spp. based on traditional morphological features has been used for speciation (Ai *et al*., 2011). This is however limited, especially in regions where the two common species are known to overlap. Similarly, the emergence of an intermediate/hybrid *Fasciola* in some endemic foci has opened a new dimension in the study of this important disease. This hybrid has long been reported from Asian countries of Japan, Korea, China and Vietnam (Itagaki and Tsutsumi, 1998; Agatsuma *et al.*, 2000; Lin *et al.*, 2007; Itagaki *et al.*, 2009) and most recently from Pakistan (Mufti *et al*., 2014). The hybrid form was also confirmed in Egypt by the aid of morphological features (Periago *et al*., 2008) and molecularly using mitochondrial and ribosomal gene markers (Amer *et al*., 2011). Understanding genetic structure and status of genetic variation of the parasite populations has important implications on epidemiology and effective control of fasciolosis (Rokni *et al*., 2010). Equally, molecular study will provide information on the taxonomic status of *Fasciola* isolates against the traditional reliance on morphological characterisation.

1.3 Justification of the Study

Among all the livestock, ruminants, comprising sheep, goats and cattle, constitute the largest group reared by farm families in the country's agricultural system. Nigeria has an estimated population of 34.5 million goats, 22.1 million sheep and 13.9 million cattle (Lawal-Adebowale, 2012). However, about 90 per cent of the country's cattle population and 70 per cent of the sheep and goat populations are concentrated in the northern part of the country (Lawal-Adebowale, 2012).

Most studies aimed at determination of prevalence of *Fasciola* spp. conducted in Nigeria (Nwosu and Srivastava, 1993; Omowaye *et al*., 2012; Adang *et al*., 2015) and Southern Lake Chad region (Jean-Richard, 2014), have been based on examination of liver at post-mortem. Hence, comparatively, fewer reports exist on the detection of *Fasciola* by coprology, the traditional detection method in the laboratory, than by post-mortem examination in the abattoir. The exact prevalence of fasciolosis is most likely underestimated due to the lack of comprehensive epidemiological surveys performed in potentially endemic areas. Furthermore, in some areas where reports exist, there is a time lag of up to a decade or more, thereby making such reports obsolete and probably not in tune with current realities.

The faecal egg count provides an estimate of the degree of infection with *Fasciola* spp. in the hosts. However; it is deficient in estimation due to intermittent release of bile into the intestine. Also, the inability to sample bile for eggs in living animals, makes bile a bad candidate for sampling in clinical applications (Radostits *et al*., 2006). Thus, estimating both faecal and bile

egg counts will provide important information on the quantum of infection and exact relationship between eggs in faeces and bile (Radostits *et al*., 2006).

The characterisation of *Fasciola* spp. by the use of morphological keys has been applied in different parts of the world including Africa. Such include the study by Biu *et al*. (2013) in Nigeria, Chauke *et al*. (2014) in Zimbabwe and Shaldoum *et al*. (2015) in Egypt. Despite the inability of morphological keys to accurately differentiate between the two *Fasciola* species especially regarding immature/juvenile flukes (Ashrafi *et al*., 2006), available evidences have shown that it is still important when careful morphometry is carried out. In fact, for research activities in Africa and Asia, where both species overlap, Mas-Coma *et al*. (2009a) recommended a minimum morphometrical study of adult flukes parameters such as body length (BL), maximum body width (BW), BL/BW ratio, distance between the ventral sucker and the end of the body (VS-P) and the distance between the ventral sucker and the union of the vitelline gland (Vit-P) distance and eggs for inclusion within genetic characterisation studies. Where available, species variations among isolates from different geographical locations presumably provide an idea of ecological adaptation as with other helminthes such as *Haemonchus contortus* (Rahman and Hamid, 2007; Kumsa *et al*., 2008).

Molecular identification of parasites including *Fasciola* is important as it helps with information on the epidemiology, genetic variation and diagnosis (Liu *et al*., 2014). For *Fasciola* spp, this often employs the use of genetic markers such as internal transcribed spacers (ITS 1&2) and 5.8S of the nuclear ribosomal DNA, 28S ribosomal ribonucleic acid (rRNA), 18S rRNA, mitochondrial NADH dehydrogenase I (NDI) and Cytochrome C Oxidase I (COI) genes.

Because of its highly repeated and conserved regions, the nuclear ribosomal DNA is especially designed for molecular studies (Chilton, 2004; Choe *et al.,* 2011). These markers have successfully been used to differentiate between the two common species including the intermediate form.

Despite the widespread use of moleular techniques for genetic characterisation and for determining inter- and intra-species variability, there is dearth of information on their application in Nigeria. Most importantly, there is need to use molecular techniques to determine the available *Fasciola* species infecting cattle, sheep and goats, which are the most important animal hosts in Nigeria. The need to characterise the isolates of cattle and sheep origins from different geographical regions of Nigeria cannot be overemphasized in view of the available evidences on the restriction of the two important species of *Fasciola* to specific climatic zones. These climatic zones are represented by some regions in Nigeria. Similarly, the emergence of a hybrid/intermediate form of *Fasciola* in some endemic regions including Africa underscores the need to determine the isolates present in Nigeria, particularly the Northern part of the country where about 90% and over 70% of the cattle and small ruminants are reared (Lawal-Adebowale, 2012). Because of the larger number of mitochondrial NADH dehydrogenase subunit 1 (nad1) haplotypes detected in Chinese aspermic *Fasciola* populations than in aspermic populations from other neighboring countries, Ichikawa-Seki *et al*.(2017a) proposed that aspermic *Fasciola* flukes should be termed as 'hybrid' *Fasciola*.

The snail intermediate hosts of *Fasciola* spp. belong to the genus *Lymnaea*. *Lymnaea truncatula* and *Lymnaea natalensis* are the major vector hosts in temperate and tropical regions respectively

(Bhatia *et al*., 2006; Radostits *et al*., 2006). In the previous report of Schillhorn van Veen (1980) as cited in Brown (2005), *L. natalensis* was described as the main intermediate host if not the only one in large areas of Africa for *Fasciola*, particularly west Africa. The previous report of Dar *et al*.(2010) on the experimental transmission of *Fasciola hepatica* by *Lymnaea natalensis* in Egypt and the finding of the sporocysts of *Fasciola gigantica* in *Achatina fulica*, an edible snail not known for the transmission of parasite in Nigeria (Igbinosa *et al*.,2016), may need further investigation of these snails. Morphological characterisations have successfully been applied to Lymnaeaid snails from France (Hurtrez-Boussès *et al*., 2005), Brazil (Carvalho *et al*., 2004) and Nigeria (Falade and Otarigho, 2015). Similar characterisation molecularly using genetic markers such as ITS-1, ITS-2, 16S ribosomal (Correa *et al*., 2010) and 18S ribosomal gene (Howell *et al*., 2012) have either confirmed *Lymnaea natalensis* or differentiated among the various species of *Lymnaea*.

1.4 Aim of the Study

The research was to determine the prevalence and characterise *Fasciola* spp from snails, and slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs.

1.5 Objectives of the Study

The objectives were to:

i. determine the prevalence of *Fasciola* spp. in bile and faeces of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs.

ii. characterise the *Fasciola* isolates morphometrically using body length (BL), body width (BW), body length to width ratio (BL/BW); Cone Length (CL).

iii. morphologically identify snail samples from Maiduguri, Gombe and Jos.

iv. molecularly characterise *Fasciola* spp. from the slaughtered cattle, sheep and also sampled snail tissues using PCR.

v. molecularly characterise *Lymnaea* species from the study areas

vi. sequence the DNA amplicons and determine their evolutionary relationship with reference sequences in the GenBank.

1.6 Research Questions

i. What is the prevalence of *Fasciola* in bile and faeces of slaughtered cattle and sheep from Gombe, Maiduguri and Jos abattoirs?

ii. Can morphological features (body length (BL), body width (BW), body length to width ratio (BL/BW); Cone Length (CL)) characterise *Fasciola* spp from the study areas?

iii. Can morphological keys identify snails from the study locations?

iv. Can PCR characterise *Fasciola* spp from the slaughtered cattle, sheep and the sampled snail tissue?

v. Can PCR characterise *Lymnaea* species from Maiduguri, Gombe and Jos?

vi. Can DNA amplicons be sequenced and evolutionary relationships determined?

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Fasciola* species and Evolutionary Trend

Fasciolosis, first reported in 1379, has been recognized as a clinically and economically important disease for centuries (Rojo-Vázquez *et al.,* 2012). The generic name *Fasciola* is from L. dim. fascia, meaning a band of a fillet. While, Jehan de Brie recognized *Fasciola hepatica* in 1379 (Gupta, 2014), Dittmar and Teegen (2003) demonstrated for the first time the existence of a direct association between a *F. hepatica*-infection and pre-historic human skeleton and domesticated animal (bovine samples) remains. This was shown at a 4,500 year old archaeological site in the Saale-Unstrut valley, Germany during a palaeoparasitological study of a site of the corded ware culture.

The first evidence of fasciolosis in Nigeria was reported by Burke (1939) among 3000 goats that died of the condition in the then Borno Province. In addition, *F. gigantica,* originally described from *Giraffa Camelopardalis* from Sub-Saharan Africa found in a travelling menagerie in England (Cobbold, 1855), was later re-described from cattle in Senegal (Raillet, 1895). That was also the earliest evidence for the existence of *Fasciola* spp from Africa.

Fasciolosis is caused by two species *Fasciola hepatica* and *Fasciola gigantica* (Myers *et al.,* 2016, WHO, 2016). Taxonomically, the genus *Fasciola* belongs to the; Kingdom: Animalia; Phylum: Helminthes; Sub-Phylum: Platyhelminthes; order: Echinostomida; Suborder: Distomata; Superfamily: Fascioloidea; Class: Trematoda; Sub-Class: Digenea; Family: Fasciolidae; Sub-Family: Fasciolinae; Genus: *Fasciola* (Myers, 2006; Gupta, 2014). Lotfy *et al.*

(2008) similarly determined the evolutionary origins, diversification, and biogeography of liver flukes (Digenea, Fasciolidae) by the use of molecular phylogenetic study utilizing 28S, internal transcribed spacers 1 and 2 (ITS-1 and ITS-2) of nuclear ribosomal DNA, and mitochondrial nicotinamide dehydrogenase subunit 1 (nad1) which included seven of the nine recognized species in the family. In the said study, the fasciolids examined consisted of monophyletic group with the most basal species recovered from African elephants. It was therefore hypothesized that, fasciolids migrated from Africa to Eurasia, with secondary colonization of Africa. Furthermore, phylogenetic analyisis of *Fasciola gigantica* isolates collected from sheep and cattle in Northwestern Iran and subjected to restriction fragment length polymorphism (RFLP) revealed the existence of two haplotypes G1 and G2 (Aryaeipour *et al*., 2014). These haplotypes were earlier hypothesized to be representing separate species (Walker *et al*., 2012).

Recent report by Mas-Coma (2015) on the origins and geographical spread of *Fasciola hepatica* and *Fasciola gigantica* in ruminant pre-domestication times and the livestock post-domestication period revealed that the origin of *F. gigantica* was probably in the warm, eastern Africa, where the Lymnaeaid snail *Radix natalensis* assured the transmission and this origin was probably due to its adaptation to ancestors such as of Alcelaphinae, Reduncinae and Bovinae, during the second pecoran episode, resulting in an explosive radiation during the early Miocene. Conversely, the origin of *F. hepatica* was probably in the Eurasian Near-East, as a derivation from the same ancient fasciolid or a *F. gigantica*–close old form introduced with ruminants from Africa during a major sea level lowering in the early Miocene. The origin of *F. hepatica* is likely the result of colonization of and subsequent adaptation to a new, more northern and temperate-

colder region, as well as the result of two-host capture phenomena to smaller Lymnaeaid species of another lineage such as *Galba* and to mid-sized ruminants.

Strain variations have been reported among the two important species of *Fasciola* involved in zoonoses i.e. *F. hepatica* and *F. gigantica* (Gupta, 2014). While *F. hepatica* exhibits a uniform infectivity to various definitive hosts including man and can infect them in all parts of the world that it occurs, reports of human infection with *F. gigantica* are just isolated cases, despite its high prevalence in animals. The relative infrequent occurrence of human infection with *F. gigantica* was attributed to the existence of different strains of *F. gigantica*, some of which are highly infective for domestic ruminants but have lower infectivity for man (Gupta, 2014).

A third species; *Fasciola jacksoni* occurs in the liver and less frequently the lungs, kidneys, pericardia, and intestines of *Elephas maximus indicus* and *Elephas maximus maximus* in the Indomalayan region, causing cirrhosis, hemorrhages, and connective tissue proliferation (Bhatia *et al.*, 2006; Heneberg, 2013). *Fasciola jacksoni* is a small rounded to pear shaped parasite, measuring 12-14 mm long and 9-12.5 mm broad. A suggestion for the reassignment of this species to the genus *Fascioloides* was made by Lotfy *et al.* (2008) owing to its closer resemblance to *Fascioloides magna* based on phylogenetic analysis. Similarly, recent analysis of the conserved (28S rDNA) and highly variable (ITS1, ITS2, and ND1) loci of mitochondrial and nuclear DNA suggested high similarity of *Fasciola jacksoni* to *Fascioloides magna*, with supporting evidences such as morphological similarities, host spectrum overlaps, and similarities in disease onset and progression (Heneberg 2013). Thus, similar suggestion for the reclassification of *Fasciola jacksoni* as *Fascioloides jacksoni* was also made (Heneberg, 2013).

An uncommon species; *Fasciola nyanzae* was previously reported from hippopotamus (Dinnik and Dinnik, 1961), with no much subsequent information in the literature on other aspects of existence such as life cycle, biology and epidemiology of the parasite. Other species that have been reported in the literature include *Fasciola halli* (Sinitsin, 1933) and *Fasciola californica* (Sinitsin, 1933) characterised by body comparatively armed with spines but the posterior part devoid of spines on dorsal surface (Gupta, 2014).

Recent researches also revealed the existence of an intermediate form of *Fasciola*; which is thought to be a hybrid between *Fasciola gigantica* and *F. hepatica* and has been reported from Egypt, Pakistan and China among other countries (Periago *et al.,* 2008; Mufti *et al*., 2014; Liu *et al.,* 2014). However, the specific status of *Fasciola* spp. ('intermediate form') still remains unclear (Liu *et al.,* 2014).

2.2 Hosts of *Fasciola* spp

Fasciolosis is a parasitic liver infection of humans, wild and domestic ruminants caused by the liver fluke, *Fasciola* (Damwesh and Ardo, 2013). The hermaphrodite parasite, more commonly and anciently called as liver fluke, resides in the bile ducts of variety of mammalian hosts belonging to the Families cervidae, capridae, bovidae, equidae, etc, and getting an opportunity, does not even spare man (Gupta, 2014). The disease affects herbivores with a worldwide distribution in a large variety of grass-grazing animals as sheep, goats, cattle, buffaloes, horses and rabbits. In Egypt, donkeys and camels as well, are hosts for *Fasciola gigantica* (Mahmoud *et al.,* 2010). The major species of liver fluke include *Fasciola gigantica* and F. *hepatica*, the former being more prevalent in cattle and the latter in sheep and goats (Lapage, 1968; Blood *et*

al., 1980 as cited in Ozung *et al.,* 2011). *F. gigantica* appears to be better adapted to cattle than to sheep in that it is more infective and lives longer in the former host (Mas-Coma *et al*., 2009a).

2.3 Fasciolosis in Humans

The first report of human fasciolosis dates back to 17th century; which was later followed by the detection of *Fasciola* in gall bladder of a man during post-mortem and during operation in an eight-year-old girl in 1872 (Malpighi, 1697; Bidloo, 1697; Fortassin 1804; Duval 1842; Partridge, 1846; as cited in Gupta *et al.,* 2014).

Human cases of fasciolosis have often been detected among patients without obvious signs attributable to the disease. Thus, two previous cases reported from Venezuela and Iran were both in an asymptomatic geriatric patients with no specific symptoms (Incani *et al.*, 2003; Moghadami and Mardani, 2008). Most human cases have been restricted to South America and Asia. However, findings revealed that they have been cases from African countries of Egypt and Ethiopia (Bayu *et al.,* 2005), with under-reporting probably responsible for lack aof information from other regions.

Inoue *et al.* (2007) had demonstrated the existence of apparent discrepancy between egg size and genotype of *Fasciola* spp. in a rare occurrence of fasciolosis in 78 year old Japanese man. Thus, obtained morphometric data of worm was consistent with that of *Fasciola hepatica*, while the egg size with that of *Fasciola gigantica*. However, sequences of the nucleotides of ITS-1, ITS-2 and COI genes of the extracted DNA from the egg revealed the egg as that of *Fasciola hepatica*,

thereby making egg size an unreliable criterion for differentiation between the two species especially in regions where they overlap.

2.4 Morphology and Molecular Biology of *Fasciola* spp

Gupta (2014) described the morphology of the adult *Fasciola* as leaf-shaped and fleshy. *F. hepatica* is 'laurel leaf' shaped, grayish-brown in colour measuring 20-30 mm in length by 8-15 mm in width. The tegument is armed anteriorly with backwardly projecting spines on both dorsal and ventral surfaces, and is smooth posteriorly. The anterior end is conical and marked off from the body by distinct shoulders. Oral and ventral suckers are distinctly visible, the latter situated at the level of shoulders. *F. gigantica* is much larger, resembling a 'neem' leaf in shape and size measuring 30 to 70 mm in length and 5-12 mm in width. The anterior conical end is short and continuous to body resulting in total obscurity of shoulders, while the ventral sucker is prominent and entire tegument is covered with spines. *Fasciola jacksoni* is a small rounded to pear-shaped parasite, measuring 12-14 mm long and 9-12.5 mm broad.

The two common species of *Fasciola* (*F. hepatica* and *F. gigantica*) occur primarily in the bile duct and liver of sheep, goat, cattle and buffalo, mule, pig and may rarely be found in unusual hosts such as man and horse (Bhatia *et al.,* 2006; Tasawar *et al.,* 2007). *F. hepatica* may also occur in the lungs of unusual hosts (Bhatia *et al.,* 2006). However, *F. jacksoni* in addition to being found in the liver and bile duct inhabits the duodenum of Indian elephants. The specie inhabits the liver of its host. Although it has an elongated body compared to the other species, morphological features such as cephalic cone, branched intestinal caeca and the reproductive organs suggest that it is a derived fasciolid (Lotfy *et al.,* 2008).

In Africa, Amor *et al*. (2011) reported the use of molecular technique in the characterisation of *Fasciola hepatica* from Mauritania based on mitochondrial and nuclear ribosomal DNA sequences. Similarly, the same study had shown that *F. gigantica* has previously been characterised from Burkina Faso, Senegal, Kenya, Zambia and Mali, while *F. hepatica* has been reported from Morocco and Tunisia. Furthermore, Chauke *et al*. (2014) and Taha (2014) have both used PCR for the characterisation of *Fasciola* spp. from Egypt and Zimbabwe respectively.

2.5 Life Cycle of *Fasciola* spp

Moazeni and Ahmadi (2016) reported that *Fasciola hepatica*, the most widely studied *Fasciola* has been known for more than 630 years, with considerable research work carried out on the life cycle. The life cycle of *Fasciola* (Figure 2.1), including the role of snails as its intermediate host was explained by Leukart and Thomas in 1883, while Adolph Lutz in 1892 described its mode of transmission to definitive host in Hawaii (Gupta, 2014). However, despite this early knowledge and understanding of the life cycle of *Fasciola*, some aspects such as the exact route and time of entry into the bile ducts still remain controversial and therefore require initiative techniques such as radio-labeling assays to help in better understanding (Moazeni and Ahmadi, 2016). *Fasciola hepatica* has enormous biotic potential, involving sexual, hermaphroditic, or parthenogenic reproduction in the final mammalian host and clonal asexual multiplication in intermediate molluscan hosts (Sargison, 2012). Climatic and geographical variables are known to be important in determining the risk of fluke infection, because of their effect on the survival and rate of development of the parasite on pasture and in the intermediate host, and on the intermediate host itself (Howell *et al.* 2015).

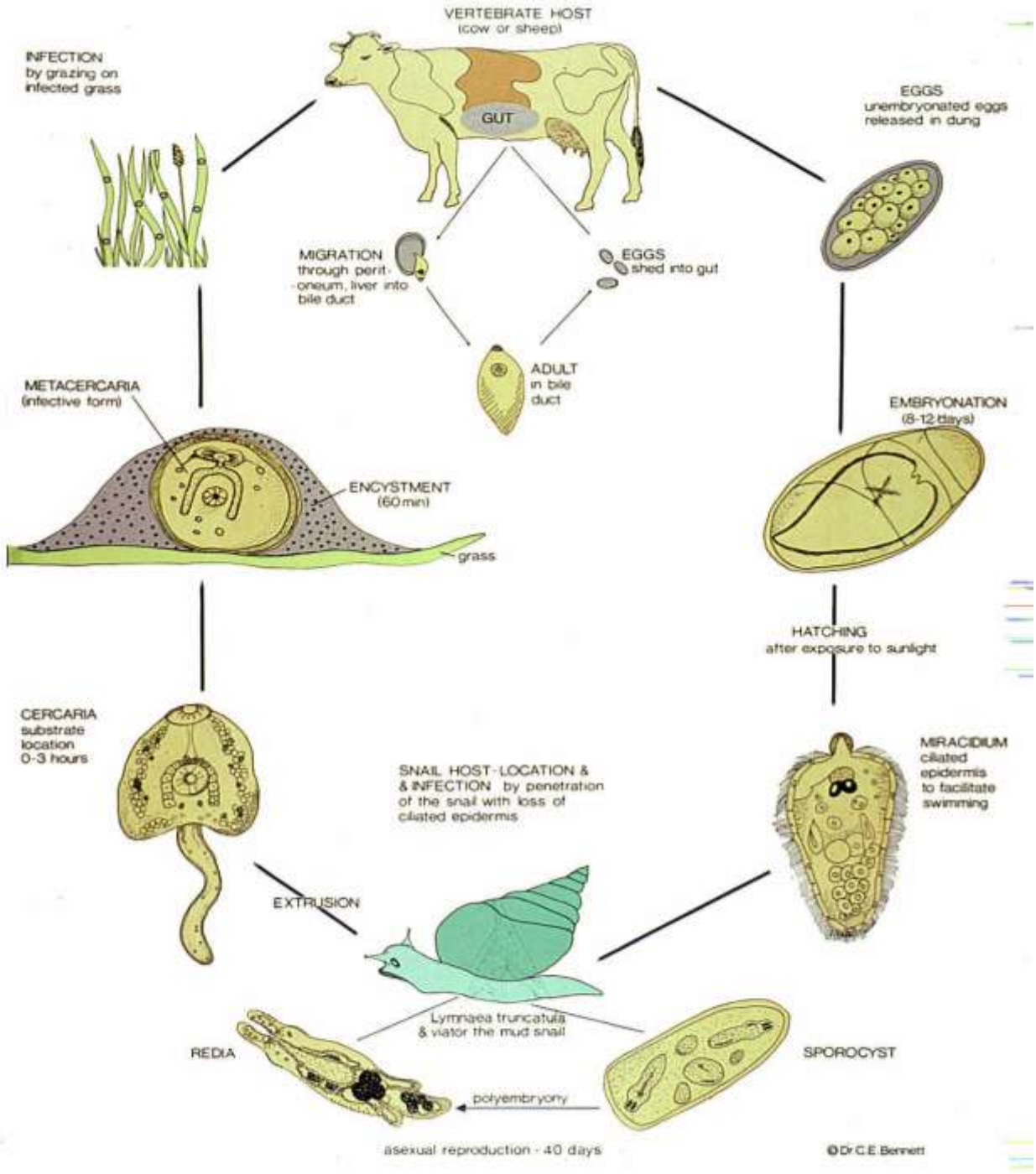


Figure 2.1: Life cycle of *Fasciola spp* (Bennett, 1999)

2.6 Prevalence and Epidemiology of *Fasciola* Infection

The prevalence of *Fasciola* spp from Nigeria is summarized in Table I. Using different methods such as bile and faecal sedimentation, post-mortem examinationa at abattoir and serology, the prevalence ranged from 1.2% to 47.4% in cattle, 0.32 to 17.0% in sheep, 0.23% to 9.0% in goats and 2.2% in vegatables put up for sales. From outside Nigeria, it ranged from 0.09 to 95.33% in cattle, 3.28 to 93.0% in sheep, 1.1% to 28.75% in goats, 33.70% to 96.0% in buffaloes and 0.0% to 13.04% in humans using coprology, post-mortem examination and serology (Table II). Case reports have also been documented in dog and humans.

Kusiluka and Kambarage (1996) discussed the epidemiology of trematode parasites and asserted that climate, management system, parasite, definitive and intermediate hosts, each play an important role in the epidemiology of the diseases caused by trematodes. The intermediate host for *Fasciola gigantica* is *Lymnaea natalensis*, although *Lymnaea trucantula* has been found to serve as intermediate hosts in the highlands of East and Central Africa. The optimum temperature range for the survival and development of *L. natalensis* is 15-26^{o}C. In fact, the prevalence of *Fasciola* spp. infection depends on several factors related to the biology of the vectors, biology of the parasite and the management of flocks and herds (Khoramian *et al.*, 2014). Light, suitable temperature of 20-25^{o}C and availability of oxygen are essential for the embryonation and hatching of *Fasciola* spp. eggs (Kusiluka and Kambarage, 1996). Equally, no development occurs below 10^{o}C and temperature above 35^{o}C result in death of larvae. The humid environment of sub-Saharan countries has been described as being favourable for the embryonation and hatching of *Fasciola* eggs throughout the year (Kusiluka and Kambarage, 1996). Additionally, faeces and manure pits are known to serve as reservoirs of infection, while

concentration of animals at watering points during the dry season serves as a favourable factor for transmission.

In a study on the epidemiology and impact of *Fasciola hepatica* in a high-yielding dairy herd in England, Scotland and Wales, Howell *et al*.(2015) reported higher rainfall, grazing boggy pasture, presence of beef cattle on farm, access to a stream or pond and smaller herd size as factors associated with an increased risk of exposure to the infection. Kithuka *et al.* (2002), in an analysis of abattoir data in Kenya, found local climatic factors, cattle trade, rustling and population numbers, and the presence of the snail intermediate hosts as probable main factors influencing the incidence of fasciolosis in the various regions of the country. The liver flukes have a versatile survival strategy with certain stages of the parasites and their intermediate hosts having a relatively well-developed ability to persist through adverse weather conditions such as drought and freezing (Hansen and Perry, 1994).

Whereas some fasciolids have retained circumscribed geographic distributions, others such as *F. hepatica* have become cosmopolitan (Mas-Coma *et al.,* 2005). The spread of this species is in part caused by its adaptability to different Lymnaeaid snail hosts and to introduction of infected livestock or of susceptible snail hosts into new areas. *Fasciola hepatica* has succeeded in expanding from its European original geographical area to colonize five continents, despite theoretical restrictions related to its biology and in turn dependent upon environmental and human activities (Cywinska, 2005). The geographic distribution of *Fasciola* species is dependent on the distribution of suitable species of snails such as *Lymnaea natalensis* and *Lymnaea truncatula,* the most common intermediate hosts and usually associated with herds and flocks

grazing wet marshy land areas (Chakiso *et al.*, 2014). Both *Lymnaea* species are needed for the parasite's life cycle to be completed. Climate and global changes appear to increasingly affect snail-borne helminthiases such as fascioliasis, which are strongly dependent on environmental factors (Mas-Coma *et al.*, 2005; Mas-Coma *et al*., 2009b).

Fascioliasis is a good example of an emerging/re-emerging parasitic disease in many countries as a consequence of many phenomena related to environmental changes as well as man-made modifications (Mas-Coma *et al.*, 2005).The high levels of genetic polymorphism in helminths, and the existence of triploidy in some populations of *F hepatica*, have enabled the evolution of complex life cycles that are dependent on overlap of suitable environments for free-living stages, intermediate and final hosts, while also conferring the ability to quickly take advantage of favourable opportunities afforded by relatively short-term animal and pasture management changes and effects of micro- and macro-climatic variation on their biotopes. The same evolutionary potential inevitably enables populations of these parasites to survive despite a variety of unfavourable conditions, such as exposure to fasciolicidal anthelmintics (Sargison *et al.*, 2012).

Fasciola hepatica is found in more than 50 countries, in all continents except Antarctica. It is found in parts of Latin America, the Caribbean, Europe, the Middle East, Africa, Asia, and Oceania. *Fasciola gigantica* is less widespread (CDC, 2013). Human cases have been reported in the tropics, in parts of Africa and Asia, and also in Hawaii. For example, the areas with the highest known rates of human infection are in the Andean highlands of Bolivia and Peru (CDC, 2013). *F. hepatica* is also an important pathogen of humans in certain regions of the world where

farm management practices allow infected animals to roam amongst vegetation consumed by humans (Molina-Hernández *et al.,* 2015). From Turkey, Yildrim *et al*. (2007) attributed variations in prevalence of fasciolosis in cattle from different regions to some factors such as environmental and climatic conditions, snail population, and choice of diagnostic method and patency of infection. The level of agricultural activity, nutritional deficiency, pasture management, micro- and macro- climate of the area, presence of intermediate host (water snail) and vectors in an area as well as the immunological status of the host determine seasonal incidence of fascioliasis (Harris and Charleston, 1974; Onyali *et al*., 1990; Suarez and Busetti, 1995 as cited in Ozung *et al.,* 2011). The distribution of *F. hepatica* infection was primarily determined by patterns of climatic conditions favourable for the snail intermediate hosts, free living stages of the parasite, grazing habits and feeding systems of the host animals. Animals grazing in wet, marshy or swampy areas, favoured by the intermediate host, are most likely to become infected as marshy environment, is especially hospitable for fluke eggs, hosts snails and larval flukes (Boray *et al.,* 1969; McCann *et al.,* 2010 as cited in Gebeyehu *et al.,* 2014). The farmers' use of a cut-and-carry system in which animals are fed vegetation from wet marshy areas, was proposed to have created an opportunity for the transmission of the infective stages of *Fasciola,* and ultimately, infection. Comparatively, highest prevalence of infection was found among cattle, when slaughtered goats, sheep and cattle were investigated for the presence of fasciolosis in Ethiopia (Abdulhakim and Addis, 2012). The obtained result was justified by the indiscriminate feeding pattern in cattle compared to goats, which are selective grazers, which in turn reduces the possibility of the contact of goats to infective stages of *Fasciola,* which are abundantly found around marshy areas. Similarly, the dramatic decrease observed in the prevalence of fasciolosis in native Korean goats from the previous prevalence two decades ago,

was attributed to improved farm management practices, indoor confinement, formula feed and frequent deworming programmes implemented on goat farms (Gebeyehu *et al.,* 2013) and possibly, variations in response among breeds, as some goat breeds have been reported to be more resistant to helminth infections than others (Tasawar *et al.,* 2007).

Table 2.1: Review of Prevalence of *Fasciola* Infection in Nigeria

Location of Study	Host(s) Sampled	Prevalence (%)	Method Employed	Reference
Gombe	Cattle	25.90	Post-mortem examination	Adang *et al*.(2015)
Kano	Cattle	1.2%	Post-mortem examination	Danbirni *et al*.(2015)
Zaria	Cattle	17.09,7.3	Coprology/Serology	Aliyu *et al*.(2014)
Yola	Sheep and goats	0.32,0.23	Post-mortem examination	Ardo and Aliyara (2014)
Zaria	Sheep, goats and Vegetables	17.0,9.0,2.2	Sedimentation technique	Esonu, 2014
Sokoto	Cattle	27.68	Coprology/Bile sedimentation	Magaji *et al*.(2014)
Maiduguri	Cattle	14.8	Post-mortem examination	Biu *et al*.(2013)
Port-Harcourt	Cattle	45.70	Post-mortem examination	Gboeloh (2012)
Jos	Cattle	3.88	Bile examination	Omawaye *et al*.(2012)
Maiduguri	Sheep, goats	0.49,0.28	Retrospectiuve Post-mortem examination	Mbaya *et al*.(2010)
Ibadan	Cattle	33.5, 38.9, 47.4	Faecal examination, Bile examination and AGPT	Adedokun *et al*.(2008a)
Imo State	Cattle, sheep and goats	(7.0,3.4,2.8)	Post-mortem examination	Okoli *et al*.(2002)
Maiduguri	Cattle, sheep and goats	21.1,3.9	Bile examination	Nwosu and Srivastava (1993)

Table 2.2: Review of Prevalence of *Fasciola* Infection outside Nigeria

Location of Study	Host(s) Sampled	Prevalence (%)	Method Employed	Reference
Lake Chad	Cattle	41.9, 46.0	Coprology	Greter *et al*.(2016)
Botswana	Cattle	0.09	Retrospective Post-mortem examination	Mochankana and Robertson (2016)
Ethiopia	Cattle	24.4	Post-mortem examination	Yusuf *et al*.(2016)
Phillippines	Cattle and buffaloes	95.33, 96	FEA-SD,qPCR	Gordon *et al*.(2015)
Bangladesh	Cattle	66.14	Coprology	Karim *et al*.(2015)
Turkey	Cattle and Sheep	66.6,93.0	Serology	Akca *et al*.(2014)
Korea	Cattle	9.4	Serology	Gebeyehu *et al*.(2014)
Southern Lake Chad	Goats, sheep and cattle	12.0,23.0,68.0	Post-mortem examination	Jean-Richard *et al*.(2014)
Iran	Sheep and goats	7.7,5.4	Post-mortem examination	Khanjari *et al*.(2014)
Iran	Cattle, sheep and goats	3.68, 3.28,2.76	Post-mortem examination	Khoramian *et al*.(2014)
Egypt	Cattle, humans	9.8, 13.04	Serology	Nossair and Abdella (2014)
Korea	Goats	1.1	Serology	Gebeyehu *et al*.(2013)
Spain	Sheep	59.3	Coprology	Martinez-Valladares *et al*. (2013)
Egypt	Dog	Case report	Coprology	Salib *et al*.(2013)
Ethiopia	Cattle, sheep, goats	28.6,20.8.13.6	Post-mortem examination	Abdulhakim and Addis (2012)
Kenya	Cattle	8.0,9.3	Post-mortem examination	Kithuka *et al*.(2012)
Kenya	Cattle, goats, sheep	25.90,23.40,33.3	Coprology	Mungube *et al*.(2012)
Egypt	Cattle, buffaloes, sheep, humans	28.60,33.70,17.2,0.0	Coprology	Hussein and Khalifa (2010a)
Pakistan	Goats	28.75	Coprology	Tasawar *et al*.(2007)
Turkey	Cattle	65.2,24.5	Serology/Coprology	Yildrim *et al*.(2007)
Zimbabwe	Cattle	15.4	Retrospective Post-mortem examination	Pfukenyi *et al*. (2006)
Mexico	Cow	50, 30,100	Coprology	Cruz-Mendoza *et al*. (2005)
England and Wales	Dairy cattle	48.00, 86.00	ELISA	Salimi-Bejestani *et al*. (2005)
Venezuela	Humans	Case report	Coprology	Incani *et al*.(2003)

2.7 Pathogenesis of Fasciolosis

The pathogenesis of fasciolosis is associated with liver damage that is inflicted by migrating and feeding immature flukes as well as host inflammatory immune responses to parasite-secreted molecules and tissue damage alarm signals (Molina-Hernández *et al*., 2015). The course of pathogenesis in different hosts is similar but may vary in severity with the number of metacercariae ingested, the species involved and the stage of the parasitic development (Gupta, 2014). This is aptly demonstrated by the fact that lesions produced by *F. gigantica* are more severe with fewer flukes as compared to *F. hepatica*, which may be attributed to the longer duration of migration in hepatic parenchyma, larger size and spines present all over the tegument of *Fasciola gigantica*.

Losos (1995) as cited in Damwesh and Ardo (2013) described three distinct but partially overlapping syndromes in fasciolosis which include:

2.7.1 Acute fasciolosis

The acute phase commences between 2-6 weeks after ingestion of substantial number of metacercariae, and depending on the species involved. In *F. hepatica*, it may be 2000 metacercariae for sheep while for *F. gigantica* 300 metacercariae can produce similar condition (Gupta, 2014). In large ruminants, buffalo and cattle, ingestion of 1000 metacercariae of *F. gigantica* produces acute disease. Acute fascioliasis in animals is usually characterised by anorexia, dullness, diarrhea, muscular atrophy, subcutaneous oedema and impaired immune systems (Boray, 1969; Blood *et al.,* 1980; Reid, 1995 as cited in Ozung *et al.,* 2011). Rupture of the liver capsule may occur with hemorrrhage into the peritoneal cavity and animal may die within a few days of onset of clinical symptoms (Bhatia *et al.,* 2006). Furthermore, the liver

becomes enlarged, pale, and friable with numerous haemorrhagic tracts on the surface and throughout the substance. There may be fibrinous clots on the liver surface and throughout the peritoneal cavity (Ajanusi, 1987; Bhatia *et al.,* 2006). In small ruminants, multiple adhesions of adjacent organs with liver are present. Sheep may die suddenly without exhibiting any clinical manifestations, or following weakness, anorexia and pain (Gupta, 2014)

2.7.2 Subacute fasciolosis

This occurs when the host ingests moderate doses of metacercariae over a longer period, resulting in presence of different migratory stages of flukes in the liver. While some have reached bile ducts and caused cholangitis, others are still migrating in liver tissues like that of acute disease but of less severity (Gupta, 2014). Here, there is anaemia due to the presence of young flukes emerging from the liver (Damwesh and Ardo, 2013). This type of fasciolosis may be seen superimposed on an existing chronic infection (Bhatia *et al.,* 2006). Enlarged liver with haemorrhagic tracks all over the surface and rupture of sub capsular haemorrhage which is rare are features of the subacute fasciolosis. Infiltration of white cells and fibrosis is more in evidence (Bhatia *et al.,* 2006). Clinical manifestations including anaemia, hypoalbuminaemia, eosinophilia and elevated alanine aminotransferase (ALT) and aspartate transaminase (ASL) serum levels and in animals, particularly sheep harbouring *Clostridium novyi* in liver, that can lead to necrotic hepatitis (Gupta, 2014)

2.7.3 Chronic fasciolosis

It is the most common form of fasciolosis in sheep, cattle and other animals and even man and it occurs most commonly in light or moderate repeated dose infections (Bhatia *et al.,* 2006; Gupta,

2014). This is a wasting disease and characterised by the presence of flukes in the bile duct and characterised by debility and anaemia with subcutaneous edema in the lower portion of the abdomen, face and thorax. Clinical signs are often mild and may present as loss of productivity, while in severe cases, submandibular oedema may be seen (Mazeri et *al.,* 2016). Animal becomes weak, inactive with distended abdomen, stiff gait, and lack of appetite, black scouring and bottle jaw due to the presence of watery swellings under the mandibles (Bhatia *et al.,* 2006). Pale mucous membrane and bottle jaw have been reported from goats (Talukder *et al.,* 2010)

2.8 Economic Impact of Fasciolosis

Over 700 million production animals are at risk of the infection, and worldwide economic losses estimated at greater than US3.2 billion per annum (Spithill *et al*., 1999). Fasciolosis was identified among other pathological conditions as a leading cause of liver condemnation in cattle, sheep and goats in a slaughterhouse survey of liver lesions in Arusha, Tanzania (Mellau *et al.,* 2010). However, in fallow deer, the disease was described as not being an economically important disease as in cattle and sheep, as the fallow deer are more tolerant of infection, showing no clinical evidence although in some enclosures all the animals may carry flukes (Munro, 1994 as cited in Vengust *et al.,* 2003).

Within Europe, the amount spent annually on anthelmintic drugs for parasitic helminths of ruminants has been estimated to be 400 million Euros (Selzer, 2009; Morgan *et al.,* 2013 as cited in Molina-Hernández *et al.,* 2015). Similarly, recent report from South Africa showed that the total financial loss due to *Fasciola* infection in a survey of three abattoirs from Cape Town, was ZAR 44, 930 (3456.2 USD) (Jaja *et al*., 2017). In Nigeria, economic losses in cattle due to

mortalities, decreased weight gain, reduced milk production, abortion, unthriftness, condemnation of infected liver and cost of treatment have been reported (Fabiyi *et al.,* 1980; Adama *et al*.2011 as cited in Damwesh and Ardo, 2013). Some of these losses especially mortalities and abortion presumably occur in sheep (Adama *et al*., 2011) to a larger extent, since the infection is mainly acute and to a lesser extent in goats. Available information in the literature indicated that most data on economic losses from Nigeria have been skewed towards the determination of loss due to liver condemnation at abattoir, with little or few reports on aspects of losses such as cost of treatment. The World Bank report (WHO, 2006; as cited in Aliyu *et al.,* 2014) reported that total gross liver loss of 8.292 kg was observed with about 75% loss of value in 29.952 kg of partially condemned liver in a single abattoir over a three-year period in South-western Nigeria.

2.9 Immune Responses to Fasciolosis

Immunology in fascioliasis is a combination of interplay involving host effector mechanisms and parasites defence/immune-evasion mechanisms, as the mammalian hosts are known to use both humoral and cellular immune mechanisms, with juvenile fluke as the main target (Gupta, 2014). Martinez-Moreno *et al.* (1997) studied both cellular and humoral immune responses to *Fasciola hepatica* excretory-secretory products following primary and secondary infections in goats and reported that goats infected in either case had similar IgG response and that, humoral and cellular responses to *F. hepatica* excretory-secretory products (ESPs) in goats have no protective effect on the establishment of flukes and the development of disease in either primary or secondary infections.

Immune suppression/modulation by the parasites prevents the development of protective immune responses as evidenced by the lack of immunity observed in naturally and experimentally infected animals (Molina-Hernández *et al*., 2015). Some animals' exhibit natural resistance against *F. gigantica*, like Indonesian thin-tail (ITT) sheep (Wiedosari and Copeman, 1990 as cited in Gupta, 2014), eliminating the infection during the prepatent phase. In fact, three independent trials aimed at directly comparing *Fasciola gigantica* and *Fasciola hepatica* infection of ITT sheep resulted in higher worm burden recoveries and greater physiological damage to ITT sheep with an innate and adaptive comparative ability to resist the early stages of infection with *F. gigantica* infection was demonstrated in the study (Pleasance *et al*., 2011). *Fasciola hepatica* secretes a myriad of molecules that direct the immune response towards a favourable non-protective Th2-mediated/regulatory environment (Dalton *et al.,* 2013). Molecules, such as cathepsin L peptidase (FhCL1), peptidase and other molecules, such as peroxiredoxin (FhPrx) and helminth defence molecule (FhHDM-1), exhibit various immunomodulatory properties that could be harnessed to help treat immune-related conditions in humans and animals (Dalton *et al.,* 2013).

In vitro biochemical studies have predicted that ES products of *F. hepatica* have roles in feeding behavior, detoxification of bile components, and the evasion of the immune system (Morphews *et al.,* 2007). In well characterised cattle models, the immune response to *F. hepatica* infection is pro-inflammatory lasting for about 4–6 weeks post infection. This immune response appears to be switched off at around the time adults enter the bile duct. The presence of flukes modifies Th1 helper T cell responses, inhibiting the induction of the protective immune system, which makes the host more susceptible to further infections. It is likely that liver fluke secretions modify host

macrophage-based signaling events to switch the proinflammatory immune response "off" before the infection has been appropriately controlled (Morphews *et al.,* 2007). *In vivo* host bile analysis of *F. hepatica* indicated that the major proteins present outside the parasite in host tissues were a variety of proteases, mainly the cathepsin L proteases and this supports previous *in vitro* studies, suggesting that these proteins play a major role in *F. hepatica* survival in the host gall bladder and bile duct (Morphews *et al.,* 2007). Sheep trypsin inhibitor complex (TIC) identified in host bile has been shown to be highly immunogenic (Lake-Bakaar *et al.,* 1979 as cited in Morphews *et al.,* 2007) as it inactivates excessive trypsin, and its increase in liver fluke-infected bile may be part of a defense to directly counteract parasite survival roles of *F. hepatica* proteases. An immunogenic TIC may function as a host biomarker for liver fluke infection via antibody-based assays in bulk milk, blood, or feces. Newly excysted juveniles emerge from their metacercarial cysts in the duodenum where early ES products are released, and migrating juveniles pass through the peritoneal cavity on route to the liver and will again release ES products into the surrounding host fluid. However, in both of these instances, the relative abundance of newly excysted juveniles and juvenile ES products in relation to host protein may severely hamper proteomics analysis of these host fluids (Morphews *et al.,* 2007).

The tegument of trematode parasites including *Fasciola* that live in the mammalian hosts' circulation or biliary system can protect them from hosts' immune attacks by evolving evasion mechanisms that include the rapid turnover of the surface membrane to prevent the attachment of immune effector cells, and by immune mimickry or disguise through the adsorption of hosts' antigens onto the parasites' surfaces (Sobhona *et al.,* 2000). Equally, the tegument is responsible for the regulation of ions and fluid balance, which in turn keep the homeostatic equilibrium

within the parasites' bodies. In a recent study, the role of apoptosis of peritoneal leucocytes in the early stages of *Fasciola hepaitica* infection in sheep was investigated by Escamilla *et al*. (2017). The *Fasciola hepatica* induced peritoneal leucoytes apoptosis observed was thought to allow the larvae evade the host's immune response. Similarly, the biomarker annexin V helped in early detection than caspase-3.

2.10 Diagnosis of Fasciolosis

Parasitological diagnosis is based on identification of eggs in stool, duodenal contents or bile, also by the recovery of adult worm during surgical exploration, after treatment or at autopsy (Nossair and Abdella, 2014). However, the eggs may be present in very small number at irregular intervals, hence difficult to be found. Besides, the eggs may be transiently present in stool after ingestion of raw or undercooked liver from infected animals (Nossair and Abdella, 2014), giving rise to pseudo-infection.

Diagnosis by serological methods like Enzyme Linked Immunosorbent Assay (ELISA) and Western blots can detect serum antibodies to specific antigens of *Fasciola spp*. using adult fluke extracts or excretory/secretory (ES) materials and can be as early as 2 weeks after infection (Santiago and Hillyer, 1988; Fagbemi and Guobadia, 1995 as cited in Adedokun *et al.*, 2008b). The detection of anti- 27 kDa *Fasciola gigantica* glycoprotein as early as two weeks post-infection using ELISA was described as a feasible diagnostic tool for the early detection of bovine fasciolosis (Ghosh *et al*., 2005).

Other serological methods like AGPT and agar gel diffusion test (AGDT) have also been demonstrated to be simple and valuable for detection of *Fasciola* sp. antibodies particularly where there are less diagnostic facilities (Bui khanh linh *et al.,* 2003 as cited in Adedokun *et al.,* 2008b).

In a comparative study on the methods used for diagnosis of fascioliasis, a total of 64 individual faecal and serum samples collected from naturally infected sheep and beef cattle herds with previous histories of *F. hepatica* infection were used to determine outcomes of faecal egg count (FEC), serology and coproantigen ELISA (cELISA) compared with the performance of polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) (Arifin *et al.,* 2016). Obtained results revealed that *F. hepatica* eggs were present in 28 animals, while coproantigen and specific anti-*F.hepatica* antibodies were detected in 36 and 53 animals, respectively.Similarly, only 3 and 6 samples were positive by PCR and LAMP, respectively. A combination of FEC and cELISA was selected as the composite reference standard (CRS) to calculate specificity and sensitivity, and when compared to the CRS, PCR had a sensitivity of 10.7% and specificity of 100%, while LAMP had sensitivity and specificity of 17.9% and 97.2%, respectively. Thus, PCR and LAMP were highly specific in this study, but both had poor sensitivity compared with FEC and cELISA.

2.11 Control of Fasciolosis

Fascioliasis is controlled by a combination of anthelmintic therapy and management measures (Mahmoud *et al.,* 2010).

Strategies involving drainage, or fencing off delineated wet and boggy areas and grazing sheep and cattle away from snail habitats during high-risk periods, combined with strategic anthelmintic treatments during the spring have generally proved to be successful in controlling, but not eliminating, fasciolosis (Sargison *et al.,* 2012). Triclabendazole has been reported to be highly effective, even though few cases of resistance have been reported. Knowledge of anthelmintic efficacy is a prerequisite for effective helminth control (Sargison *et al.,* 2012). Another set back to the control of fasciolosis is the number of wild animals serving as reservoir hosts (Bhatia *et al*., 2006). Mezo *et al.* (2013) had demonstrated in the first report on the importance of the wildboar as a possible reservoir of *Fasciola hepatica* infection in Spain that, *F. hepatica* from cattle, sheep and wild boars from the same geographical area presents a similar body development and gravidity and that *F. hepatica* in Galicia (Spain) had a normal development in wild boars, presenting its own characteristics in shape and size in comparison with other host species.The control measures against snails and the treatment of infected animals should be carried out simultaneously in a given locality (Bhatia *et al*., 2006).

The use of biologicals for the control of *Fasciola spp* was previously attempted in a study by Dias *et al.* (2012) involving the effect of *Pochonia chlamydosporia* fungus on *Fasciola hepatica* eggs. Mycelia of the fungus were administered to experimental animals orally and the faeces containing the fungus used against *Fasciola hepatica* eggs *in vitro*. The ovicidal *P.chlamydosporia* fungus was effective in destroying *F.hepatica* eggs within seven days of interaction; suggesting that this fungus could be employed as agent for the control of helminth eggs.

Major advances have been made in identifying potential vaccine molecules for the control of fasciolosis in livestock, but yet the level of efficacy required for commercialization has not been reached (Ajanusi, 2016). The pathogenesis of fasciolosis is associated with liver damage that is inflicted by migrating and feeding immature flukes as well as host inflammatory immune responses to parasite-secreted molecules and tissue damage alarm signals (Molina-Hernández *et al*., 2015). The known vaccine potential of *F. hepatica* Glutathiones (GST) may relate to an anti-inflammatory GST being secreted by newly excysted juveniles that are first exposed to the immune system (Morphews *et al.,* 2007). Glutathiones from *Fasciola hepatica* had been found to be effective in vaccinating cattle and sheep against fasciolosis (Degheidy and Shalaby, 2010). The role of glutathione-S-transferase as a potential vaccine candidate was evaluated by Degheidy and Shalaby (2010) in goats following vaccination with *Fasciola gigantica*, glutathione-S-transferase (FgGST) isolated and purified using affinity chromatography, and infected with 120 metacercaria two weeks later. There was 64.1% worm reduction, reduced size of recovered flukes and tegumental changes which included swelling of the surface and between the spines in the apical cone and mid-body regions in the majority of the flukes examined. In another study on buffalo calves, Nambi *et al.* (2005) revealed that recombinant fatty acid binding protein (rFABP) of *Fasciola gigantica* expressed in *Escherichia coli* and used as vaccine in Freund's adjuvant induced both humoral and cell-mediated immune response with moderate level of protection in terms of reduced fluke burden (35.8%) and liver damage as assayed by aspartate aminotransferase and sulfhydryl group levels as well as anti-fecundity effect. Consequently, these findings increased the understanding on the possibility of the use of GST and FABP as potential vaccine candidates for use against fasciolosis.

The effect of vaccination with functional recombinant thioredoxin glutathione reductase (rFhTGR) from *Fasciola hepatica* and produced in calves was evaluated in two vaccination experiments consisting of the first administration in combination with Freund´ıs Incomplete Adjuvant (FIA) in a three-inoculation scheme on weeks 0, 4 and 8 and the second trial given mixed with Adyuvac 50 or Alum as adjuvants on weeks 0 and 4 (Maggioli *et al*., 2016). The vaccine formulations induced a mixed IgG1/IgG2 response wth non-significant reduction in worm counts, suggesting that rFhTGR failed to induce a protective immunity in challenged calves.

In rabbits, an attempt was made to develop a vaccine against fasciolosis using immunoaffinity fraction of *F. gigantica* excretory-secretory products (Abou-El-Doubal *et al.,* 2015). The fraction was found to possess 87.67% of the initial antigenic activities with 2051.5 fold increase in specific activity compared to crude extract. Furthermore, the fraction resulted in 85% reduction in worm burden, in addition to high antibody IgG levels as proved by ELISA, which was observed in vaccinated rabbits at two weeks post infection and which remained stable to the end of the experiment (Abou-El-Doubal *et al.,* 2015). A significant expression of IL-4 and INF-γ was observed in vaccinated rabbits starting one week until thirteen weeks post infection. The level of IL-4 was significantly higher than the level of INF- γ throughout the experiment as measured by ELISA. Collectively, the results suggest promising prospect. Recently, an upregulation of Th2 and downregulation of Th1 responses in the liver and hepatic lymph nodes at 9 and 18 days post-infection respectively in cathepsin L1 (CL1)-vaccinated and unvaccinated sheep was shown by Pacheco *et al*.(2017). From the study, CL1-vaccinated sheep had increased expression of IFN-γ

than unvaccinated sheep at 9 dpi, thereby coinciding with lower hepatic lesions in the vaccinated group.

Two polypeptides of 27 and 23.5 KDa, obtained from the characterisation of the isolated ES fraction of *Fasciola gigantica* excretory-secretory products in rabbits, were examined for their immunogenic and immunoprophylactic properties (Abdel-Rahman *et al.,* 2014). A reduction of 85.7 % in worm burdens was observed in vaccinated rabbits, in addition to fewer liver lesions in all vaccinated rabbits than in control. Therefore, the isolated fractions proved as successful vaccine candidate against fasciolosis.

In mice, *Fasciola gigantica* cathepsin L1 (CatL1), an important and predominant protease expressed in caecal epithelial cells and secreted into the excretory-secretory products (ES),was evaluated for vaccine potential through the use of recombinant pro-*F.gigantica* CatL1 (rproFgCatL1) and recombinant mature *F.gigantica* CatL1 (rmatFgCatL1) expressed in *Escherichia coli* and performed in the imprinting control region (ICR) by subcutaneous injection with 50g of rproFgCatL1 and rmatFgCatL1 combined with Freund's adjuvant (Kueakhai *et al*., 2015). Signiificantly increased Th1 and Th2 responses in rproFgCatL1- and rmatFgCatL1-immunized groups, with higher levels of Th2 (IgG1) than Th1 (IgG2a) in addition to significantly decreased serum aspartate aminotransferase (AST) and alanine transaminase (ALT) in rmatFgCatL1-immunized group characterised the result obtained in the study, indicating that rFgCatL1 has a vaccine potential against *F. gigantica* in mice, and may be suitable for investigation in higher animals (Kueakhai *et al*., 2015).

2.12 Fasciolosis and Co-infections

The relationship between fasciolosis and the presence of bacterial agents particularly, *Mycobacterium spp.* as co-infection has been a subject of discussion of recent. This is thought to be due to how fasciolosis regulate the immune system of the host. Infection with *Fasciola hepatica* results in polarization of the host's immune response and generation of type 2 helper (Th2) immune responses, which are known to be inhibitory to Th1 responses (Flynn *et al.,* 2007). In fact, a complete down regulation of Th1 responses was seen to occur with an up regulation of the Th2 cytokines IL-4 and IL-5, with the magnitude of the effect dependent on the parasite burden in infected murine models (O'Neill *et al.,* 2000 as cited in Flynn *et al.,* 2007). The relationship between the presence of pathogenic bacteria in the bile versus fascioliasis using parasitological criteria such as worm and egg load, bacteriological bile culture, transmission electron microscopy studies and biochemical changes (liver enzymes) was investigated by William *et al.* (2008) in naturally and experimentally infected sheep and cattle in Egypt . Bacteriological bile cultures revealed the presence of *Escherichia Coli* only in 4 sheep, while naturally infected sheep and cattle had *E. Coli, Klebsiella pneumonia, Pseudomonas* and *Proteus.* In the presence of fluke infection, bacteria are thought to flourish due to changes in the biliary environment (William *et al.,* 2008).

Thompson and Howell (1979) studied the effects of three different doses of BCG on the subsequent establishment of *Fasciola hepatica* metacercariae in rats and reported that there was no significant variation between BCG-treated rats and the control, inspite of profound evidence for the existence of cell-mediated immune response in BCG-treated groups.This was later confirmed by Martinez-Moreno *et al.* (1997) in goats, where both cellular and humoral immune

responses to *Fasciola hepatica* excretory-secretory products did not influence the establishment of *Fasciola hepatica*. In another study, the influence of *F. hepatica* infection on the outcome of two bovine tuberculosis diagnostic assays routinely used in control schemes was determined, in addition to establishing if the timing of helminth infection has an influence on the response to tests (Flynn *et al.,* 2007). The study also determined a possible mechanism that could be responsible for altered responses in co-infected animals. The results revealed that, the predictive capacity of tests was compromised in co-infected animals as the SCITT detection rates fell from 80% in group 4 animals (infected with avirulent *Mycobacterium bovis*) to 40% in group 3 animals (infected with avirulent *Mycobacterium bovis* and subsequently with *Fasciola hepatica*) to 0% in group 2 animals (infected with *Fasciola hepatica* and subsequently with avirulent *Mycobacterium bovis*) and that *F. hepatica* infection altered macrophage function. Similarly, interleukin-4 and gamma interferon expression in whole-blood lymphocytes restimulated *in vitro* with *M. bovis* antigen was also altered in co-infected animals. The observation of altered interferon (IFN)-γ responsiveness in animals co-infected with avirulent *Mycobacterium bovis* (Flynn *et al.,* 2007) was subsequently demonstrated with a virulent *M. bovis* following aerosol infection (Flynn *et al.,* 2009).

Varied responses have also been found among ruminants particularly sheep and cattle co-infected with *Fasciola* and *Mycobacterium spp*. Claridge *et al.* (2012) had demonstrated in a study, a significant negative association between exposure to *Fasciola hepatica* and diagnosis of bovine tuberculosis. The magnitude of the single intradermal comparative cervical tuberculin test used in the diagnosis was reduced in cattle experimentally co-infected with both organisms, with an under-ascertainment rate of about one-third among a total of 3,026 dairy herds investigated in

England and Wales. In another study, Munyeme *et al.* (2012) demonstrated the existence of nexus between bovine tuberculosis and fasciolosis in cattle at the Kafue basin ecosystem in Zambia. Among 72 animals co-infected with fasciolosis and tuberculosis, among a total of 600 positive animals in the study, a significant association was found between fasciolosis and tuberculous lesions in the study. Simple regression analysis revealed fasciolosis as a strong predictor for tuberculous lesions as animals with fasciolosis were five more times likely to have tuberculous lesions.

2.13 Identification of *Fasciola* spp

2.13.1 Morphological identification of *Fasciola* spp

The shape particularly prominence of the shoulder region, in addition to geographical distribution has traditionally been used to differentiate between the two main species of *Fasciola*. Thus, *Fasciola hepatica* is often associated with stumpy body compared to the rather slender body of *Fasciola gigantica*. Most morphometric studies report an average length of more than 30 mm for *Fasciola gigantica* and below that for *Fasciola hepatica* (Biu *et al*., 2013; Shaldoum *et al*., 2015). However, these values are not absolute as overlap was substantially demonstrated previously in a study involving more than 500 *Fasciola* isolates from cattle and buffaloes in Burkina Faso, Bolivia and Iran (Ashrafi *et al.,* 2006). Fully mature *F. hepatica* is grey-brown in colour and around 3.5 cm long and 1.0 cm wide (Urquhart *et al*., 1987).

2.13.2 Molecular identification of *Fasciola* spp

Molecular identification of *Fasciola hepatica* and *F. gigantic*a is done by a variety of methods including random amplified polymorphic DNA (Ramadan *et al.*, 2010), conventional PCR

protocol (Gabrielli *et al.,* 2014; Phalee and Wongsawad, 2014), restriction fragment length polymorphism RFLP (Yakhchali *et al.,* 2015a), random amplified polymorphic DNA (RAPD) (Garedaghi and Khakpour, 2012), multiplex PCR (Le *et al.,* 2012). Species-wise, Chaudhry *et al.* (2015) demonstrated molecularly through the sequencing of internal transcribed spacer 2 rDNA that *Fasciola gigantica* was the most predominant *Fasciola species* in Pakistan.

There has been an increase in the application of molecular techniques in the study of *Fasciola* spp. as shown by the available information in literature. Some of the techniques used include; restriction fragment length polymorphism PCR-RFLP (Dar *et al*., 2012), random amplified polymorphic DNA (RAPD-PCR) (Chauke *et al*., 2014), conventional PCR (Mufti *et al*., 2014) and Duplex PCR (Le *et al*., 2012). The techniques have been applied to studies from China (Peng *et al*., 2009), Thailand and Vietnam (Le *et al*., 2012), Pakistan (Mufti *et al*., 2014), Iran (Shafiei *et al*., 2014) and Spain (Alasaad *et al*., 2007).

Similarly, in the first report of the occurrence of *Fasciola hepatica* in *Equus caballus* host species from Tunisia based on the ribosomal internal transcribed spacer regions, Amor *et al.* (2011) showed that samples morphologically identified as *Fasciola spp.* and characterised, had sequences identical to those of *F. hepatica* sequences selected as reference, on which basis the isolates were classified as *Fasciola hepatica*. Furthermore, the first molecular typing of *Fasciola spp*. in donkeys in Egypt was conducted by Badawy *et al.* (2014), with isolates morphologically identified as *Fasciola gigantica* later confirmed as so by the sequences of the 28S rRNA using restriction fragment length polymorphism.

The recent characterisation of 28S rRNA of *Fasciola* from donkeys in Egypt using restriction fragment length polymorphism (RFLP) represents the first molecular typing of *Fasciola* in

donkeys in Egypt and probably one of the few studies so far conducted (Badawy *et al.*, 2014). Results of such studies further increase awareness on the status of non-ruminants serving as possible final host for *Fasciola*.

The detection of *Fasciola* DNA in the intermediate snail host has been used for molecular studies. The prevalence of *Fasciola gigantica* infection in field-collected snails of *Radix gedrosiana* in northwestern Iran was determined using restriction fragment length polymorphism (Imani-Baran *et al*., 2012). The study showed that infected snails had a limited distribution over the water bodies located in the central part of the region and that PCR-RFLP was a reliable approach to detect *Fasciola* infection in pond snails, and may be useful to establish control measures for livestock and human fasciolosis in the region.

Most of the molecular techniques utilized for taxonomic, phylogenetic and evolutionary investigation of *Fasciola species* are based on conventional polymerase chain reaction (PCR) method (Teofanova *et al*., 2012). Because of the close relationship between *Fasciola hepatica* and *Fasciola gigantica* as they only diverged evolutionarily recently, Mas-Coma *et al*. (2009a) outlined techniques, such as RAPD and microsatellite markers for obtaining information at the population level, as well techniques offering the highest resolution for accurate genotyping of specimens such as appropriately selected markers of nuclear rDNA and MtDNA for differentiating the two species. Ribosomal DNA (rDNA) has been widely used for taxonomy status establishment and/or inter- and intraspecific differences. It consists of alternating conservative and variable regions. The ribosomal DNA genes (18S, 28S) are conservative in nature and are used for revealing the relationships between different or closely related species

((Teofanova *et al*., 2012). The 28S rDNA is 4,171 bp long and highly conserved in both *F. hepatica* and *F. gigantica*, with few inter-specific nucleotide differences (Mas-Coma *et al*., 2009a). For clarifying intraspecific genetic structure of populations, only a variable ribosomal regions could be reliable for analyzing such as the most commonly utilized variable regions are ITS 1, ITS 2 and D-domains (divergent regions in 28S and 18S genes).

The MtDNA genes sequence analysis assists sometimes extremely hard differentiation of two species and/or subspecies closely related in accordance with their morphological and physiological characteristics (Teofanova *et al*., 2012). Following an extensive review on the characterisation of *Fasciola spp* from around the globe, Mas-Coma *et al*. (2009a) proposed that for fasciolids from Europe, the Americas and Oceania where *F. gigantica* is not present, a minimum of the complete sequence of the ITS-2 is ideal. However, a combination of ITS-2 and one mtDNA marker for the characterisation of each fluke specimen is strongly recommended. For those from the Americas, partial sequences of the mtDNA nad1 or cox1 become insufficiently informative when analyzing *F. hepatica* population dynamics. Therefore, faster evolving molecular markers will be needed for local population dynamic studies. The use of at least three markers is recommended, including the complete sequences of each ITS-2, nad1 and cox1 for fasciolids from Africa and Asia. However, for laboratory strains from the regions, the four-marker characterisation approach by means of the complete sequences of each ITS-2, ITS-1, nad1 and cox1 is needed.

2.14 Snail Intermediate Hosts of *Fasciola* spp

Snail intermediate hosts for *Fasciola* are of the Family Lymnaeaidae (PAHO, 2003). Various species of *Lymnaea, Amphipepla, Simlimnaea, Galba, Fossaria, Stagnicola* and *Pseudosuccinea* serve as intermediate hosts in different countries (Bhatia *et al.*, 2006). The species included in *F. hepatica* life cycle are amphibious, whereas those for *F. gigantica* are aquatic. Important snail hosts for the transmission of *F. hepatica* are *Fossaria bulimoides, F. modicella, Pseudosuccinia columella, Stagnicola caperata* and *S. montanensi* in North America; *F. viatrix* and *Lymnaeaa diaphora* in South America; *L. tomentosa* in Australia and New Zealand; *L. truncatula* in Africa, Asia and Europe; *L. viridis* in Asia, the state of Hawaii (USA), *F. cubensis* and *P. columella* are the main intermediate hosts in the Caribbeans, Columbia and Venezuela. The intermediate hosts for *F. gigantica* are different aquatic snails belonging to the super species *Lymnaeaa (Radix) auricularia*. In India and Pakistan, *L. auricularia rufescens*; in Malaya, *L. a. rubiginosa*; in Iran *L. a. geodrosiana*; in Iraq, *L. lagotis euphratica*; and *L. a natalensis* in Africa serve as intermediate host for *F. gigantica* (Gupta, 2014).

Lymnaea natalensis is the main intermediate host if not the only one in large areas of Africa for *Fasciola* (Schillhorn van Veen, 1980 as cited in Brown, 2005). Previous parasitological and malacological study conducted on the mountain Elgon National Park in Uganda, revealed an abundance of the snail, *Lymnaeaa (Radix) natalensis* at higher altitudes, which became much rarer, and ceased to be found above 1800 m (Howell *et al*., 2012). Conversely, *Lymnaeaa (Galba) truncatula,* another snail intermediate host in the transmission of *Fasciola*, was found only at altitudes above 3000 m. In a retrospective study on the experimental infections of *Lymnaeaa truncatula* with *Fasciola hepatica*, Vignoles *et al.* (2002), determined if the

populations of snails living in highland or lowland countries had the same ability to sustain trematode larval development and found out that highland snails had better ability to sustain parasite larval development and that they would be better intermediate hosts in the life cycle of *F. hepatica* than the snails in the lowland. These reports further justified environmental effect on the distribution and in turn the ability of snails to transmit *Fasciola* in different locations.

Dar *et al.* (2003) determined cercarial shedding from *Galba truncatula* infected with *Fasciola gigantica* of distinct geographic origins. In the study, *Galba truncatula* snails experimentally infected with either of two different isolates of *Fasciola gigantica*, originating from Egypt or China, showed survival rates of 90.0% and 60.2% for Egyptian and Chinese groups respectively 30 post-exposure, while the cercaria-shedding snails within the Egyptian and Chinese isolates was 79.8% and 22.4%. The obtained results could be explained by the fact that *G. truncatula* might be a natural intermediate host for *F. gigantica* in Egypt, hence the greater adaptability of the Egyptian miracidia of *F. gigantica* to unusual snail hosts. These results demonstrate the influence of the geographic origin of the parasite on the success of trematodes infecting snails.

2.14.1 Identification of snails

2.14.1.1 Morphological identification of Lymnaea natalensis

Many members of the family Lymnaeaidae are of medical and veterinary importance, serving as intermediate hosts to various blood, liver and intestinal flukes which may affect man directly or accidentally as parasitic zoonoses (Monzon *et al*., 1993). Dung *et al*.(2013) identified *Lymnaea species* from the Philippines on the basis of the possession of elongated and cylindrical shell, shell height of 11-20mm and width of 5-8mm, high spire, large aperture that is not extended, but

moderately expanded, outer margin S-shaped, while *L.columella* was generally twisted. In the description of freshwater snails of Africa and their medical importance, Brown (2005) described members of the family Lymnaeaidae as having small to large shell, dextral, with pointed spire varying widely in height, thin-walled and fragile and found in fresh water worldwide, though with relatively few species in tropical Africa. Similarly, the shell spire of *Lymnaea natalensis* generally appear much less high than the aperture; surface often with spiral rows of short transverse grooves, but lacking the spiral ridges characteristic of *L. columella*, with variation in shape occurring throughout Africa (Brown, 2005). Additionally, *L. natalensis* is found frequently in permanent streams, shallow seeping water, impoundments such as small dams; moderate pollution may be favourable, but only rarely in seasonal pools (Bitakaramire, 1968; Smith, 1982; Ndifon & Ukoli, 1989 as cited in Brown, 2005). The distribution of *Lymnaea natalensis* indicate that the most northerly living populations in the Sahelian zone are known in South-west Sudan, Lake Chad, the Niger Basin in Mali and Senegambia (Malek and Chaine, 1981;Madsen *et al.*, 1987 as cited in Brown, 2005), in addition to widespread distribution in tropical Africa with rarity in north-east coastal area. From Nigeria, among a total of 99 Lymnaeaid snails collected from Osun and Oyo States, Falade and Otarigho (2015) identified *Lymnaea natalensis* on the possession of morphological parameters of shell height (SH) of 11.4-13.30 mm, shell width (SW) of 7.84-7.96mm, aperture height (AH) of 6.72-9.12mm, aperture width (AW) of 4.60-5.22mm, spiral length (SL) of 3.61-4.30mm and aperture circumference (AC) of 20.83-22.89mm.

From the Philippines, Monzon *et al*.(1993) studied the taxonomic relationship between the endemic Philippine species, *Lymnaeaa (Bullastra) cumingiana* and five other Lymnaeaid species

(*L. (Radix) quadrasi* (Philippines), *L. (Radix) rubiginosa* (Indonesia), *L. (Radix) rubiginosa* (Thailand), *L. (Radix) viridis* (Guam) and *L. (Radix) viridis* (Hong Kong) in the Indo-Pacific region, and reported that; obtained results strongly suggested *L. cumingiana* as a distinct species among the rest, while *L. quadrasi. L. rubiginosa* (Indonesia) and *L. rubiginosa* (Thailand) exhibited great affinity towards each other.

Shell morphometric is a useful tool for describing, identifying, characterizing and recognizing intraspecific morphological variations and a first step in mollusc taxonomy and ecological studies (Mandahl-Barth, 1962; Abdel-Malek, 1958; Wullschleger and Jokela, 2002; Schniebs *et al*., 2013 as cited in Falade and Otarigho, 2015). The taxonomy and identification of snails is based on conchological and anatomical characters especially in species and subspecies, where the shells are frequently important (Mandahl-Barth, 1962)

2.14.1.2 Molecular identification of Lymnaea natalensis

Lymnaea (Synonyms: *Galba, Radix) species* have been subjects of research for long, considering their role in the transmission of fasciolosis in different geographical regions of the world, where the disease is known to occur. The application of molecular tools has equally been on the increase in recent past for the identification of *Lymnaea*. This has been undertaken utilizing specific genetic markers such as 18S ribosomal DNA, ITS 2 nuclear ribosomal DNA, ITS 1 ribosomal DNA, the mitochondrial 16S ribosomal DNA and cytochrome oxidase sub-unit 1 (COI) gene.

A survey was conducted to determine the abundance of Lymnaeaid snails from Peru, and to ascertain, which species were present (Bargues *et al.,* 2012). Procedurally, the ITS-2 and ITS-1 ribosomal DNA and 16S and cox1 mitochondrial DNA were sequenced using dideoxy chain-termination method. The results revealed three morphologically similar, but distinct Lymnaeaid species which were *Galba truncatula, Lymnaeaa neotropica* and *L. schirazensis*, although *Galba truncatula* was the most abundant. Similar study on morphological and molecular characterisation of Lymnaeaid snails and their potential role in transmission of *Fasciola* spp. in Vietnam, revealed the existence of three Lymnaeaid species;*Austropeplea viridis* (morphologically identified as *L. viridis*), *Radix auricularia* (morphologically identified as *L. swinhoei*) and *Radix rubiginosa* (morphologically identified as *Lymnaeaa* sp.) (Dung *et al.,* 2013). The ITS 2 sequences of the Lymnaeaid snails showed three distinct types (450, 470 and 451 bp). These studies emphasize the associated confusion inherent in the identification of the snail morphologically and the controversy surrounding the transmission of *Fasciola spp.* by these snail intermediate hosts. In another study on cryptic and sympatric Lymnaeaid species from the *Galba/ Fossaria* group in Mendoza Province, Northern Patagonia, Argentina, Standley *et al.* (2013) reported two species of Lymnaeaid, *Galba truncatula* and *G. viatrix*, based on morphological keys in contrast to the three (*Galba truncatula* and *G. viatrix* and a *G. neotropica*) which were sympatric with *G. viatrix*) observed through sequencing of cytochrome oxidase sub-unit 1 (COI) gene and the 16S ribosomal RNA gene (16S) of the snails.

In a previous study, the genetic markers ITS1, ITS2 rDNA and mitochondrial 16S ribosomal gene (16S rDNAmt) were employed by Carvalho *et al*. (2004) to differentiate the *Lymnaea species* (*Lymnaeaa columella, L. viatrix* and *L. diaphana)* from some localities of Brazil,

Argentina, and Uruguay and to verify whether the molecular results corroborates the classical morphological method. These markers were both applied in a conventional polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). PCR-RFLP analysis of the ITS1, ITS2, and 16S using 12 restriction enzymes revealed characteristic patterns for *L. columella* and *L. diaphana* which were concordant with the classical morphology.

The 18S ribosomal DNA has been previously used for sequence analysis of some common European *Lymneid species* (Bargues and Mas-coma, 1997) and for analysis of *Lymnaea natalensis* and *Lymnaea trucantula* from the Mount Elgon National Park in Uganda (Howell *et al*., 2012). For the European lymaneid snails, sequence alignment and secondary structures of the 18S rRNA gene of *Lymnaeaa stagnalis, L. auricularia, L. peregra, L. palustris, L. glabru,* and *L. truncatulu* were analyzed, with the gene proving to be a good marker for both specific determination and supra-specific Lymnaeaid phylogeny. Similarly, Bargues *et al.* (2007) after their investigation concluded that the ITS 2, ITS 1 and COX 1 (cytochrome c oxidase subunit 1) nucleotide sequences are clearly useful markers for the differentiation of Lymnaeaid snails. Phylogenetic trees obtained by comparison with four other molluscan species (a polyplacophoran, two bivalves, and a stylommatophoran gastropod) showed the presence of four well-defined subgenera among the genus *Lymnaea sensu lato:* (1) *Lymnaeaa (Radix), (2) Lymnaeaa (Galba), (3) Lymnaeaa (Leptolimnaea),* and *(4) Lymnaeaa (Lymnaeaa).* From the Ugandan analysis, restriction fragment length polymorphism successfully differentiated between *Lymnaea natalensis* and *Lymnaea trucantula* based on the exhibition of 3-banded and 2-banded restriction profiles respectively following restriction analysis.

The important role of Lymnaeaid snails in the transmission of *Fasciola spp*, epidemiology and control demonstrate the need to develop new techniques to facilitate specimen classification, genetic characterisation of natural populations and laboratory strains, and to elucidate the systematics and taxonomy of the Lymnaeaidae (Mas-Coma *et al*., 2009a). For the classification, characterisation of Lymnaeaid specimens and establishment of valid species and assessment of species inter-relationships, Bargues and Mas-Coma (2005) outlined the ITS-2 and secondarily ITS-1 as the most useful genetic sequences markers. However, the 18S rRNA which appear more conserved with few variable positions has also been successfully applied to studies aimed at identification of *Lymnaea spp* previously (Bargues and Mas-Coma, 1997; Stothard *et al*., 2000). Similarly, the markers first and second internal transcribed spacers (ITS1 and ITS2) rDNA and the mitochondrial 16S ribosomal gene (16S rDNAmt) were used to differentiate the speci*es Lymnaeaa columella, L. viatrix, and L. diaphana* (Carvalho *et al*.,2004), while Bargues *et al*.(2007) utilised the small subunit (18S) gene region of the ribosomal DNA, the internal transcribed spacers (ITS-2 and ITS-1) and the mitochondrial DNA (Cytochrome c oxidase subunit I (COI) of wild-caught Lymnaeaid snails of *L. cubensis, L. viatrix* var. A ventricosa, *L. viatrix* var. B elongata and *G. truncatula* for the identification of the snail vectors and concluded that the ITS-2, ITS-1 and COI nucleotide sequences are clearly useful markers for the differentiation of Lymnaeaid species.

From the review of results of molecular characterisation of lymaneid snails conducted in different parts of the globe, Mas-Coma *et al*. (2009a) proposed that a minimum of the complete sequence of ITS-2 is needed to verify (or allow) precise specimen classification, while the complete sequence of the ITS-1 might help in species and subspecies assessment and

characterisation. Equally, complete sequence of the 18S gene may be used for analyses of supraspecific relationships, mainly when concerning species phylogenetically distant from one another. On the other hand, mitochondrial DNA markers such as cox1 and 16S may be used for comparison of close species within the same genus, and to differentiate populations or to analyze genetic exchange between populations or distribution of populations within the same species.

2.14.2 Control of snail

The destruction of snails which serve as intermediate hosts for *Fasciola* is key to the achievement of strategic *Fasciola* control programmes. Snail control strategies such as the use of proper drainage network and removal of vegetation on which the snails feed have been described as not practicable especially over large areas due to costs (Bhatia *et al*., 2006). Thus, use of mollusicides such as sodium pentachlorophenate and copper sulphate in a dilution of 1 part in 150 million parts of water will destroy the snails in 12 hours. Di-nitro-o-cyclohexyl-phenol (DCPH) at a concentration of 3 to 5 parts per million of water was described as being more effective than copper sulphate (Bhatia *et al*., 2006).

Taha *et al.* (2014) studied the effect of sodium hypochlorite on the intermediate host of *Fasciola gigantica, Lymnaea natalensis* using scanning electron microscopy. Increased mortality rate, which was dependent on the concentration and length of exposure, was observed, in addition to extensive alterations of the surface of the snail foot region, with damage in some areas, after treatment with 10 ppm of the solution for 15 minutes. Thus, it was concluded from the study that sodium hypochlorite may be useful for eliminating the snail intermediate hosts of *Fasciola*. In an ethnobotannical study, the molluscicidal activity of *Bauhinia variegata* leaf and *Mimusops elengi* bark was studied against vector snail *Lymnaeaa acuminate*, with the ethanolic extracts of both

plants more toxic than the other organic extracts and the possibility of their usage as potent molluscicide clearly established (Singh *et al*., 2012)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Areas

3.1.1 Maiduguri abattoir

The Maiduguri abattoir is located at the Kasuwa Shanu area of Maiduguri. Maiduguri (Figure 3.1)**,** also called Yerwa, or Yerwa-Maiduguri. It is the capital and largest city of Borno State, northeastern Nigeria. It is located on the north bank of the seasonal Ngadda (Alau) River, the waters of which disappear in the *Firki* ("black cotton") swamps just southwest of Lake Chad, about 70 miles (113 km) northeast (Encyclopaedia Britannica, 2015). Maiduguri is located between latitude 115^{o} N and longitude 135^{o} E (Elumere, 1987). Maiduguri is bordered by Konduga Local Government area to the northwest and Jere Local Government area to the south (Ahmed, 2014). It has the months of March – April as the hottest period of the year and temperature ranging between 30^{o}C - 40^{o}C. The area is semi-arid and is usually cold and dry during the months of November –January. The area is characterised by a short rainy season from June to September, a prolonged dry period between November and May (Udoh, 1981).

3.1.2 Gombe abattoir

The Gombe abattoir is located in Gombe (Figure 3.1), Gombe State, Nigeria. Gombe is situated between latitude 10^{o} 08 N and 11^{o} 24^{1} E and longitude 11^{o} 02^{1} N and 11^{o} 18^{1} E. It shares common boundary with Akko Local Government Area in the Southern and Western parts of the State; Yamaltu-Deba to the Eastern and Kwami to the Northern parts of the State. It is the capital of Gombe State and occupies an area of about $45km^{2}$ (Ministry of Land and Survey, Gombe, 2008, as cited in Adang *et al*., 2015). The town experiences two seasons, the rainy season, from

April to October and dry season, from November to March (Adang *et al*., 2015). Annual rainfall ranges between 1000 mm to 1200 mm. Average daily temperature is 34^{o}C in April and 27^{o}C in August. The relative humidity ranges from 70% - 80% in August and decreases to about 15% - 20% in December. The natural vegetation is typically that of the Sudan Savanna, and is composed of shrubs, herbs, grasses and sparsely distributed trees. This provides enough grazing land and pasture for cattle rearing.

3.1.3 Jos abattoir

The Jos abattoir is located at the abattoir area close to Namuwa Junction in Jos South Local Government Area of Plateau State (Figure 3.1). Jos South has its headquarters in Bukuru town at 9^{o} 48’ 00’N 8^{o} 52’ 00’’ E. It has an area of 510 km^{2} south of the state capital and a population of 311, 392 (Agada *et al*., 2014). Jos (north and south Local Government Areas) is formed on a basement complex of rocks, which has produced the characteristic iceberg landscape. The landscape of Jos is that of Guinea Savannah; mostly rocky, but with chains of hills and many captivating rock (Pam *et al*., 2013). The near temperate climatic condition is greatly influenced by its strategic location on the Plateau, making Jos climate the nearest equivalent to the temperate climate in Europe and America. Temperature ranges from 11^{oC}-30^{oC}, with an annual rainfall of 150 cm, lasting between 6 and 7 months. The months of December through February are particularly cold and dry (Pam *et al*., 2013).

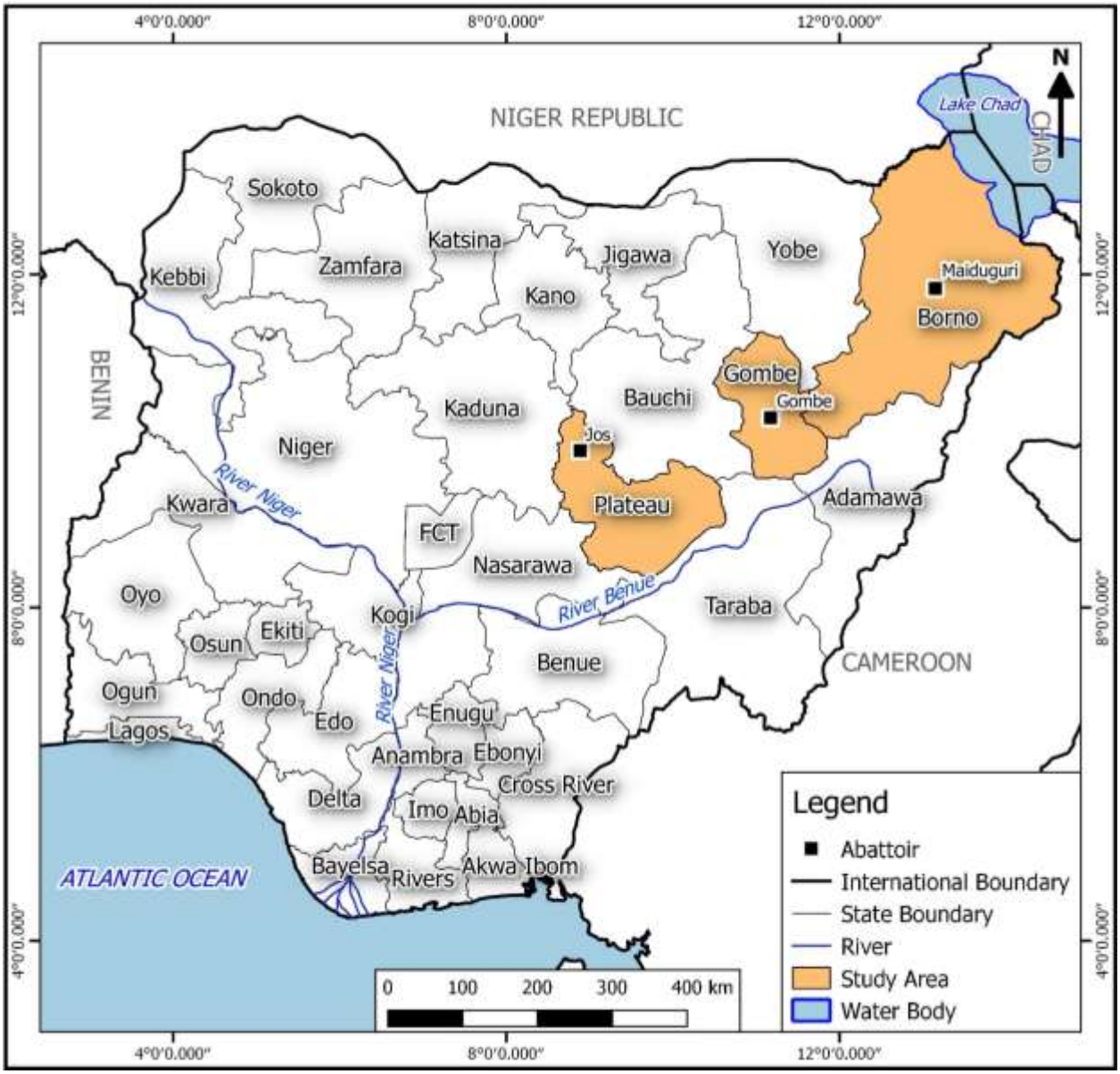


Figure 3:1: Map of Nigeria showing location of study areas

(Modified from the Administrative Map of Nigeria;Courtesy:http://www.theodora.com/maps)

3.2 Study Design

The design was a prospective cross-sectional study and involved slaughtered cattle and sheep of different breeds from Maiduguri, Gombe and Jos abattoirs.

3.3 Sample Size Determination

The number of samples collected was determined using the formula of Thrusfield (2005). Due to lack of previous data on the combined prevalence of fasciolosis in bile and faeces of cattle and sheep from the study locations, an assumed prevalence of 50% was used in calculating the sample size.

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

Where:

q = complementary probability (1-P).

n = minimum sample size.

P = Assumed prevalence of fasciolosis (50.0%)

d = desired absolute precision 0.05.

Z = appropriate value for the standard normal deviate set at 95% confidence interval (1.96).

Therefore:

$$N = \frac{1.96^2 \times 0.5 \times 0.5}{0.05^2}$$

$$= \frac{0.9604}{0.0025}$$

$$= 384.16 = 385.$$

However, sample size was increased by 4% to increase precision and to compensate for losses in transit.

3.4 Background of Sampled Animals

Information was sought from the butchers, livestock marketers and abattoir workers to have an idea of the system under which slaughtered animals were kept. The ages of the sampled animals were estimated as described by Chibuzo (2006). Cattle < 2½ years were classified as young, while those ≥ than that age were regarded as adutlts. Sheep < 1½ years were equally classified as young and those greater than or equal to (≥) that age were the adults. Breeds' identification was done according to the identification keys as provided by Blench (1999) for traditional livestock breeds of West Africa. Similarly, the sexes were identified on the appearance of the external genitalia.

Despite lack of comprehensive information on the husbandry system under which the slaughtered animals were kept, available information gathered revealed that most of the slaughtered sheep were kept under semi-intensive and intensive systems of management within their respective localities. Similarly, substantial number of the slaughtered cattle was sedentary; being kept mostly under fattening conditions, with significant number drawn from transhumant population from neigbouring countries of Cameroun, Chad and Niger republics in the case of slaughtered animals at the Maiduguri abattoir. Cattle and sheep of different ages, sexes and breeds were sampled during the study.

3.5 Sample Collection and Transportation

Sampling lasted for a period of eight months from January 2016 - August 2016. Faecal and bile samples as well as adult *Fasciola* were collected from cattle and sheep from the selected three abattoirs; Maiduguri, Gombe and Jos using convenient sampling method. The number of animals sampled per day ranged from 10 to 20, corresponding to 20-40 samples per day. Visit was made

to the abattoir every day of the week during the sampling period except on Sundays. Samples from Maiduguri were collected in the months of April and May, while those Gombe in the months of February, March and June. January, February, July and August were the months of sample collection from Jos. Samples of snails from water bodies and land from the three locations were similarly collected during the period of study. The water bodies were selected based on availability and convenience and they ranged from 3-5 per study area.

3.5.1 Faecal and bile sampling

Faeces and bile were collected from cattle and sheep as described by Magaji *et al.* (2014), with minor modifications. Briefly, about 15 grams of faeces were collected directly from the rectum of each of the sampled cattle/sheep into a universal sample bottle, using a gloved hand. 15 mls of bile was collected directly from the gall bladder into a universal sample bottle. Samples (bile and faeces) collected per animal were labelled immediately and kept on ice packs in cold box. Basic information of the sampled animals such as sex, breed, age, location and date of collection were immediately entered into a register at the collection sites. Samples from Jos and Gombe were transported immediately after each day's collection to the Parasitology Division, National Veterinary Research Institute, Vom and that from Maiduguri to the Department of Veterinary Parasitology and Entomology, University of Maiduguri for processing. However, samples that could not be processed immediately or within the day of collection were preserved in 10% buffered formal saline.

3.5.2 Collection of *Fasciola* species

Adult *Fasciola* were collected directly from the bile duct of slaughtered cattle/sheep and washed thoroughly through several changes in physiological saline and later transported in physiological saline or 70% ethanol (Dar *et al*., 2012) depending on the intended use. Samples meant for molecular studies were preserved in 70% ethanol until used, while those for morphometry were transported in physiological saline.

3.5.3 Snail sampling

Snail samples were collected as described by Sharif *et al*. (2010) and Luka and Mbaya (2015). Briefly, samples from aquatic environments were collected using improvised scoop nets; while those on plain land, vegetation cover and debris were handpicked using gloved hand. Specimens meant for identification were preserved in 10% buffered formal saline, while those for molecular analyses were preserved in 70% ethanol (Gabrielli *et al*., 2014) until used.

3.6 Sample Processing

3.6.1 Determination of prevalence of *Fasciola* spp. in faeces

The processing was as earlier described (Magaji *et al*., 2014). Four grams (4 g) of faeces were placed into labelled test tubes containing 6 mls of distilled water, and then strained to give a suspension, which was also strained through a tea strainer into a clean labelled Petri dish. The resultant filtrate was poured into a test tube and one milliliter (1 ml) of 10% formalin was added, after which the suspension was allowed to stand for 5 minutes, followed by addition of diethyl-ether (1 ml), followed by addition of Dietyl-ether (1 ml). The test tube containing the suspension was corked and shaken to mix, and centrifuged at 2000 g for 8 minutes, after which the

supernatant was decanted, leaving a drop of it with the sediment. Part of the sediment was placed on a glass slide and covered with cover slip, drops of methylene blue were added and then viewed at ×10 magnification of a stereomicroscope for *Fasciola* eggs, until the entire sediment has been examined and all eggs counted (Cheesbrough, 1980).

Prevalence was determined by expressing the number of positive samples as a % of total samples collected. The number of eggs counted was expressed per grams of faeces (Ortiz *et al*., 2000).

3.6.2 Determination of prevalence of *Fasciola* spp. in bile

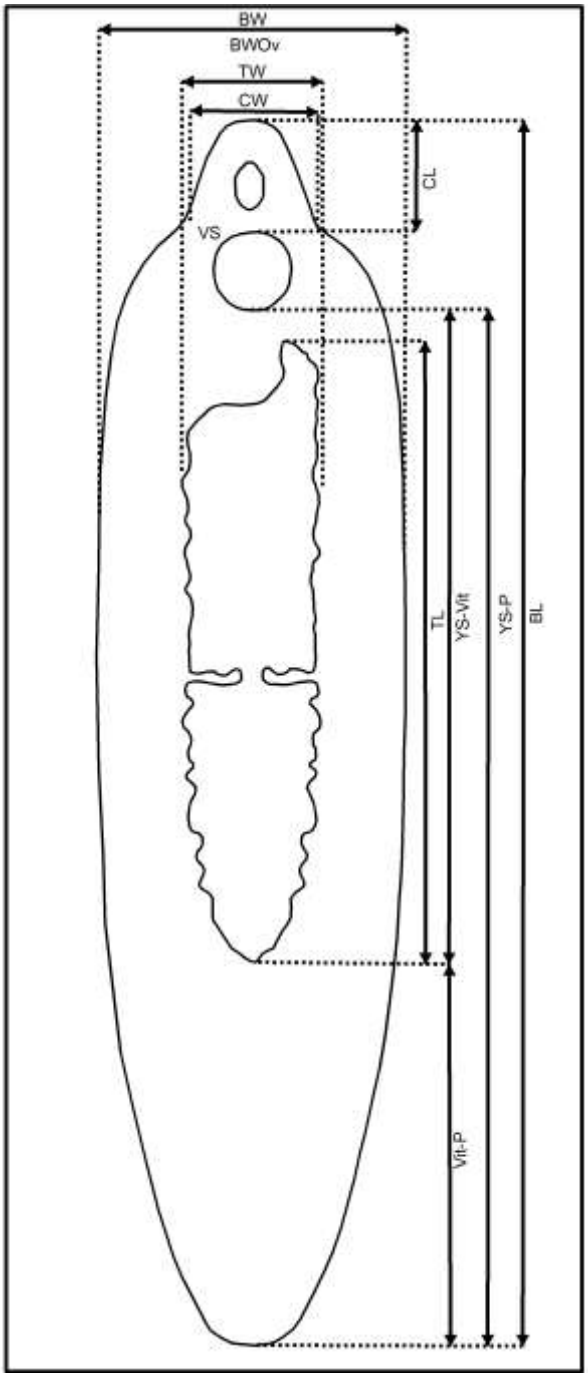
Approximately 4 mls of the collected bile was poured into a labelled test tube and the procedure used for faecal sample analysis was repeated, followed by addition of 1ml of 10% formalin. After 5 minutes, diethyl-ether (1 ml) was added. The test tube containing the solution was corked, shaken to mix, and then centrifuged at 2,000 g for 10 minutes. The supernatant was decanted leaving few of it with the sediment. Parts of the sediment was placed on a clean glass slide and drops of methylene blue added before being viewed under a microscope using ×10 magnifications for *Fasciola* eggs, until the entire sediment has been examined (Cheesbrough, 1980). Prevalence was determined by expressing the number of positive samples as a % of total samples collected. The number of eggs counted was expressed per millilitres of bile used (Ortiz *et al*., 2000).

3.6.3 Morphometry and morphological identification of *Fasciola* spp

Fasciola spp. transported to the laboratory in physiological saline solution were incubated at 37^{o}C for 2 hours to allow them expel gut contents and placed between 2 microscopic slides held

by a rubber band before being examined under a stereoscopic microscope (Biu *et al.*, 2013). Landmarks for the linear characters were spotted and marked under the stereoscope (Olympus, Japan) prior to measurement using metre rule to the nearest millimeters, while areas and ratios were calculated from the measured characters.

The adult *Fasciola* spp. were assessed and characterised as previously described by Ashrafi *et al*. (2006), Biu *et al*. (2013), Shaldoum *et al*. (2015) with slight modification, using linear morphometric characters and ratios: body length (BL), body width (BW), cone length (CL), cone width (CW), distance between the ventral sucker and the posterior end of the body (VS-P), the distance between ventral sucker(VS) and union of vitelline gland(Vs-Vit) and the BL/BW and CL/CW ratios (Figure 3.2).



Source: Adapted from Ashrafi *et al*. (2006)

Figure 3:2: Guide used for linear biometric measurements (mm) of adult *Fasciola* spp: (body length (BL), body width (BW), cone length (CL), cone width (CW), distance between ventral sucker (VS) and union of vitelline gland (Vs-Vit), distance between the ventral sucker and the posterior end of the body (VS-P).

3.6.4 Molecular characterisation of internal transcribed spceer ITS 1 and 28S rDNA genes of *Fasciola* spp using PCR

Genomic DNA of *Fasciola* spp was extracted by the modified phenol-chloroform method (Sambrook and Russell, 2002). 400 µl of lysis buffer and 10 µl of proteinase K were added to 100 mg of homogenized adult *Fasciola* tissue and the tube placed on a heating block at 60^{o}C for 1 hour, with vortexing every 20 minutes. 400 µl of Phenol: chloroform (AMRESCO, Fountain Parkway, Solon, USA) was added to the lysate and vortexed briefly, after which it was spinned at 13,000 g for 10 minutes at room temperature to separate the phases. Thereafter, the upper layer was removed carefully with a pipette and added to a new 1.5 ml tube, making sure that the white interphase was not extracted along. To the recovered layer, 400 µl of chloroform was added and vortexed briefly, after which it was spinned at 13,000 g for 5 minutes to separate the phases. The upper layer was carefully removed with a pipette and added to a new 1.5 ml tube, ensuring that the white interphase was not extracted. 1000 µl of 100% ethanol and 40 µl of 3M sodium acetate mix was added and the tubes were inverted several times to allow for mixing, then incubated at 20^{o}C over night, and spinned at 14,000 g for 10 minutes. The ethanol was removed by pipetting and 400 µl of ethanol again was added before being spinned at 14,000 g for 5 minutes, followed by another spinning for 2 minutes at 14,000 g to remove all traces of ethanol, while the DNA was dried out by leaving the tube open for 10 minutes, after which the pellet was resuspended in 100 µl sterile water and preserved at -20^{o}C.

Series of PCR were run in order to determine the optimal comditions that ensured reproducible results. The PCR conditions used in the study were adopted after annealing condition of 55^{o}C gave the best result. The internal transcribed spacer 1 (ITS1) fragment was amplified by PCR as

described by Itagaki *et al.* (2005), using the primer set (5'-TTGCGCTGATTACGTCCCTG-3' and 5'-TTGGCTGCGCTCTTCATCGAC-3') as forward and reverse primers, while a fragment of 618bp of the 28S rDNA gene was amplified by PCR using the primer set described by Marcilla et al.(2002);Forward: 5'-ACGTGATTACCCGCTGAACT-3' and reverse: 5'-CTGAGAAAGTGCACTGACAAG-3'). The PCR reaction for both genes was carried out in a 25 µl reaction mixture containing 2 µl of the genomic DNA (diluted 1:30), 1.5 U of *Taq* DNA polymerase, 50 mM of each dNTPs , 2mM of $MgCl_2$, 2.5 µl of PCR reaction buffer (10×) and 0.2 µM of each primer. PCR mixtures were subjected to an initial denaturation step at 94^{o}C for 5 min, followed by denaturation at 94^{o}C for 30 seconds. Annealing was at 55^{o}C for 30 seconds, while 30 cycles of extension for 30s at 72^{o}C was used, with final extension for 5 minutes at 72^{o}C.

Electrophoresis of the PCR product was done on 1.5% agarose gel. To make the gel, 3 g of agarose was weighed and dissolved in 75 mls Tris acetate (TAE) buffer, before heating the solution in a microwave oven until the agarose was completely dissolved. It was allowed to cool at 50-55^{o}C. The gel casting tray was prepared by sealing the ends of gel chamber with tape and a 20-well comb placed at the edge and middle of the gel tray. 5 µl of ethidium bromide was added after cooling the gel to room temperature; before pouring the gel into gel tray, after which it was allowed to cool for 15 minutes at room temperature. The combs were removed and the gel placed in electrophoresis chamber and covered with TAE buffer. Both the DNA sample and standard (Ladder) were loaded onto the gel wells, and ran for 1.5 hours at 90 V. DNA bands were visualized using Gene Spectrophotometer (Genequant Pro Biochrom Limited, England). The size of each band was determined by a 100-bp plus ladder molecular weight marker.

3.6.5 Molecular characterisation of the NADH dehydrogenase subunit 4 (NAD4) of *Fasciola* spp using PCR

For the *Fasciola* samples analysed at the Instituto de Ganaderia de Montana, University of Leon, DNA was extracted from the basal zone tissue of adult flukes using SPEEDTOOLS TISSUE DNA EXTRACTION KIT according to manfacturer's instrunctions (Biotools B&M Labs, Madrid, Spain). The extraction kit composed of pre-lysis buffer, lysis buffer, proteinase K (lyophilised), proteinase suspension buffer, DNA binding columns, wash buffers and elution buffer.Samples were concentrated until 10 µL.

For the analyses of *Fasciola* samples at the Instituto de Ganaderia de Montana, University of Leon, Spain, two different primer sets targeting the mitochondrial DNA (NAD4) were designed for the two *Fasciola* species thus:

Fasciola hepatica:

Forward: 5'-GCTTGTTTGGCATTGTTAGGG-3'

Reverse: 5'-CAACCAGCCCATCAATCCC-3'

Fasciola gigantica:

Forward: 5'-GGGATTCAGTCTTGGAGGGA-3'

Reverse: 5'-CCGCCATAAACACCACACCT-3'

PCR reaction was done in a total volume of 10 µL master mix (Biotools, DNA AmpliTools HotSplit Master Mix) composed of 3 µL primers (10uM) (Forward + Reverse; one pair of primer for each *Fasciola* species, *F. hepatica* and *F. gigantica*, amplifying mitochondrial DNA), 5 µL sterilized H_2O and 2 µL DNA. The PCR conditions consisted of 40 cycles at 95ºC for 5 minutes

(initial denaturation), 95ºC for 30 seconds (denaturation), 66 ºC for 30 seconds (annealing), 72 ºC for 60 seconds (extension) and 72 ºC for 5 minute (final extension).

PCR products were analysed by electrophoresis on 1.5 % Agarose + TAE 1% gels and dyed with Gelred. The gels were visualizated by Gel Doc™ XR+ Gel Documentation System. Only PCR products with thick bands were purified and sequenced for confirmation of species.

3.6.6 Morphological identification of snails

Snails collected and preserved in 10% formalin for morphological studies were identified at the Museum of Natural History, Department of Biological Sciences, Ahmadu bello University, Zaria-Nigeria using identification keys such as shape of the shell, number of whorls, direction of the spires, size of the shell, presence or absence of operculum and micro-habitat of collection as earlier described (Brown, 2005). A total of one hundred and sixty one (161) snail samples consisting of one hundred and fourty four (144) for morphological identification and seventeen (17) for molecular studies were collected during the study from ecological sites consisting of water bodies (ponds, dams, lakes, water pools, streams) and terrestrial environment (plain land, rocks) from the three study locations of Maiduguri, Gombe and Jos.

3.6.7 Molecular characterisation of *Fasciola* spp. in snail tissues using PCR

Small snails such as *Lymnaea* spp, *Melanoides tuberculata* and *Biomphalaria* spp. were preserved wholly and individually in 70% ethanol per sampling location. However, about 15 grams of tissues from the foot region of the larger snails such as *Pila ampullacea* and *Limicolaria* spp. was taken and preserved in 70% ethanol.

3.6.7.1 Molecular characterisation of 28S rDNA of Fasciola spp *in snails*

Snails preserved in 70% ethanol were homogenised in a mortar using pestle. The phenol-chloroform method of extraction (Sambrook and Russell, 2002) was adapted for DNA extraction. 20ul of 100% ethanol and 20ul of 3M sodium acetate were added and mixed by inverting the tube several times, followed by incubation at -20^{o}C over night, after which the tube was spinned at 14000 g for 30 minutes. The ethanol was removed and 400 µl of ethanol was added before being spinned at 14000 g for 5 minutes. The tube was again spinned for 30 sec at 14000 g to remove all traces of ethanol, while the DNA was dried out by leaving the tube open for 3-10 minutes, after which the pellet was resuspended in 20ul sterile water and stored at 20^{0}C until used.

The choice of primer, PCR optimization, DNA amplification and gel electrophoresis are as described in study above

3.6.7.2 Molecular identification of Lymnaea natalensis using PCR

The DNA of *Lymnaea (Radix) natalensis* was extracted using the same protocol described above. The primer set 18SLYMFOR 5'-AGTAGTCATATGCTTGTCTCAAAGATTAAGCCA-3' and 18SLYMREV, 5'-TGCGCGCCTCTGCCTTCCTTGGATGTGGTAGCCGT-3' previously used for the amplification of the 18S nuclear ribosomal region of DNA of *Lymnaea* (Stothard *et al*., 2000; as cited in Howell *et al*.,2012), was similarly adopted in this study. Optimised PCR conditions are as detailed earlier (3.6.4).

The same protocol outlined above was used for the amplification of the DNA of the snails and the gel electrophoresis

3.7 DNA Sequencing

For species confirmation, a total of twenty (20) DNA amplicons consisting of 2(ITS), 3(28S rRNA), 3(18S) and 12 (NAD4) genes were sequenced at the laboratorio de técnicas instrumentals, University of Leon, Spain, using the dye terminator cycle sequencing (Applied Biosystems, Madrid, Spain). The reverse primers of the pairs used for the respective genes were used in this study. Sequencing reaction was prepared in a 2.0 ml tube. All reagents were kept on ice and used in the following order: dH_2O 0 - 9.5 µl, DNA template 0.5 – 10.0 µl, Primers 2.0 µl, DTCS Quick start master mix 8.0 µl. The following conditions were used for the sequencing reaction: 96^{o}C for 20 sec, 50^{o}C for 20 sec X 30 cycle and 60^{o}C for 4 min. Sequence chromatographs and sequences were edited using ApE (Plasmid Editor) version 2.0.49.0 (June, 2017) and Molecular Evolutionary Genetics Analysis (MEGA 6.06) tool (Tamura *et al*., 2013). Edited sequences were aligned in order to get consensus sequence. The sequences were further used for Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi)) search (Altschul *et al*.,1990) in National Centre for Biotechnology Information (NCBI) database using the Blastn category in order to search for similar sequences desposited in the GenBank from the NCBI databse.

3.8 Nucleotide Submission to GenBank

Edited sequences of three *Lymnaea* (*Radix natalensis)* and five *Fasciola* spp were submitted to GenBank for assignment of accession numbers through the GenBank submission portal for the submission of ribosomal RNA (rRNA) or rRNA-ITS (https://submit.ncbi.nlm.nih.gov/subs/genbank/), after sequences were curated and annotated.

3.9 Phylogenetic Analysis of *Fasciola* spp and *Lymnaea (Radix) natalensis* Sequences

Phylogenetic analysis was carried out to determine the evolutionary relationship of the sequences of the *Fasciola* spp (ITS 1 and 28S rRNA) and *Lymnaea* (*Radix) natalensis* (18S rRNA). The Molecular Evolutionary Genetic Analysis (MEGA 6.06) was used (Tamura *et al*., 2013).

Sequences of the genes (ITS 1 and 28S rRNA) from this study were copied separately to notepad along with similar sequences of the respective genes from GenBank as determined from Blastn search and then saved in FASTA format. Sequences of other genetically similar organisms corresponding to the genes studied were retrieved from GenBank for rooting and used as outgroup.Thus, *Fasciola* spp sequences were rooted using sequence of *Paragonimus westermanii* (AF219379) and the *Radix natalensis* using *Biomphalaria tenagophila* (AY030220). Therefater, sequences were imported into MEGA and subjected to multiple sequence alignment (MSA) using the Clusta W algorithm for pair wise alignment. Genetic distance between pairs of sequences was calculated using maximum composite likelihood model and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was used for the phylogenetic tree

construnction. Bootsrap value of 1000 replicates was used to statistically determine the branching of the tree, while a bootsrap value of 80% was considered significant and as an evidence of phylogenetic grouping.

3.10 Data Analyses

Data generated from the field were summarized as Mean±SEM and presented in Tables. Chi square test was used to check for possible association between the prevalence and the various parameters, while student unpaired *T-test* was used to determine the statistical significance difference between/among the various parameters in the two species of *Fasciola*. Statistical Package for Social Science (SPSS, Version 16) was used.

Nucleotide variation among a sequence of a species was determined using pairwise comparison using maximum composite likelihood model in MEGA 6.06 (Tamura *et al*., 2013). Pairwise comparison of sequence difference (D) among *Fasciola* spp and *Radix natalensis* consensus sequences were calcaulated using the formula D=1-(M/L), where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (Chilton *et al*., 1995).

CHAPTER FOUR

4.0 RESULTS

4.1 Number of Faecal and Bile Samples Collected from Cattle and Sheep from Maiduguri, Gombe and Jos Abattoirs

Bile and faecal samples were collected from a total of 1,188 cattle and 1,177 sheep in the three locations. A total 4,730 samples consisting of 2365 of biles and faeces each were collected from the animals sampled during the study (Table 4.1)

Table 4.1: Number of Animals Sampled for Faeces and Bile from Maiduguri, Gombe and Jos Abattoirs

Location of Abattoir	Subject	Sample type		Animal Species	
		Bile	Faeces	Cattle	Sheeep
Jos	Cattle	412	412	412	
	Sheep	390	390		390
Gombe	Cattle	389	389	389	
	Sheep	398	398		398
Maiduguri	Cattle	387	387	387	
	Sheep	389	389		389
Total Sample size		2,365	2,365	1,188	1,177

The number of subjects based on availability.

4.2 Overall Prevalence of *Fasciola* spp in Slaughtered Cattle from Maiduguri, Gombe and Jos Abattoirs

Out of the 1,188 cattle sampled, 284(23.9%) were positive for infection through bile sedimentation method of detection of *Fasciola* eggs (Plate I), and this rate was significantly higher ($p<0.0001$), compared to the rate obtained through faecal sedimentation (131/11.02%). Prevalence was higher in female cattle (262/27.66%) compared to male (65/26.97% ($p=0.8292$), and in adults (269/28.22%) compared to the young (58/24.68%) cattle. Also, prevalence was highest in cross breed cattle (18/40.0%), than others ($p=0.1538$) and in February (50/39.37%) to other months ($p<0.0001$). However, based on sex, age and breed, there were no significant difference ($p>0.05$) in prevalence rates. There was however, a statistical significant variation ($p<0.0001$) based on the sample type (Table 4.2).

The mean egg count of 36.27±5.35 in female cattle from the three locations was higher than the 23.81±4.12 recorded in males during the sampling period. Similarly, adult cattle had a higher mean counts 269 (33.39±4.85) than the young 58 (30.64±6.99). The highest mean egg count of 117.7±59.12 was obtained in the Kuri breed, followed by the Wadara and Rahaji with counts of 117.3±49.58 and 40.60±9.67 respectively. However, there was no statistical significant ($p>0.05$) variation in the egg counts on the basis of sex, age and breeds of the cattle examined. On the other hand, the egg count (126.80±43.58) for the animals sampled in April was significantly ($p<0.0001$) higher than for the corresponding months of January (12.14±1.17), February (32.37±13.24), May (38.65±5.95) and June (37.47±7.23)(Table 4.3)

Table 4.2: Overall prevalence of *Fasciola* spp.eggs in faeces and bile of slaughtered cattle in Maiduguri, Gombe and Jos abattoirs

Parameter	No. Sampled	No.(%)+ve	X^2	P-value	OR	CI at 95%
Sex						
Male	241	65 (26.97)	0.0466	0.8292	0.9656	0.7024 - 1.327
Female	947	262 (27.66)				
Total	1188	327 (27.52)				
Age						
Young	235	58 (24.68)	1.188	0.2757	0.8332	0.5999 - 1.157
Adult	953	269 (28.22)				
Total	1188	327 (27.52)				
Breed						
Rahaji	416	105(25.24)	9.371	0.1538	Ref	
Bunaji	630	185(29.36)			1.231	0.9307 - 1.629
Crosses	45	18(40.00)			1.975	1.0450 - 3.731
Sokoto Gudali	7	2(28.57)			1.185	0.2264 - 6.200
Wadara	81	15(18.51)			0.6732	0.3684 - 1.230
Kuri	8	2(25.00)			0.9873	0.1962 - 4.969
Ambala	1	0(0.00)				
Total	1188	327(27.52)				
Month						
January	339	114(33.62)[a]	29.81	**p<0.0001**	Ref	
February	127	50(39.37)[b]			1.282	0.8409 - 1.953
March	67	22(32.83)[a]			0.9649	0.5525 - 1.685
April	103	19(18.44)[c]			0.4464	0.2584 - 0.7712
May	284	57(20.07)[c]			0.4956	0.3431 - 0.7158
June	268	65(24.25)[d]			0.6320	0.4414 - 0.9049
July	NCM	_				
August	NCM	_				
Total	1188	327(27.52)				
Sample type						
Bile	1188	284(23.90)[a]				
Faeces	1188	131(11.02)[b]	68.34	**p<0.0001**	2.535	2.024-3.175

Values with different superscripts in the same column differed significantly ($p<0.05$).

Key: NCM= No collection made

Table 4.3: Mean *Fasciola* egg count in Slaughtered Cattle from Maiduguri, Gombe and Jos Abattoirs

Parameter	No.Examined	No.(%)+ve	Mean±SEM	P-value
Sex				
Male	241	65(26.97)	23.81±4.12	
Female	947	262(27.66)	36.27±5.35	
Total	1188	327(27.52)	33.67±4.33	0.243
Age				
Young	235	58(24.68)	30.64±6.99	
Adult	953	269(28.22)	33.39±4.85	
Total	1188	327(27.52	33.67±4.33	0.8091
Breed				
Rahaji	416	105(25.24)	40.60±9.67	
Bunaji	630	185(29.36)	12.33±3.10	
Crosses	45	18(40.00)	1.50±1.50	
Wadara	7	2(28.57)	117.30±49.58	
Kuri	8	2(25.00)	117.7±59.12	
Ambala	1	0(0.00)	0.00±0.00	
Total	1188	327(27.52)	33.67±4.33	NA
Months				
January	339	114(33.62)	12.14 ± 1.17^{a}	
February	127	50(39.37)	32.37 ± 13.24^{b}	
March	67	22(32.83)	59.68 ± 33.17^{c}	
April	103	19(18.44)	126.80 ± 43.58^{d}	
May	284	57(20.07)	38.65 ± 5.95^{ab}	
June	268	65(24.25)	37.47 ± 7.23^{ab}	
July	NCM	_		
August	NCM	_		
Total	1188	327(27.52)	33.67 ± 4.33^{b}	**p<0.0001**
Sample type				
Bile	1188	284(23.90)	41.12 ± 5.78^{a}	
Faeces	1188	131(11.02)	15.72 ± 3.75^{b}	**0.0056**

Values with different superscripts in the same column differed significantly ($p<0.05$).

Keys: NA= Not applicable, NCM= No collection made

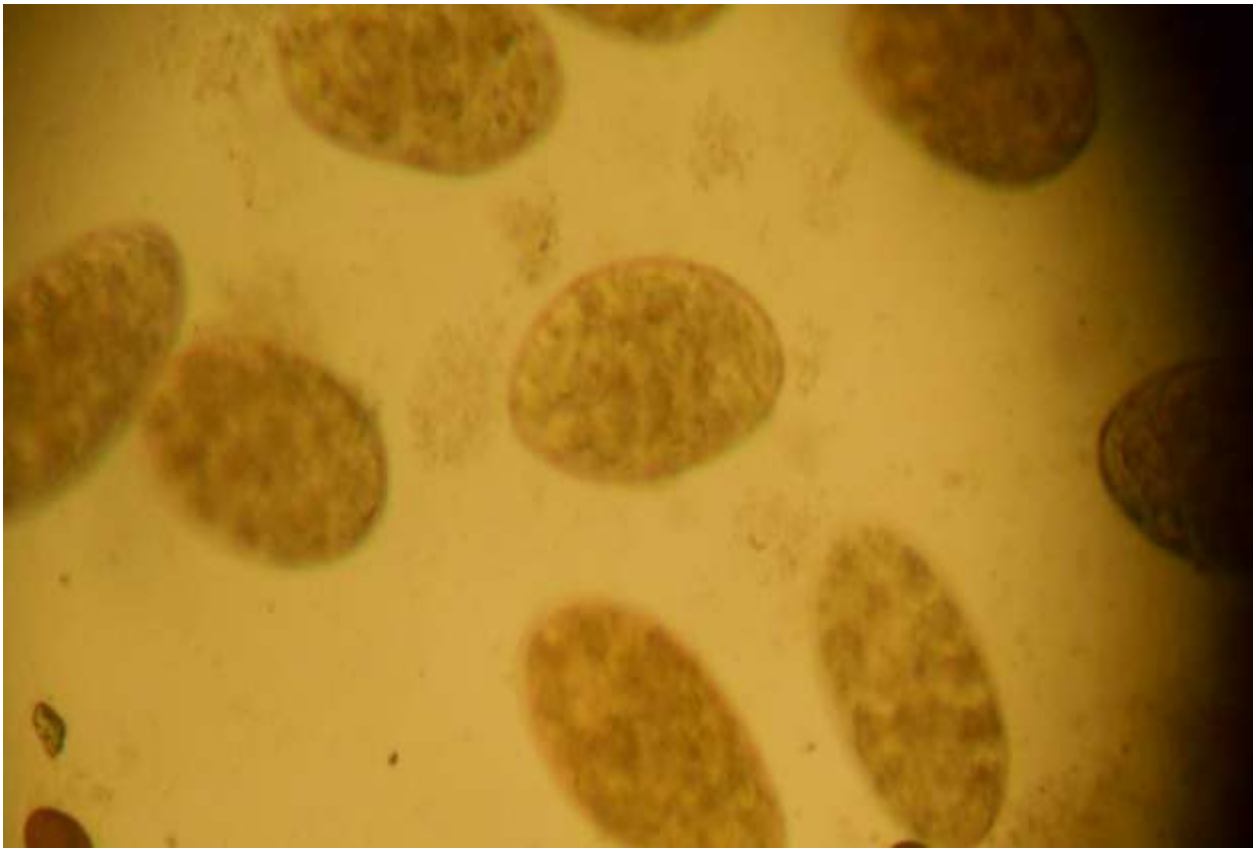


Plate I: Photomicrograph of eggs of *Fasciola* spp from bile of slaughtered cattle, from Jos abattoir, Plateau State (×400)

4.3 Overall Prevalence of *Fasciola* spp in Slaughtered Sheep from Maiduguri, Gombe and Jos Abattoirs

The overall prevalence of *Fasciola* spp in the 1,177 sheep sampled from the three abattoirs was (141/11.97%). More males (23/13.44%) had infection than females (118/11.77%) and prevalence was higher in Yankasa (126/12.24%) compared to other breeds. The prevalence rates were higher in January (4/28.57%), July (45/26.94%) and August (44/22.0%). There were no significant differences in prevalence based on sex ($p=0.607$) and breed ($p=0.3072$), but there were significant differences in prevalence based on age ($p<0.0001$), and month of sampling ($p<0.0001$). Prevalence obtained through faecal sedimentation (103/8.75%) was significantly ($p<0.0149$) higher than the rate of (72/6.11%) obtained through bile sedimentation (Table 4.4).

Adults and female sheep had respective mean egg counts of 26.31±6.58 and 29.45±7.91, higher than the corresponding counts for young (2.00± 1.00) and male (12.78±3.64) sheep (Table 4.5). Also, Balami sheep had higher mean egg count (53.88±36.93) than Yankasa (23.17±6.55) and West African dwarf (WAD) (0.00±0.00). Samples collected in February had significantly ($p<0.007$) higher count than others. The bile mean egg count (BEC)(50.88±15.11) was significantly higher ($p<0.0016$) than the faecal mean egg count (8.36±1.96) (Table 4.5).

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Table 4.4: Overall prevalence of *Fasciola* spp. eggs in faeces and bile of slaughtered sheep from Maiduguri, Gombe and Jos abattoirs

Parameter	No. Sampled	No +ve(%)	X^2	P-value	OR	CI at 95%
Sex						
Male	175	23(13.14)	0.2638	0.6075	1.134	0.7022 - 1.830
Female	1002	118(11.77)				
Total	1177	141(11.97)				
Age						
Young	76	2(2.63)[a]		**0.0055**	0.1871	0.04539 - 0.7708
Adult	1101	139(12.62)[b]				
Total	1177	141(11.97)				
Breed						
Yankasa	1021	126(12.34)	2.360	0.3072	Ref	
Balami	152	13(8.55)			0.6643	0.3652 - 1.209
WAD	4	0(0.00)				
Total	1177	141(11.97)				
Month						
January	14	4(28.57)[a]	85.17	**p<0.0001**	Ref	
February	90	4(4.44)[b]			0.1163	0.02510 - 0.5387
March	161	10(6.21)[b]			0.1656	0.04402 - 0.6227
April	194	12(6.18)[b]			0.1648	0.04498 - 0.6040
May	195	12(6.15)[b]			0.1639	0.04474 - 0.6007
June	156	10(6.41)[b]			0.1712	0.04551 - 0.6443
July	167	45(26.94)[a]			0.9221	0.2752 - 3.090
August	200	44(22.00)[a]			0.7051	0.2109 - 2.358
Total	1177	141(11.97)[c]				
Sample type						
Bile	1,177	72(6.11)[a]				
Faeces	1,177	103(8.75)[b]	5.932	**0.0149**	0.6794	0.4970 - 0.9288

Values with different superscripts in the same column differed significantly ($p<0.05$)

Table 4.5: Mean *Fasciola* egg count of slaughterd sheep from Maiduguri, Gombe and Jos abattoirs

Parameter	No.Examined	No.(%) +ve	Mean±SEM	P-value	Remark
Sex					
Male	175	23(13.14)	12.78±3.64		
Female	1002	118(11.77)	29.45±7.91		
Total	1177	141(11.97)	26.71±6.65	0.3538	
Age					
Young	76	2(2.63)	2.00±1.00		
Adult	1101	139(12.62)	26.31±6.58		
Total	1177	141(11.97)	26.71±6.65	0.6388	
Breed					
Yankasa	1021	126(12.34)	23.17±6.55		
Balami	152	13(8.55)	53.88±36.93		
WAD	4	0(0.00)	0.00±0.00		
Total	1177	141(11.97)	26.71±6.65	NA	
Months					
January	14	4(28.57)	6.00 ± 1.70^{a}		
February	90	4(4.44)	169.30 ± 166.10^{b}		
March	161	10(6.21)	12.80 ± 4.52^{a}		
April	194	12(6.18)	13.00 ± 5.27^{a}		
May	195	12(6.15)	65.38 ± 45.16^{ab}		
June	156	10(6.41)	11.50 ± 7.49^{a}		
July	167	45(26.94)	21.30 ± 3.90^{a}		
August	200	44(22.00)	21.04 ± 5.25^{a}		
Total	1177	141(11.97)	26.71 ± 6.65^{a}		
Sample type					
Bile	1177	72(6.11)	50.88 ± 15.11^{a}		
Faeces	1177	103(8.75)	8.364 ± 1.966^{b}	**p=0.0016**	

Values with different superscripts in the same column differed significantly ($p<0.05$).

Key: NA=Not applicable

4.4 Prevalence of *Fasciola* spp by Sampling Location of Abattoir Slaughtered Cattle

Of the total of one thousand, one hundred and eighty-eight (1,188) cattle examined during the study, Jos had the highest prevalence of 146 (35.43%), followed by Gombe 105 (26.99%) and Maiduguri 76 (19.63%). The prevalence obtained from three locations differed significantly ($p<0.0100$) from each other (Table 4.6). Similarly, sheep from Jos had the highest prevalence 95 (24.35%), followed by Maiduguri 24 (6.16%), while the least prevalence of 22 (5.52%) was demonstrated in sheep from Gombe (Table 4.6). The prevalence in sheep from Jos differed significantly ($p<0.0100$) with that from Gombe and Maiduguri.

Table 4.6: Prevalence of *Fasciola* spp. eggs in faeces and bile of abattoir slaughtered cattle and sheep based on sampling location

Species	Abattoir Locations						
	Maiduguri	**Gombe**	**Jos**	**P-value**	**X^2**	**OR**	**CI**
Cattle	76(19.63)[a]	105(26.99)[b]	146(35.43)[c]	**p<0.0100**	6.631	1.485	1.098 -2.007
Sheep	24(6.16)[a]	22(5.52)[a]	95(24.35)[b]	**p<0.0001**	55.25	5.504	3.378 -8.968

Values with different superscripts in the same row differed significantly (p<0.05)

4.5 Prevalence of *Fasciola* spp in Abattoir Slaughtered Cattle and Sheep in Relation to Sex

For cattle, the male/female prevalences were 12 (17.64%)/64(20.06%), 23 (27.71%)/82(26.79%) and 30 (33.30%)/116 (36.02%) for Maiduguri, Gombe and Jos respectively. For sheep, the male/female prevalences were: (1/3.70%), (23/6.35%), (5/6.75%), (17/5.24%) and (17/22.97%), (78/24.68%) from Maiduguri, Gombe and Jos respectively. The overall prevalence in male and female sheep in the 3 locations was 23(13.1%) and 118 (11.8%) respectively, and regarding location, the overall prevalence was highest in Jos 95(24.35%), followed by Gombe 24(6.16%) and Maiduguri 22(5.52%) (Table 4.7).

The prevalence in sheep from the 3 locations did not follow a consistent pattern. While in Jos and Maiduguri, the prevalence in females 78 (24.68%) and 23 (6.35%)) respectively was higher than males 17(22.97%) and 1(3.7%), the reverse was the situation in Gombe (6.75% in males and 5.24% in females). The same inconsistent pattern was observed in cattle. In Gombe, the prevalence in males 23 (27.71%) was higher that of females 82 (26.79%), while the reverse were the cases in Jos (36.02% in females and 33.33% in males) and Maiduguri (20.05% in females and 17.64% in males). However, these differences were not statistically sigfnificant ($p>0.05$), even though males in Gombe had an odds ratio of 1.047 (Table 4.7).

In both cattle and sheep, the mean egg count of males and females did not follow any discernable pattern. In some instances, the males had higher egg count (FEC&BEC), while in others females had higher counts. In all cases however, there were no significant differences in the mean egg counts of the two sexes. However, female sheep and cattle from Maiduguri had the highest mean count compared to other locations (Table 4.8)

Table 4.7: Prevalence of *Fasciola* spp. in slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to sex

Location	Species	Sex	No .Examined	No.(%) Infected	X^2	P-value	OR	CI
Maiduguri	Cattle	Male	68	12(17.64)				
		Female	319	64(20.06)	0.207	0.648	0.853	0.4320 - 1.687
	Sheep	Male	27	1(3.70)				
		Female	362	23(6.35)		1.00	0.5669	0.07357-4.368
Gombe	Cattle	Male	83	23(27.71)				
		Female	306	82(26.79)	0.027	0.867	1.047	0.6082-1.803
	Sheep	Male	74	05(6.75)				
		Female	324	17(5.24)	0.263	0.608	1.309	0.4667-3.669
Jos	Cattle	Male	90	30(33.33)				
		Female	322	116(36.02)	0.222	0.637	0.8879	0.5418-1.455
	Sheep	Male	74	17(22.97)				
		Female	316	78(24.68)	0.095	0.757	0.91000	0.4999-1.657

Table 4.8: Mean egg count of *Fasciola* spp. in slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to sex

Location	Species	Sex	Mean±SEM	P-value
Maiduguri	Cattle	Male	29.25±16.23	
		Female	73.29±15.42	0.21
	Sheep	Male	2.00±00	
		Female	37.57±21.30	NA
Gombe	Cattle	Male	32.62±9.73	
		Female	48.63±13.20	0.55
	Sheep	Male	7.80±5.56	
		Female	16.47±6.35	0.50
Jos	Cattle	Male	17.55±3.11	
		Female	13.51±1.46	0.209
	Sheep	Male	13.54±4.53	
		Female	21.07±3.48	0.333

Key: NA= Not applicable

4.6 Prevalence of *Fasciola* spp in Abattoir Slaughtered Catte and Sheep in Relation to Age

In cattle, the prevalence was higher in adults from Jos and Gombe 119 (38.26%) and 96 (24.67%) respectively than young ones 27(26.73%) and 40 (22.50%) respectively, but reverse was the case in Maiduguri (Table 4.9). For sheep, the prevalence rates in adults from the 3 locations were higher in Jos, 94 (24.80%); Gombe, 21 (5.64%); Maiduguri, 24 (6.85%) than the young 1 (9.09%), 1 (3.84%) and 0% respectively); but the difference was only significant ($p<0.05$) in Jos.

In the three (3) locations adult sheep had higher but insignificant ($p>0.05$) higher mean egg count (19.85±2.99, 15.17±5.37 and 36.34±20.29) than lambs (3.00±0.00, 3.00±0.00 and 0.00±0.00 respectively). For cattle, it was only in Maiduguri that the mean adult egg count (70.45±17.18) was markedly higher than in calves (54.10±17.09), but the difference was not significant ($p>0.05$) (Table 4.10)

Table 4.9: Prevalence of *Fasciola* spp. in slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to age

Location	Species	Age	No. Examined	No.(%) Infected	X^2	P-value	OR	CI at 95%
Maiduguri	Cattle	Young	94	22(23.40)				
		Adult	293	54(18.43)	1.116	0.290	1.352	0.7713-2.371
	Sheep	Young	39	0(0.0)				
		Adult	350	24(6.85)		NA		
Gombe	Cattle	Young	40	9(22.50)				
		Adult	349	96(24.67)	0.530	0.466	0.7495	0.3442-1.632
	Sheep	Young	26	1(3.84)				
		Adult	372	21(5.64)		1.000	0.6382	0.08255-4.933
Jos	Cattle	Young	101	27(26.73)[a]				
		Adult	311	119(38.26)[b]	4.431	**0.035**	0.5887	0.3583-0.9673
	Sheep	Young	11	1(9.09)				
		Adult	379	94(24.80)		0.308	0.3032	0.03828-2.401

Values with different superscripts in rows differed significantly ($p<0.05$). Key: NA=Not applicable (Test could not be applied to values having zeroes as either mean or standard error of the mean)

Table 4.10: Mean egg counts of *Fasciola* spp. in slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs abattoirs in relation to age

Location	**Species**	**Age**	Mean±SEM	**P-value**
Maiduguri	Cattle	Young	54.10±17.09	
		Adult	70.45±17.18	0.581
	Sheep	Young	0.00±0.00	
		Adult	36.34±20.59	NA
Gombe	Cattle	Young	42.25±25.72	
		Adult	45.80±11.59	0.925
	Sheep	Young	3.00±0.00	
		Adult	15.17±5.37	NA
Jos	Cattle	Young	13.58±2.49	
		Adult	14.58±1.53	0.773
	Sheep	Young	3.00±0.00	
		Adult	19.85±2.99	NA

Key: NA= Not applicable (Test could not be applied to values having zeroes as either mean or standard error of the mean)

4.7 Prevalence of *Fasciola* spp in Abattoir Slaughtered Cattle and Sheep in Relation to Breed

Of the predominant four breeds of cattle sampled, the Rahaji, Bunaji, Crosses and Sokoto Gudali had 105 (25.2%), 185 (29.36%), 18 (40.0%) and 2 (28.57%) prevalences respectively, while the Wadara, Kuri and Ambala had respective counts of 15 (18.51%), 2 (25.0%) and 0.0%. Sokoto Gudali and Crosses from Gombe each had 100% prevalence as against 33.33% and 25.44% in Rahaji and Bunaji respectively (Table 4.11). Of the two predominant abattoir slaughtered sheep breeds (Yankasa and Balami) sampled, Yankasa had the overall higher prevalence of 126 (12.34%), than Balami 13(8.55%) prevalence.

Of the predominant breeds of cattle namely; Bunaji, Rahaji, Sokoto Gudali and Kuri, Kuri examined had the highest mean egg count of 117.7±59.12, while no egg was detected in Ambala (0.00±0.00). In all cases, there were no significant ($p>0.05$) differences among the rates. On egg counts, Balami had higher mean egg count of 24.70±3.50 than Yankasa (19.82±2.99) and WAD (0.00±0.00). The detailed egg counts of abattoir slaughtered cattle and sheep sampled in relation to their breeds are as presented in Table 4.12.

Table 4.11:Prevalence of *Fasciola* spp. in slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to breed

Location	**Species**	**Breed**	**No. Examined**	**No.(%) Infected**	**X^2**	**P-value**	**OR**	**CI at 95%**
Maiduguri	Cattle	Wadara	81	15(18.51)				
		Rahaji	282	57(20.21)			1.115	0.5927-2.096
		Kuri	08	2(25.00)			1.467	0.2690-7.997
		Sokoto Gudali	06	1(16.66)			0.8800	0.09562-8.099
		Crosses	03	0(0.00)				
		Ambala	01	0(0.00)				
		Bunaji	06	1(16.63)	1.314	0.970	0.8800	0.09562-8.099
	Sheep	Yankasa	248	12(4.83)				
		WAD	02	0(0.00)				
		Balami	139	12(8.63)	2.347	0.309	1.858	0.8112-4.257
Gombe	Cattle	Rahaji	48	16(33.33)[a]			1.465	0.7662-2.802
		Bunaji	338	86(25.44)[b]				
		Cross	02	02(100.00)[c]				
		Sokoto Gudali	01	01(100.00)[c]	9.505	**0.023**		
	Sheep	Yankasa	386	20(5.18)				
		WAD	02	0(0.00)				
		Balami	10	02(20.00)	4.216	0.1215	0.2186	0.04352-1.098
Jos	Cattle	Rahaji	86	32(37.20)				
		Bunaji	286	98(34.26)			0.8797	0.5330-1.452
		Cross	40	16(40.00)	0.653	0.7212	1.125	0.5213-2.428
	Sheep	Yankasa	387	94(24.28)				
		Balami	3	1(33.33)		0.568	0.6416	0.05750-7.160

Values with different superscripts in the same column differed significantly ($p<0.05$)

Table 4.12: Mean egg count of *Fasciola* spp. in slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs, Nigera in relation to breed

Location	**Species**	**Breed**	Mean±SEM	**P-value**
Maiduguri	Cattle	Wadara	117.3±49.58	
		Rahaji	45.96±8.50	
		Kuri	117.7±59.12	
		Sokoto Gudali	00.00±0.00	
		Crosses	00.00±0.00	
		Ambala	00.00±0.00	
		Bunaji	102±0.00	NA
	Sheep	Yankasa	13.00±5.27	
		WAD	00.00±0.00	
		Balami	65.08±45.16	NA
Gombe	Cattle	Rahaji	81.74±52.14	
		Bunaji	40.71±9.41	
		Cross	3.33±1.85	
		Sokoto Gudali	3.00±0.00	NA
	Sheep	Yankasa	15.73±5.59	
		WAD	00.00±0.00	
		Balami	3.00±0.00	NA
Jos	Cattle	Rahaji	14.89±4.24	
		Bunaji	14.76±1.52	
		Cross	14.30±3.56	0.993
	Sheep	Yankasa	19.82±2.99	
		Balami	6.00±0.00	NA

Key: NA= Not aapplicable (Test could not be applied to values having zeroes as either mean or standard error of the mean)

4.8 Prevalence of *Fasciola* spp in Abattoir Slaughtered Cattle and Sheep in Relation to Month of Sample Collection from Maiduguri, Gombe and Jos

Although samples could not be collected in some locations in some months, of the samples collected, significantly higher rates ($p<0.05$) were obtained in January 114 (33.62%), February 32 (43.83%) and March 22 (32.83%) in cattle and January 4 (28.57%), July 167 (26.94%) and August 200 (22.00%) in sheep. Based on the species sampled from the locations, the least infection rate of 2.46% was in the month of February among sheep from Gombe (Table 4.13).

Table 4.13: Prevalence of *Fasciola* spp. in slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to months of sampling

Location	Species	Month	No. Examined	No. (%) Infected	X^2	P-value	OR	CI at 95%
Maiduguri	Cattle	April	104	19(18.26)				
		May	283	57(20.14)	0.168	0.681	0.886	0.4981-1.577
	Sheep	April	194	12(6.18)				
		May	195	12(6.15)	0.0001	0.989	1.005	0.4401-2.297
Gombe	Cattle	February	54	18(33.33)				
		March	67	22(32.83)			0.977	0.4564-2.095
		June	268	65(24.25)	3.283	0.193	0.640	0.3407-1.204
	Sheep	February	81	02(2.46)				
		March	161	10(6.21)			2.616	0.5593-12.24
		June	156	10(6.41)	1.828	0.401	2.705	0.5782-12.66
Jos	Cattle	January	339	114(33.62)				
		February	73	32(43.83)	2.735	0.098	0.649	0.3881-1.086
	Sheep	January	14	04(28.57)				
		February	09	02(22.22)			0.714	0.1013-5.038
		July	167	45(26.94)			0.922	0.2752-3.090
		August	200	44(22.00)	1.368	0.713	0.705	0.2109-2.358

4.9 Prevalence of *Fasciola* spp in Abattoir Slaughtered Cattle and Sheep in Relation to Sample Type

In cattle, the overall prevalence through bile sedimentation (23.9%) was significantly higher ($p<0.0001$) than the value (10.9%) obtained through faecal sedimentation (Table 4.14). Higher prevalence rates of 18.08%, 22.87% and 30.33% were found among cattle from Maiduguri, Gombe and Jos respectively through bile sedimentation compared to 2.58%, 11.05% and 18.93% found by faecal sedimentation.

Of the total 1,177 sheep faeces sampled and for analysed using faecal sedimentation method in the three locations, the overall prevalence of 103(8.85%) was significantly higher ($p<0.049$) than the rate 72 (6.11%) obtained through bile sedimentation. Prevalence rates of 17(4.37%), 13(3.15%) and 73(18.71%), were respectively obtained from Maiduguri, Gombe and Jos through faecal sedimentation compared to corresponding rates of 11 (2.82%), 10 (2.51%) and 51 (13.07%) obtained through bile sedimentation (Table 4.14).

On the mean egg counts of sampled cattle, bile sedimentation gave a higher egg counts compared to faecal sedimentation from all locations. However, in sheep, it was only in Jos that the bile sedimentation gave a significantly ($p<0.0003$) higher count (32.70±5.59) than faecal sedimentation (9.66±2.68) (Table 4.15)

Table 4.14: Prevalence of *Fasciola* spp. in slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to sample type

Location of Abattoir	Species	Sample type	No. Examined	No. (%) Infected	X^2	P-value	OR	CI at 95%
Maiduguri	Cattle	Bile sedimentation	387	70(18.08)[a]	50.19	**p<0.0001**	**8.325**	**4.220-16.42**
		Faecal Sedimentation	387	10(2.58)[b]				
	Sheep	Bile sedimentation	389	11(2.82)[a]	1.334	0.2481	0.6368	0.2943-1.378
		Faecal sedimentation		17(4.37)[a]				
Gombe	Cattle	Bile sedimentation	389	89(22.87)[a]	19.31	**p<0.0001**	**2.387**	**1.607-3.545**
		Faecal sedimentation	389	43(11.05)[b]				
	Sheep	Bile sedimentation	398	10(2.51)[a]	0.4029	0.5256	0.7633	0.3306-1.762
		Faecal sedimentation	398	13(3.15)[a]				
Jos	Cattle	Bile sedimentation	412	125(30.33)[a]	14.44	**0.0001**	1.865	1.349-2.578
		Faecal sedimentation	412	78(18.93)[b]				
	Sheep	Bile sedimentation	390	51(13.07)[a]	4.641	**0.0312**	**0.6533**	**0.4427-0.9641**
		Faecal sedimentation	390	73(18.71)[b]				

Values with different superscripts in the same column differed significantly ($p<0.05$).

Table 4.15: Mean egg count of *Fasciola* spp. in faeces and bile of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to sample type

Location of Abattoir	Species	Sample type	Mean±SEM	P-value
Maiduguri	Cattle	Bile sedimentation	67.87±14.03	
		Faecal sedimentation	54.82±39.20	0.723
	Sheep	Bile sedimentation	79.33±48.03	
		Faecal sedimentation	6.35±2.62	0.08
Gombe	Cattle	Bile sedimentation	60.49±15.31[a]	
		Faecal sedimentation	12.15±3.53[b]	**0.037**
	Sheep	Bile sedimentation	109.33±89.33	
		Faecal sedimentation	3.69±1.70	0.211
Jos	Cattle	Bile sedimentation	16.33±1.87	
		Faecal sedimentation	11.66±1.79	0.098
	Sheep	Bile sedimentation	32.70±5.59[a]	
		Faecal sedimentation	9.66±2.68[b]	**0.0003**

Values with different superscripts in the same column differed significantly ($p<0.05$)

4.10 Number of Adult *Fasciola* Species Collected from Slaughtered Cattle and Sheep from Maiduguri, Gombe and Jos Abattoirs

Three hundred and sixty-eight (368) flukes in all were collected in the study. Two hundred and fourty-eight (248) of them were used for morphological identification and morphometry, while one hundred and twenty (120) of them were used for molecular characterisation. A total of 229 flukes were collected from cattle from Jos, 7 from Gombe and 77 from Maiduguri. Fifty five (55) flukes were collected from sheep as follows; Jos (23), Gombe (4) and Maiduguri (28). Of the 368 adult *Fasciola* isolates collected, 313 were from cattle and 55 from sheep (Table 4.16). Of the 55 from sheep, 24 were used for morphological identification and 31 for molecular characterisation.

Two hundred and twenty three (223) out of the 313 *Fasciola* collected from cattle were used for morphological identification, while 89 were used for molecular characterization (Table 4.16).

Table 4.16: Distribution of *Fasciola* spp collected from slaughtered cattle and sheep from Maiduguri, Gombe and Jos Abattoirs and their usage.

Location	**Cattle**			**Sheep**		
Abattoir	**No. Collected**	**Usage**		**No. Collected**	**Usage**	
		Morphological identification	Molecular identification	Total	Morphological identification	Molecular identificatiom
Maiduguri	77	60	17	28	15	13
Gombe	7	5	2	4	0	4
Jos	229	159	70	23	9	14
Total	313	223	89	55	24	32

4.11 Morphometric Identification of *Fasciola* Isolates from Cattle

The lengths (mm) of *Fasciola* spp from Maiduguri (41.68±0.870) and Jos (41.46±0.842) were more nearly the same, but those from Gombe were significantly ($p<0.0287$) shorter (31.5±2.91). The cone width (CW) of isolates from Gombe (4.813±0.365) was significantly ($p<0.0001$) longer than those from Jos (3.588±0.040) and Maiduguri (3.713±0.063). The CL, Vs-Vit and VS-P did not vary significantly ($p>0.05$) among isolates from the three study locations.

The ratio BW/BL among *Fasciola* isolates from Maiduguri (5.135±0.125) was significantly ($p< 0.0076$) higher than those from Gombe (4.005±0.506), but not Jos (4.823±0.076). However, the ratio CL/CW for isolates from Jos (0.800±0.01) was significantly ($p<0.0017$ higher than for isolates from Maiduguri (0.753±0.016) and Gombe (0.67±0.023) (Table 4.17).

Table 4.17: Mean linear biometric parameters/ratios in (mm) of *Fasciola* species from slaughtered cattle from Maiduguri, Gombe and Jos abattoirs

Biometric character/ratio(s)	Maiduguri	Gombe	Jos	P -value
BL	41.68±0.87[a]	31.50±2.91[b]	41.46±0.84[a]	**p=0.0287**
BW	8.23±0.14	8.27±0.63	8.67±0.13	P<0.1969
CW	3.71±0.06[a]	4.81±0.36[b]	3.58±0.04[a]	**p<0.0001**
CL	2.79±0.06	3.26±0.30	2.87±0.04	p<0.1011
VS-Vit	18.59±0.46	17.19±1.59	19.03±0.51	p<0.6642
VS-P	33.42±0.75	27.75±2.97	32.75±0.77	p<0.2787
BL/BW	5.13±0.12[a]	4.00±0.50[b]	4.82±0.07[a]	**p<0.0076**
CL/CW	0.75±0.01[b]	0.67±0.02[c]	0.80±0.01[a]	**p<0.0017**

Values with differeent supercripts in the same row differed significantly (p≤0.05). Keys: BL= Body length, BW= body width, CW= cone width, CL= cone length, VS-Vit= distance between the ventral sucker and the union of vitelline gland, VS-P= distance between the ventral sucker and the posterior end of the body, BL/BW= body lenghth/body width ratio and CL/CW= cone length/ cone width ratio.

4.12 Morphometric Identification of *Fasciola* isolates from sheep

The body length (BL) (42.50±2.63) and body width (BW) (7.625±0.800) of isolates from Jos did not differ significantly ($p>0.05$) from those of isolates from Maiduguri (42.26±1.579 and 6.811±0.248 respectively). Conversely, the cone width (CW) (4.321±0.154) and cone length (CL) (3.189±0.130) of isolates from Maiduguri were significantly higher ($p<0.0023$; 0.0032 respectively) than the corresponding values (2.95±0.477 and 2.175±0.246) from Jos. The distance between ventral sucker (VS) and union of vitelline gland (Vs-Vit) (21.88±1.234) and the distance between the ventral sucker and the posterior end of the body (VS-P)(34.77±1.613) among isolates from Maiduguri were not significantly different ($p>0.05$), to those of Jos isolates (19.5±2.102 and 33.25±1.702 respectively). Similarly, the BL/BW and CL/CW ratios of Maiduguri (6.362±0.280 and 0.738±0.034 respectively) and Jos isolates (5.703±0.548 and 0.77±0.091 respectively) were not significantly different ($p>0.05$). No isolate was collected from Gombe due to unavailability within the sampling period (Table 4.18).

Table 4.18: Mean linear biometric parameters/ratios in (mm) of *Fasciola* species from slaughtered sheep in Maiduguri and Jos abattoirs

Biometric character/ratios(mm)	Maiduguri	Jos	P-value
BL	42.26±1.57	42.50±2.63	0.9480
BW	6.81±0.24	7.62±0.80	0.2197
CW	4.32±0.15[b]	2.95±0.47[a]	**0.0023**
CL	3.18±0.13[b]	2.17±0.24[a]	**0.0032**
VS-Vit	21.88±1.23	19.50±2.10	0.4163
VS-P	34.77±1.61	33.25±1.70	0.6806
BL/BW	6.36±0.28	5.70±0.54	0.3310
CL/CW	0.73±0.03	0.77±0.09	0.7025

Values with differeent supercripts in rows differed significantly ($p<0.05$). Keys: BL= Body length, BW= body width, CW= cone width, CL= cone length, VS-Vit= distance between the ventral sucker and the union of vitelline gland, VS-P= distance between the ventral sucker and the posterior end of the body, BL/BW= body lenghth/body width ratio and CL/CW= cone length/ cone width ratio.

4.13 PCR Ampliification of the ITS 1 and 28S rDNA Genes of *Fasciola* Isolates from Slaughtered Cattle and Sheep from Maiduguri, Gombe and Jos Abattoirs

A 680bp fragment was obtained following PCR amplification of *Fasciola* isolates from cattle and sheep in Jos (Figure 4.1), and Gombe (Figure 4.2). Eighteen (81.81%) of the 22 *Fasciola* isolates investigated for ITS 1 gene had demonstrable bands on gel (Figures 4.2-4.3).

PCR amplification of *Fasciola* spp 28S rDNA produced 618bp fragment in samples collected from cattle (lane 25-26) and sheep (lane 27-28) from Maiduguri) (Figure 4.3). Six (75.00%) of the 8 isolates that were subjected to PCR for the detection of 28S rDNA gene of *Fasciola* had bands on gel.

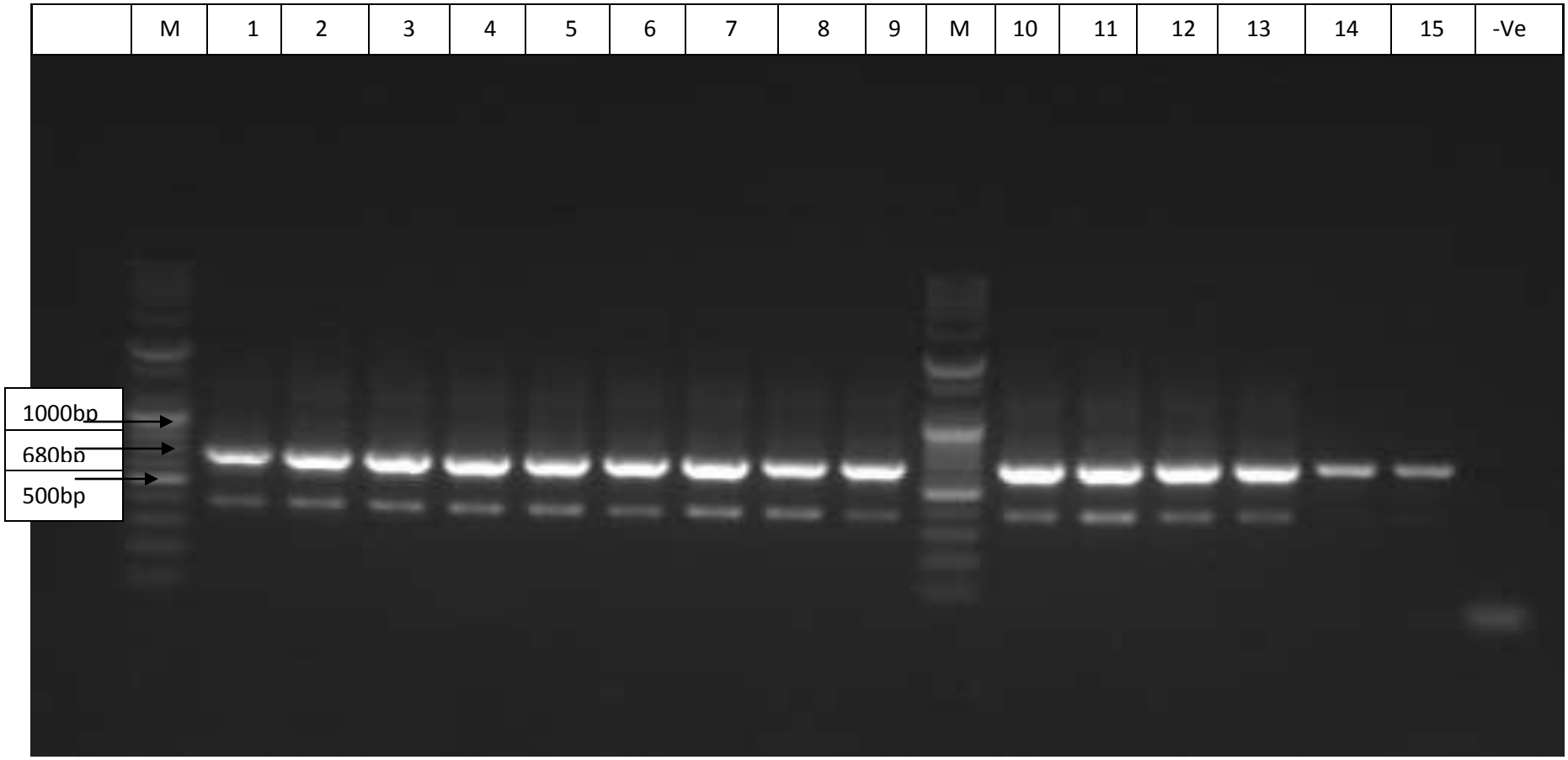

Figure 4:1: Gel electrophoresis of PCR products of ITS 1 gene of *Fasciola* isolates from abattoir slaughtered cattle (Lanes 1-11) and sheep (lanes 12-15) from Jos, Nigeria showing 680bp long PCR product, 100 bp DNA size marker (Lane M) and a negative control (–Ve)

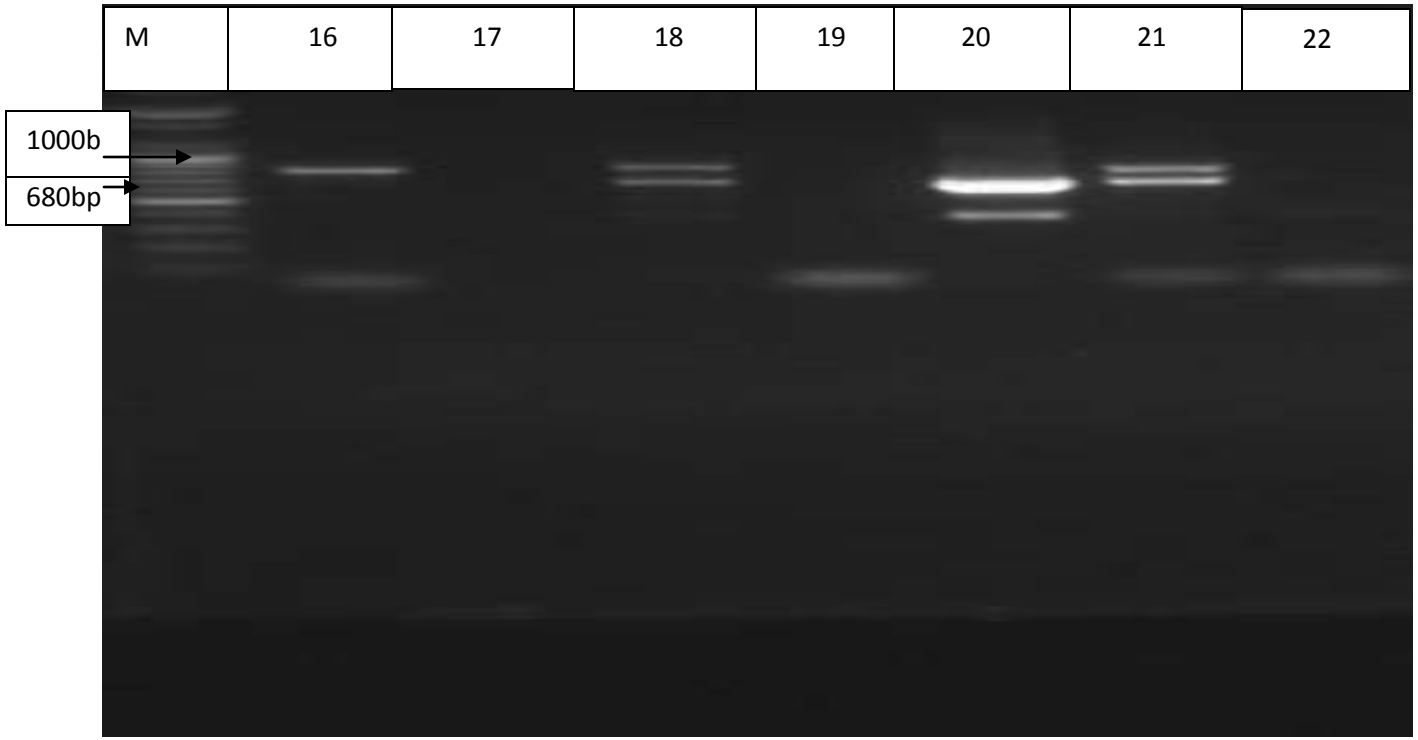

Figure 4:2: Gel electrophoresis of PCR products of ITS 1 region of *Fasciola* isolates from abattoir slaughtered cattle (Lane 21) and sheep (Lane 20) from Gombe, Nigeria showing 680bp long PCR products, lane 16 is an unspecific amplified DNA from *Lymnaea (Radix) natalensis*, lane 18 (Unidentifed snail) and 100 bp DNA size molecular marker (Lane M).

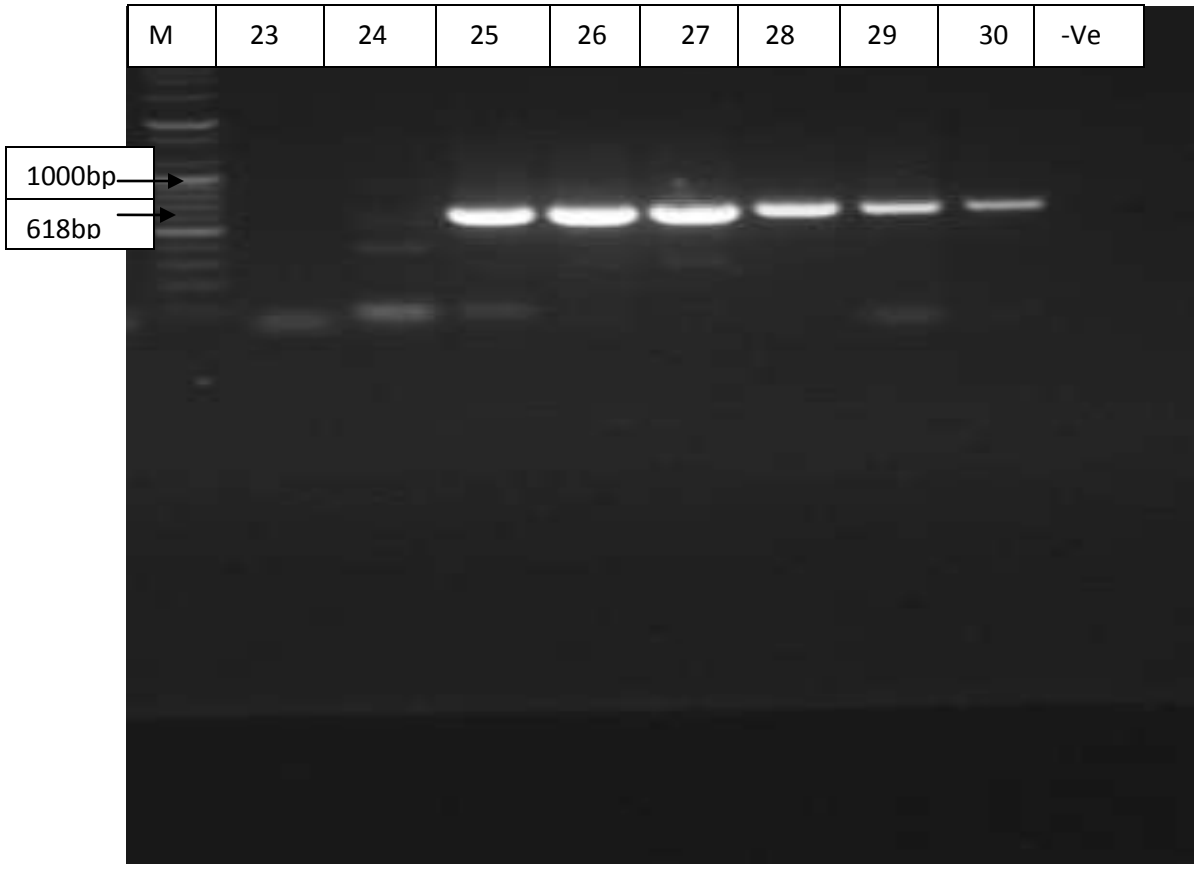

Figure 4:3: Gel electrophoresis of 28S rDNA of *Fasciola* isolates from abattoir slaughtered cattle (lanes 25-26), sheep (27-28) and from *Pila ampullacea* snail (lanes 29-30) from Maiduguri, Nigeria showing 618bp long products, 100 bp DNA size marker (Lane M) and negative control (–Ve)

4.14. PCR Ampliification of the Mitochondrial NADH Dehydrogenase Subunit 4 (NAD4) Gene of *Fasciola* Isolates from Abattoir Slaughtered Cattle and Sheep from Maiduguri, Gombe and Jos

Out of the ninety-nine *Fasciola* isolates selected among the previously described for the detection of mitochondrial NADH dehydrogenase subunit 4 (NAD4) gene, only 53 showed the presence of *Fasciola* DNA following DNA extraction. Out of the 53, the mitochondrial DNA NADH dehydrogenase subunit 4 (NAD4) gene was amplified in 25 isolates. Of the 25 isolates in which the gene was amplified, 13 had bands amplified at 304bp, strongly indicating that they were *Fasciola hepatica*, while 12 had bands amplified at 752 bp, strongly suggesting that they were *F.gigantica* (Figures 4.4- 4.9).

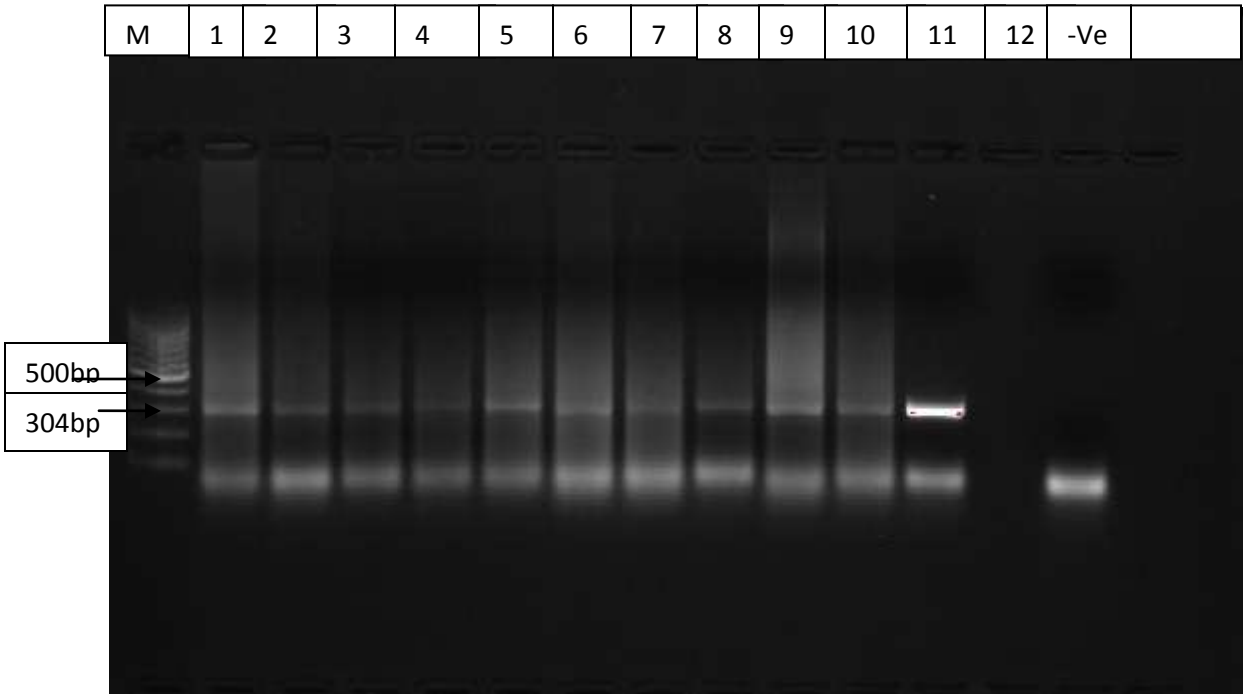

Figure 4:4. Gel electrophoresis of PCR product of NAD4 of *Fasciola hepatica* from abattoir slaughtered cattle (lane 1-6) and sheep (7-11) from Jos, Nigeria, showing 304bp long product, M is the molecular marker, while (-Ve) is the negative control

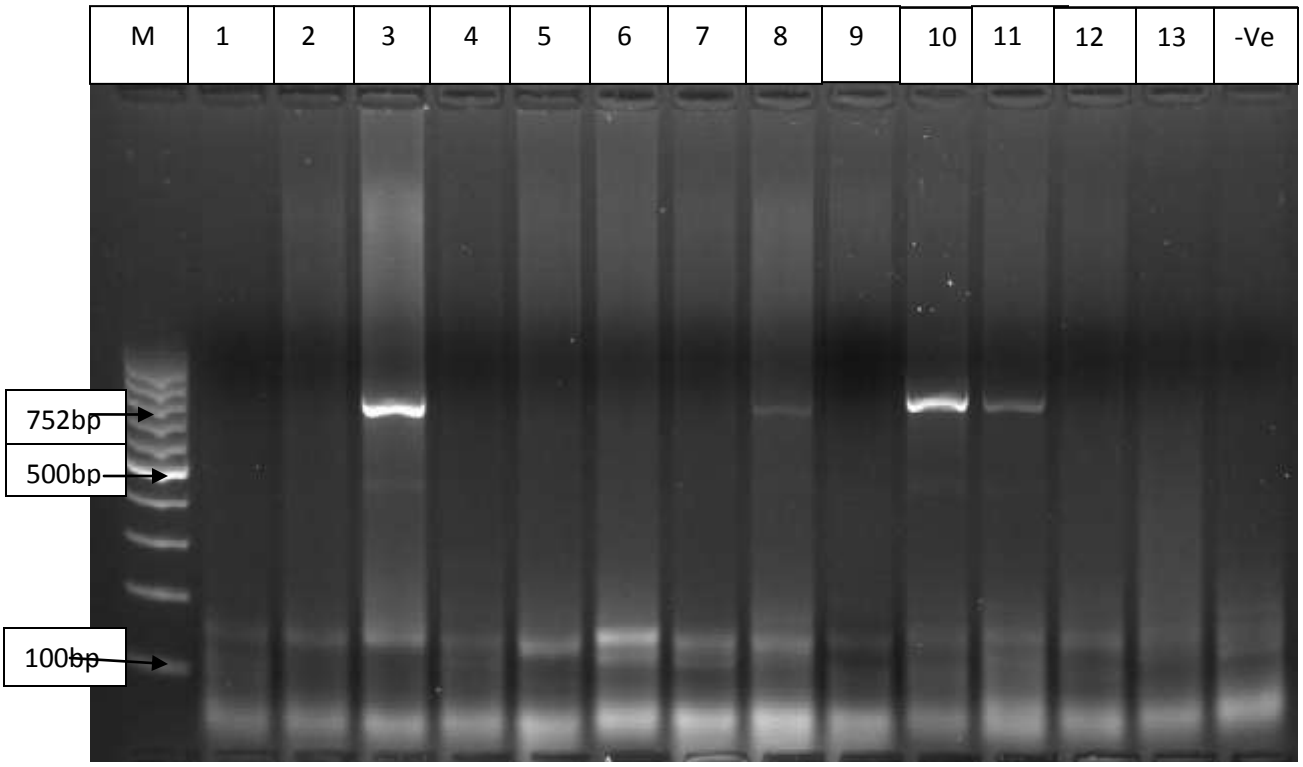

Figure 4:5. Gel electrophoresis of PCR product of NAD4 of *Fasciola gigantica* from abattoir slaughtered cattle (lanes 8, 10 and 11) and sheep (3) from Jos, Nigeria showing 752 bp long product. DNA was not amplified in the samples represented by the other lanes for the target gene. M is the 100bp molecular marker, while (-Ve) is the negative control

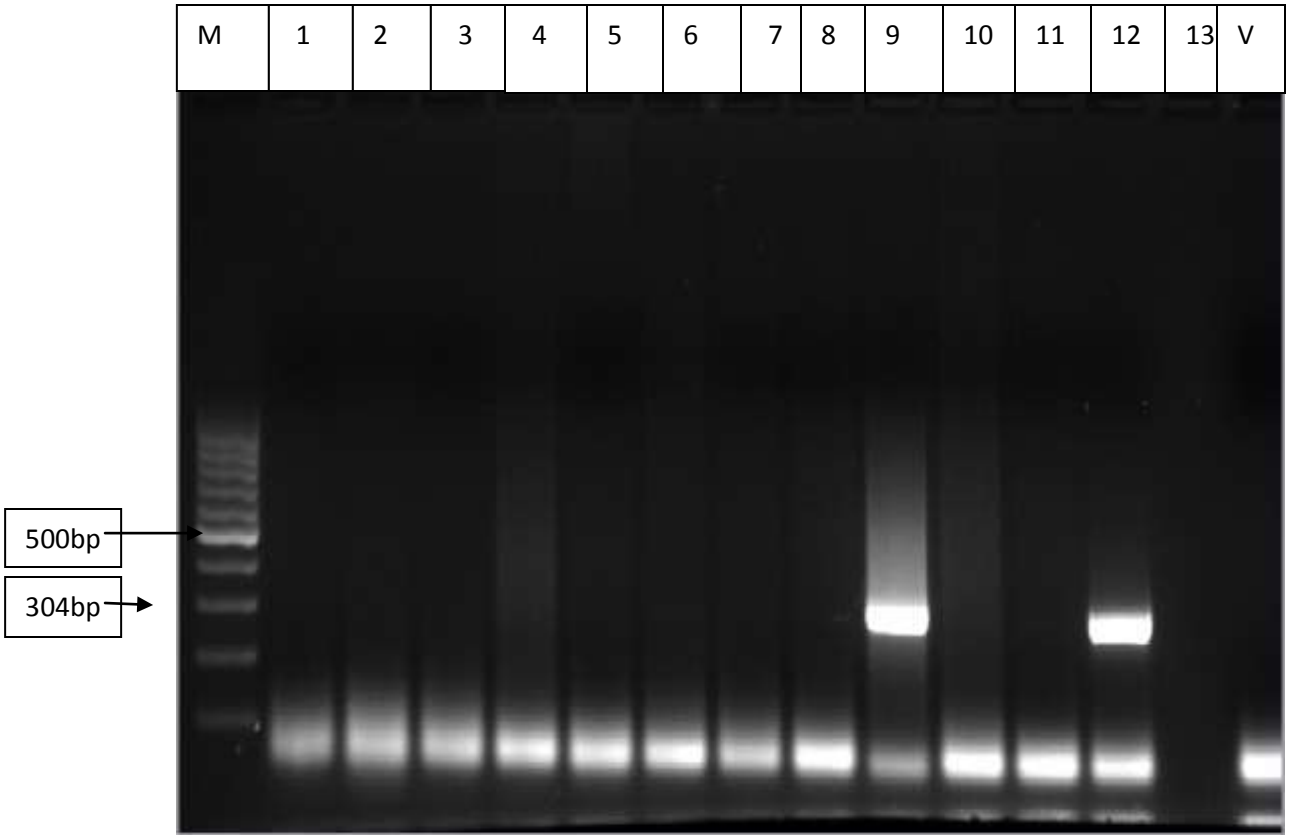

Figure 4:6. Gel electrophoresis of PCR product of NAD4 of *Fasciola hepatica* from abattoir slaughtered cattle (lanes 9 and 12) from Gombe, Nigeria showing 304 bp long product. DNA was not amplified in the samples represented by the other lanes for the target gene. M is the 100bp molecular marker, while (V) is the negative control

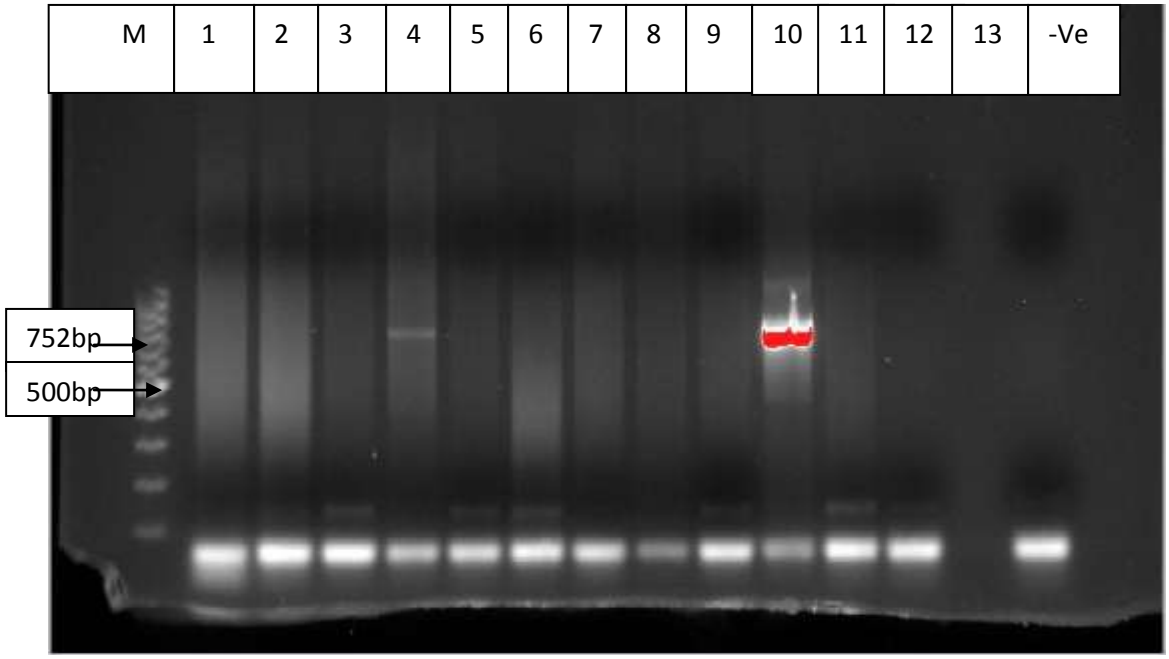

Figure 4:7. Gel electrophoresis of PCR product of NAD4 of *Fasciola gigantica* from abattoir slaughtered cattle (lanes 4 and 10) from Gombe, Nigeria showing 304 bp long product. DNA was not amplified in the samples represented by the other lanes for the target gene. M is the 100bp molecular marker, while (-Ve) is the negative control

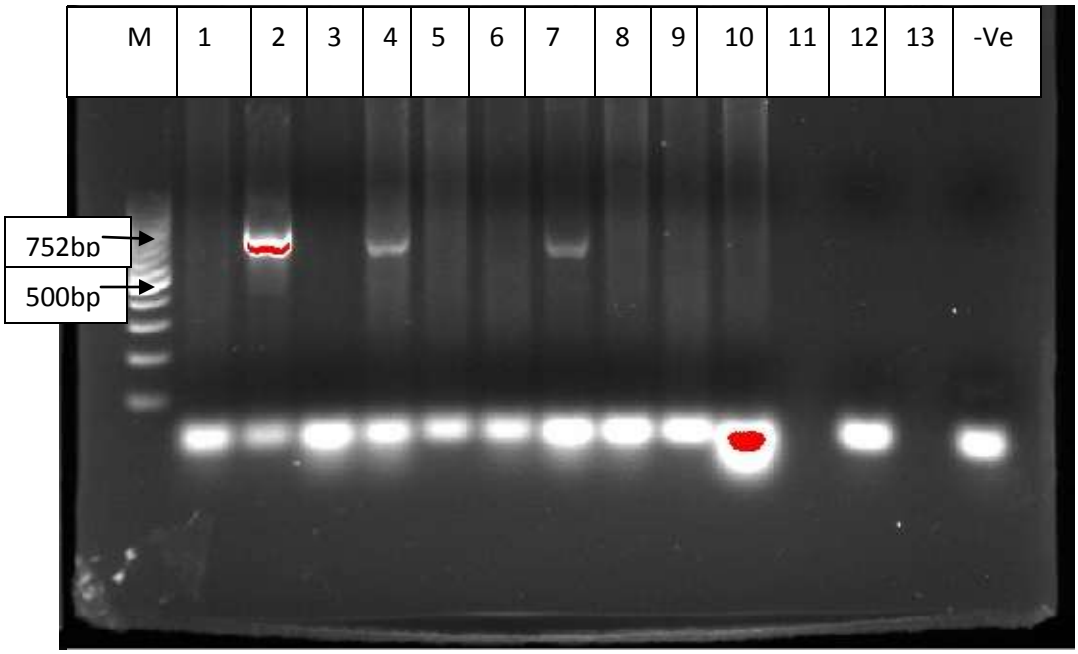

Figure 4:8. Gel electrophoresis of PCR product of NAD4 of *Fasciola gigantica* from abattoir slaughtered cattle (lanes 2, 4) and sheep (lane 7) from Maiduguri, Nigeria showing 752 bp long product. DNA was not amplified in the samples represented by the other lanes for the target gene. M is the 100bp molecular marker, while (-Ve) is the negative control

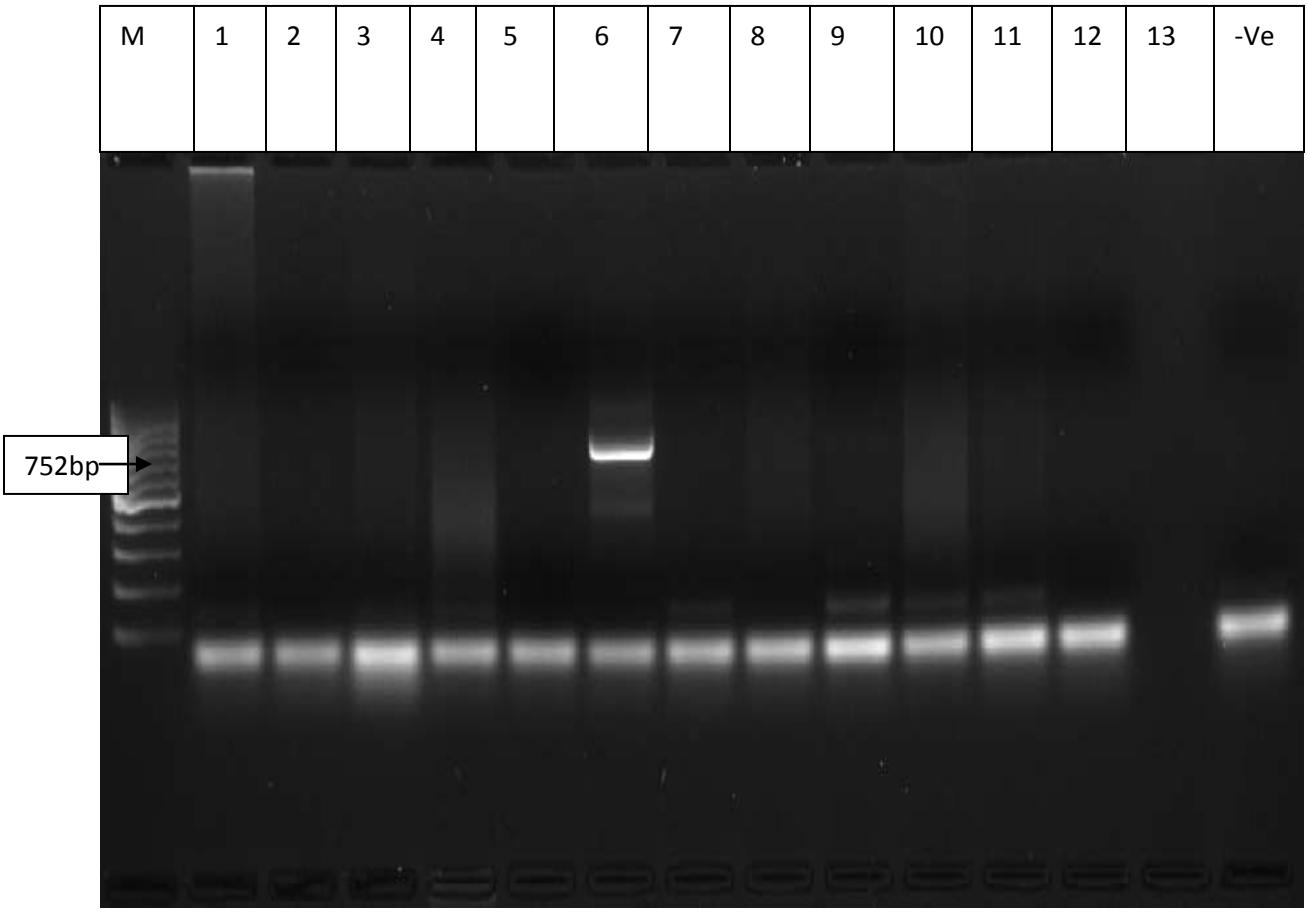

Figure 4.9: Gel electrophoresis of PCR product of NAD4 of *Fasciola gigantica* from abattoir slaughtered cattle (lane 6) from Maiduguri, Nigeria showing 752 bp long product. DNA was not amplified in the samples represented by the other lanes for the target gene. M is the 100bp molecular marker, while (-Ve) is the negative control

4.15 Results of DNA Sequencing of *Fasciola* Amplicons

The chromatograph sequence of the *Fasciola* gene (ITS 1) and 28S rRNNA as seen in ApE plasmid Editor are presented in Figures 4.10 and 4.11 respectively.The sequences were obtained following trimming of areas of ambiguities and noise representive of low quality sequences. Areas of throughput high quality sequencing can be seen as prominent bars at the background in between the arrows.

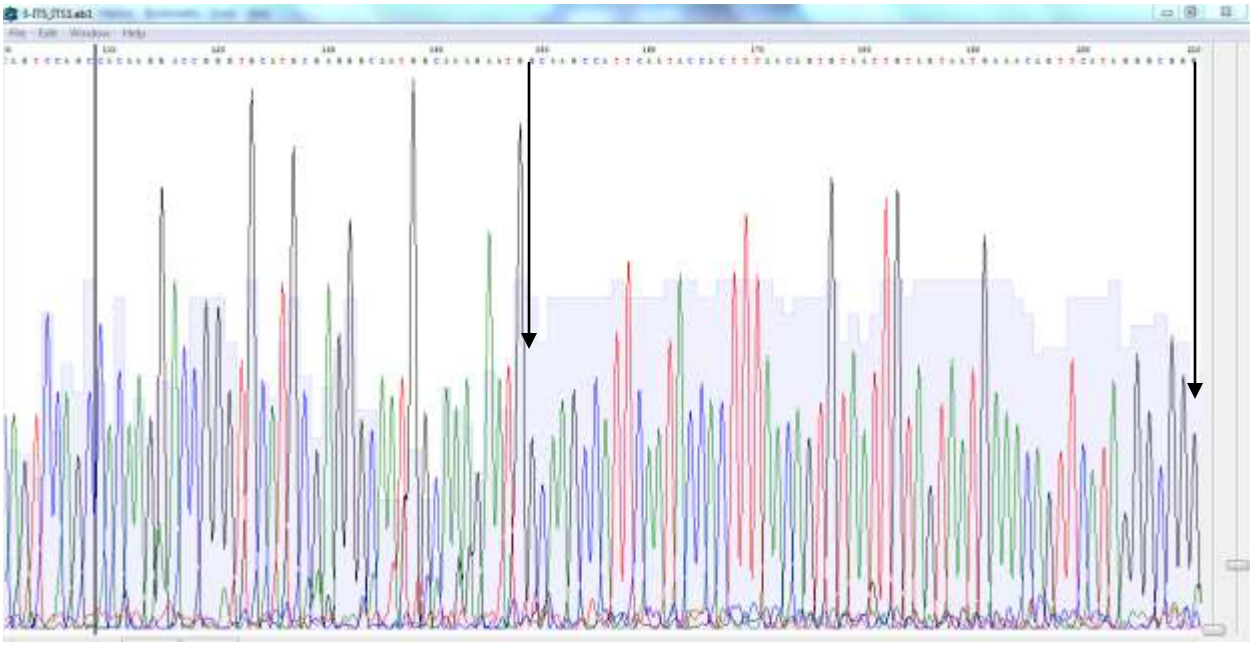

Figure 4.10: Chromatograph sequence of *Fasciola gigantica* (Seq 3) as seen in ApE based on ITS 1

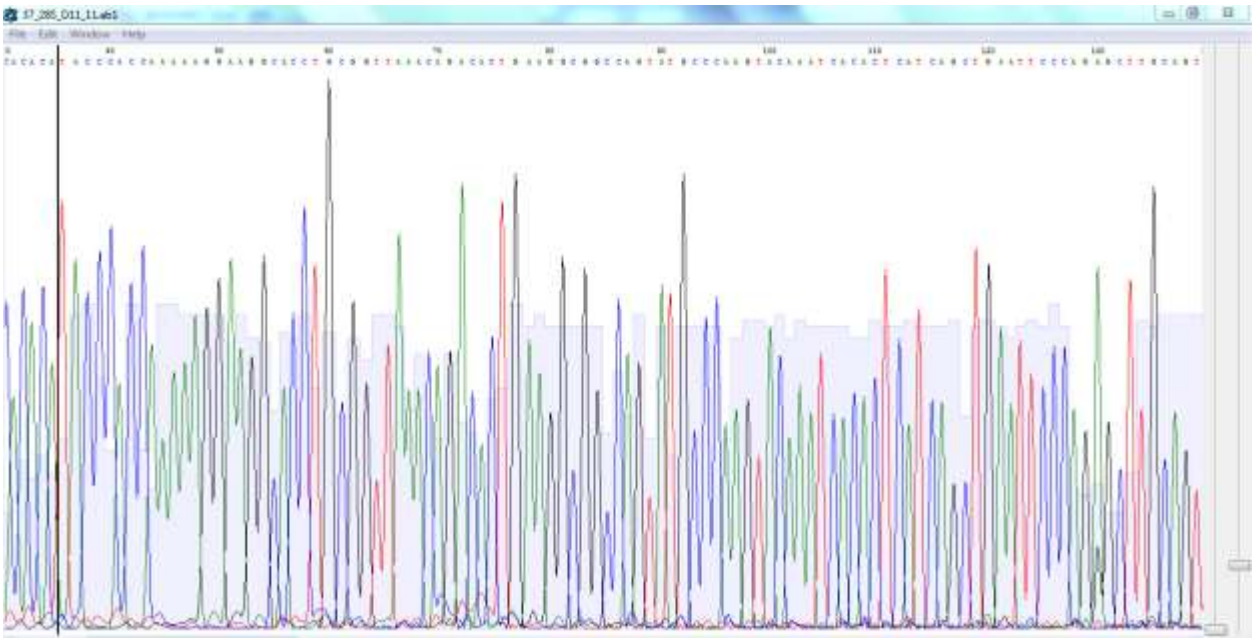

Figure 4.11: Chromatograph sequence of *Fasciola hepatica* (Seq 37) as seen in ApE based on 28S rDNA

4.16 Results of Sequence Alignment of *Fasciola* spp

4.16. 1 Single sequence alignment (SSA) of *Fasciola* spp sequences

The result of alignment of sequences (query) against sequences in the NCBI database is presented in Figures 4.12 and 4.13. In Figure 4.12, the query sequence had 99% identity with the subject sequence and 0% gaps, as only one nucleotide among the 329 had gap. The only gap is represented by an arrow. Similarly, in Figure 4.13, there was 85% identity between the query and subject sequence, with 1% gaps as 9 nucleotides out of the 495 of the query had gaps. Regions of gaps and mismacthes are represented by arrows.

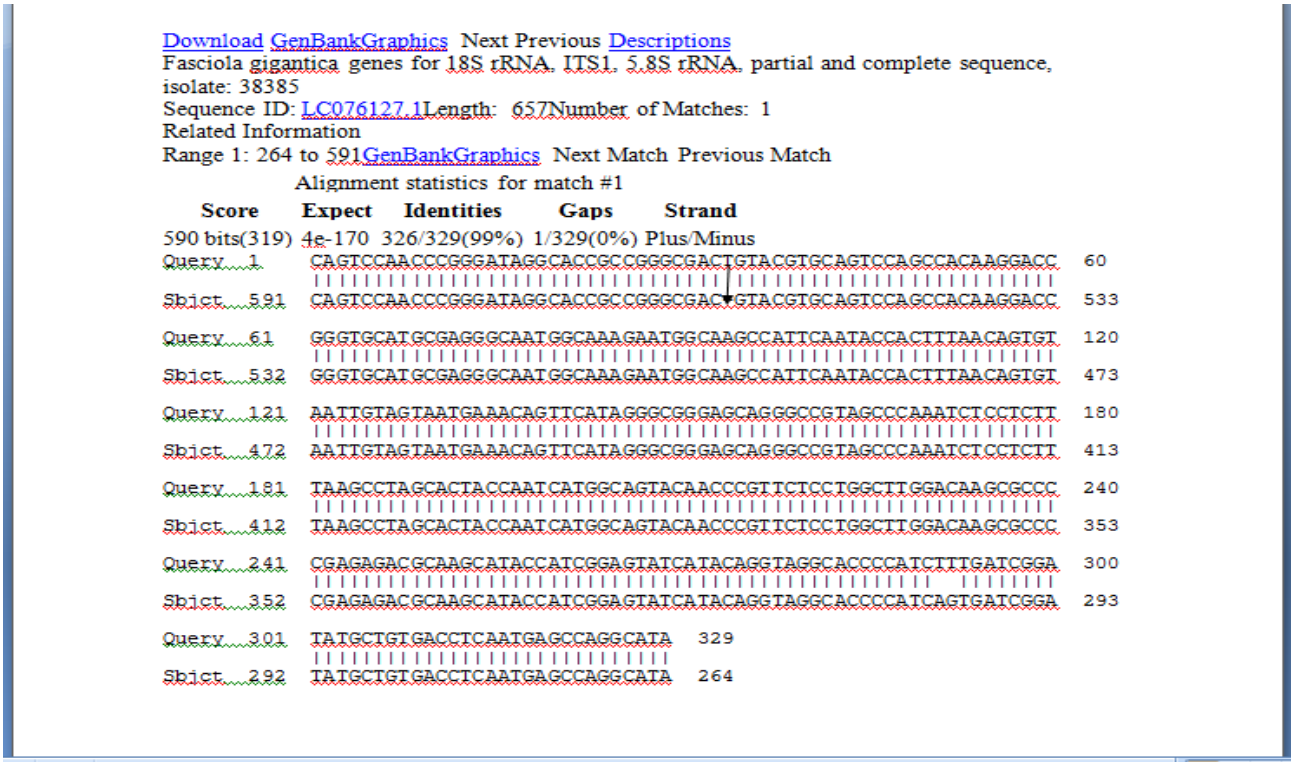

Figure 4.12: Aligned *Fasciola gigantica* isolate sequence (ITS 1) from NCBI Blastn search

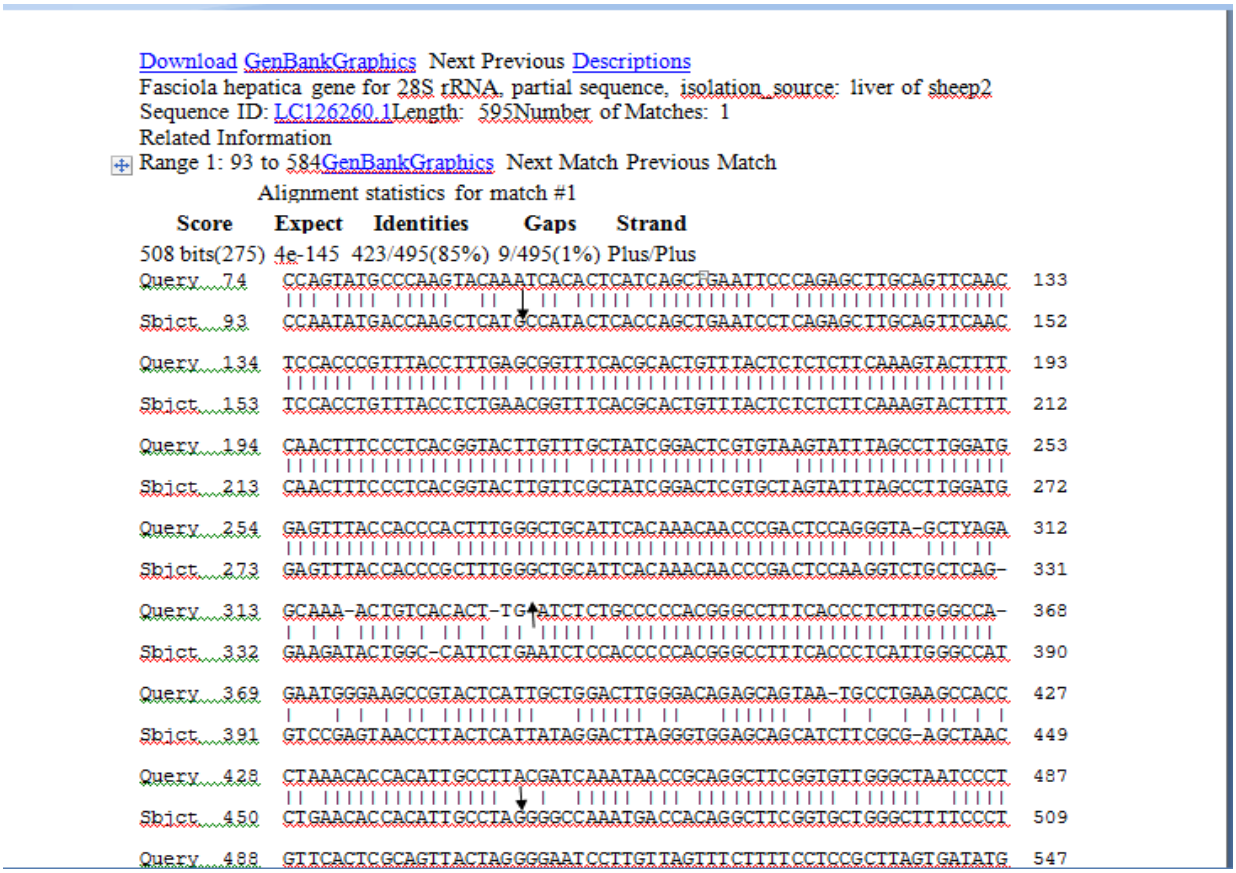

Figure 4.13: Aligned *Fasciola hepatica* isolate sequence (28S rRNA) from NCBI Blastn search

4.16. 2: Multiple sequence alignment (MSA) of *Fasciola* spp sequences

The result of the multiple sequence alignment of *Fasciola* spp sequences along with other sequences is presented in Figure 4.14. Gaps are represented by arrows for both study and subject sequences imported from GenBank.

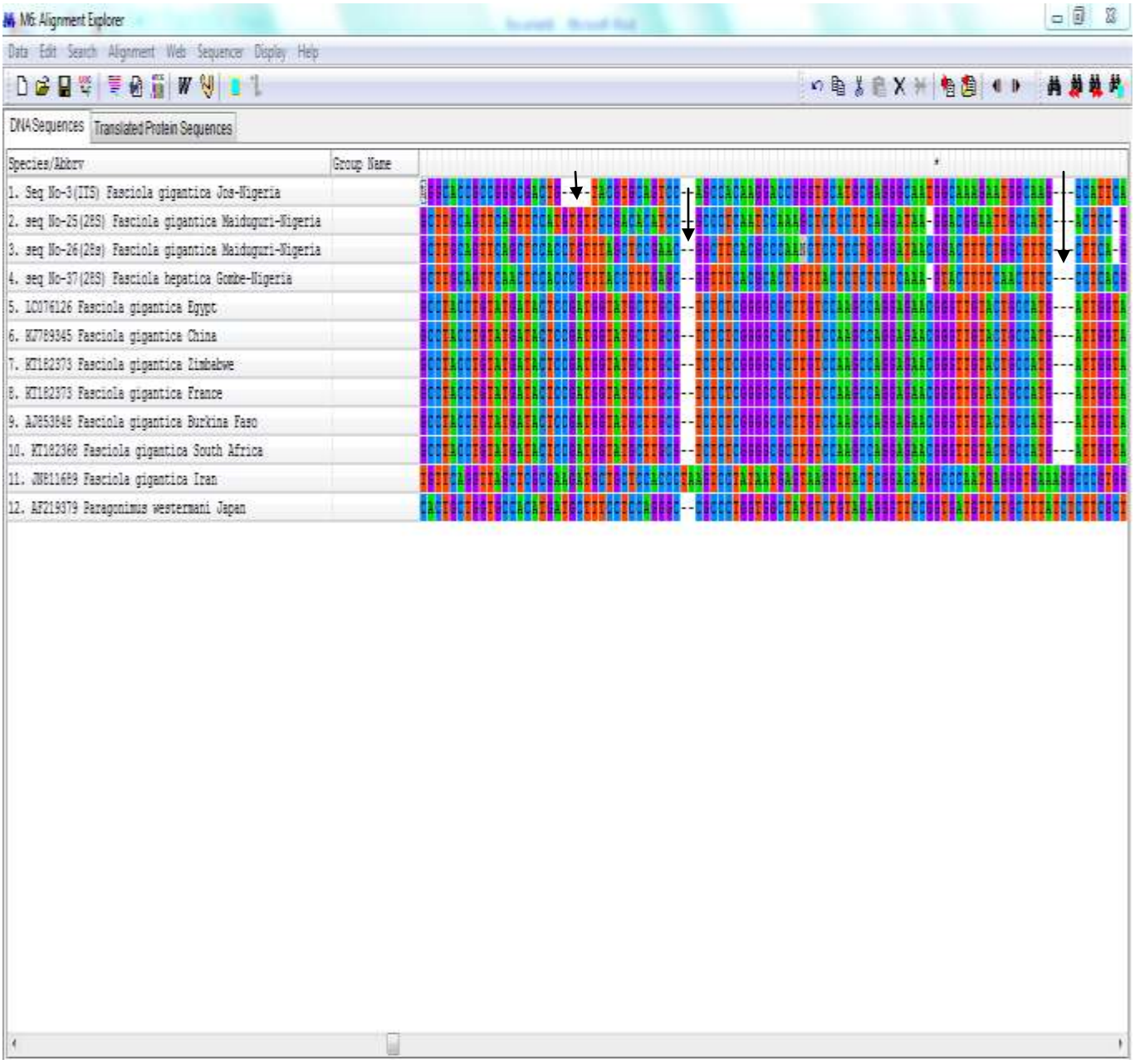

Figure 4.14: Result of multiple sequence alignment (MSA) of *Fasciola gigantica* (Seq 3, 25-26) and *Fasciola hepatica* (Seq 37) sequences along with similar sequences from GenBank in MEGA 6.06

4.17 Results of Phylogenetic Analysis

The evolutionary relationship of *Fasciola* spp. sequences with other sequences is depicted in the phylogenetic tree (Figure 4.15). Three different clades and five different subclades can be seen on the tree.

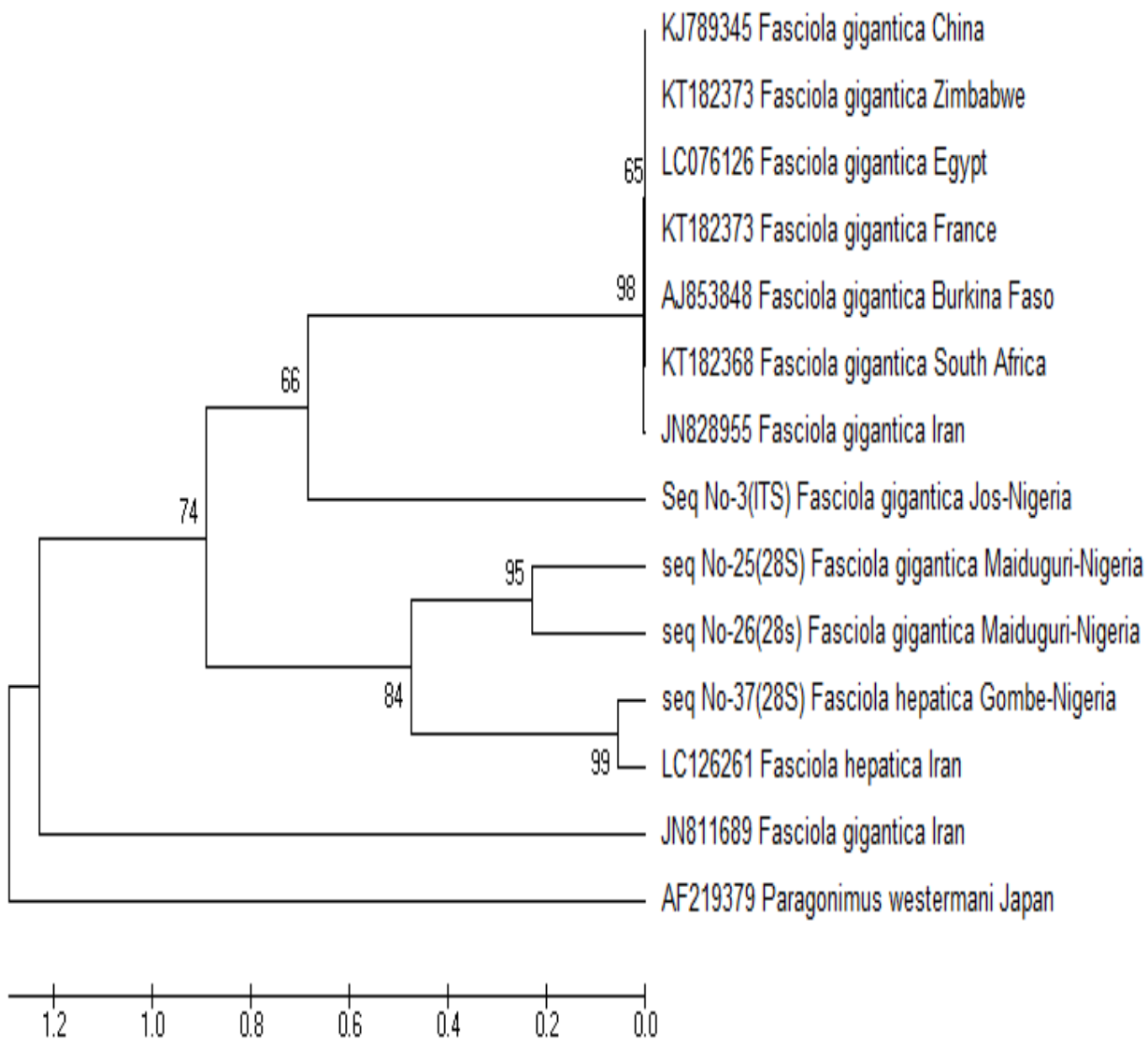

Figure 4.15: Phylogenetic tree of *Fasciola* spp. obtained by concatenating the ITS 1 and 28S rRNA sequences and other Fasciola spp sequences from GenBank using UPGMA. *Paragonimus westermanii* (AF219379) was used as an outgroup.

4.18 Morphological Identification of Snails

A total of 144 snails were collected and used for morphological study, out of which 20 representative samples preserved in 10% formol saline were identified at the Museum of Natural History, former Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria. The identified species are presented in plates II to VIII. Table 4.19 shows the distribution of collected snails used for morphological identification based on the respective Genera/Species.

Table 4.19: Sources of collected snails used for morphological identification based on their Genera/Species

Snail Genus/Species Identified	Location of Collection			
	Maiduguri	Gombe	Jos	Total
Limicolaria flammea	0	0	8	8
Limicolaria kambeul	0	0	22	22
Limicolaria turris	0	0	2	2
Biomphalaria pfeifferei	0	1	11	12
Pia ampullaceal	27	0	4	31
Lymnaea natalensis	0	12	31	43
Melanoides tuberculata	0	25	0	25
Unidentified snail	0	0	1	1
Total	27	38	79	144

Plate II: *Biomphalaria* spp collected from Gombe, Gombe State

Plate III: *Limicolaria flammea* collected from Gombe, Gombe State

Plate IV:*Limicolaria kambeul* collected from Jos, Plateau State

Plate V: *Limicolaria turris* collected from Jos, Plateau State

Plate VI: ***Lymnaea (Radix) natalensis*** **collected from Jos, Plateau State**

Plate VII: *Melanoides tuberculata* collected from Gombe, Gombe State

Plate VIII: *Pila ampullacea* collected from Maiduguri, Borno State

4.19 Molecular Characterisation of ITS 1 and 28S rDNA Genes of *Fasciola* spp in Snails from Maiduguri, Gombe and Jos using Polymerase Chain Reaction (PCR)

A total of seventeen (17) snail samples were purposively picked from the total collection based on species and analysed at the DNA Labs Kaduna, Nigeria for the presence of *Fasciola* DNA in the snails using ITS 1 and 28S rDNA genes. Table 4:20 shows the genera/species of snails analysed for the presence of *Fasciola* DNA.

The ITS 1 gene of *Fasciola* spp was amplified only in one snail (Lane 18: Unidentified snail) among others investigated for the presence of the gene (lanes 16,17,18,19 and 22) as seen in Figure 4.2. The 28S rDNA was amplified in 3 *Lymnaea natalensis* (Lanes 31-33, *Biomphalaria pfeifferi* (lane 37) and *Melanoides tuberculata* (lane 38) as shown (Figure 4.16).

Table 4:20: Distribution of the Genera/Species of collected snails from Maiduguri, Gombe and Jos analysed for *Fasciola* spp DNA

Snail Genera/Species	**Location**			
	Maiduguri	Gombe	Jos	No.Collected
Lymnaea natalensis		2	3	5
Biomphalaria pfeifferi		2	1	3
Limicolaria flammea		1		1
Melanoides tuberculata		1	1	2
Pila ampullaceal	2	1	2	5
Unidentified snail			1	1
Total	2	7	8	17

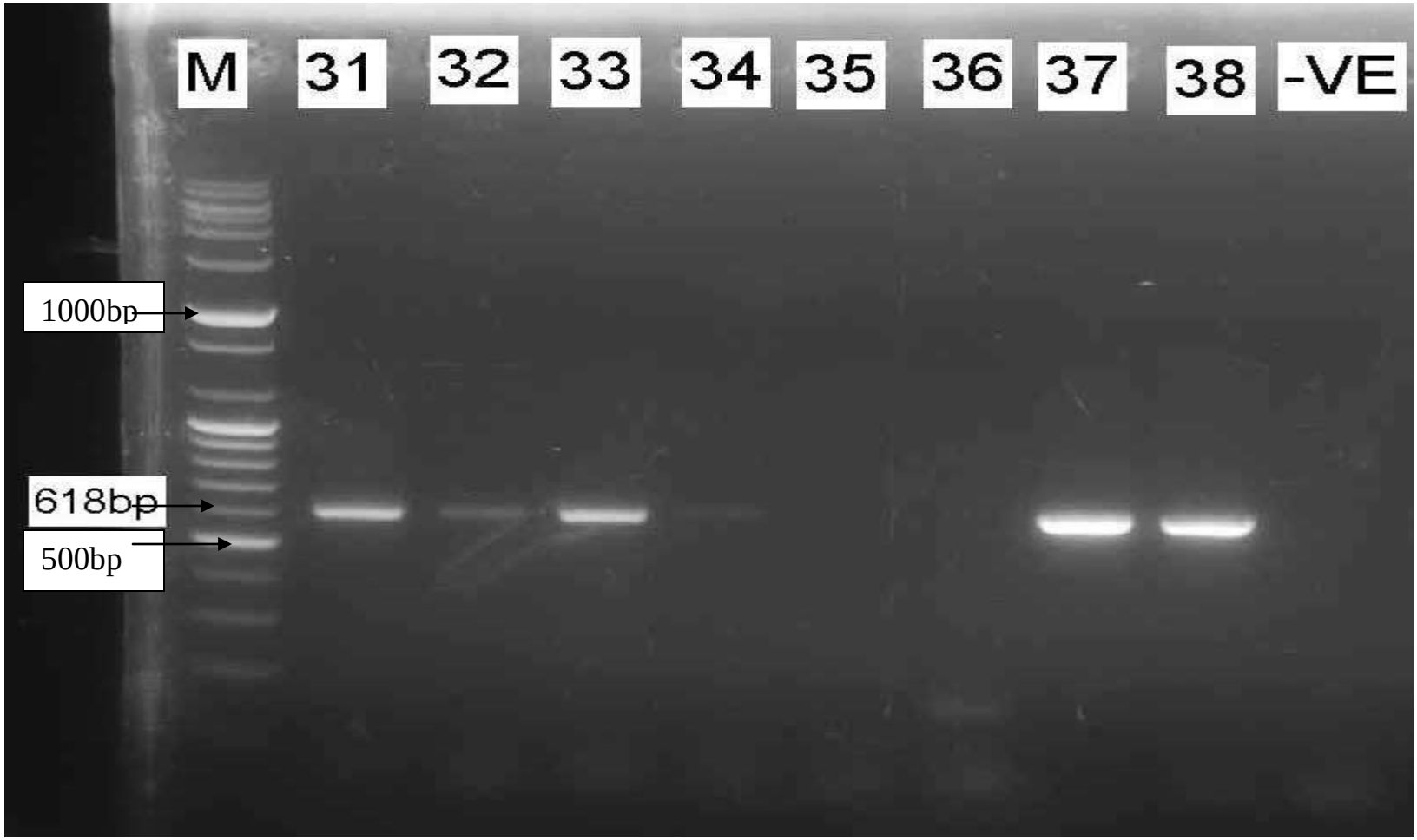

Figure 4.16: Gel electrophoresis of PCR products of 28S rDNA gene of *Fasciola spp* in snails; *Lymnaea natalensis* (Lanes 31-34) from Jos, *Pila ampullacea* (lanes 35-36) from Maiduguri, *Biompahalaria pfeifferei (*lane 37) and *Melanoides tuberculata* (lane 38) from Jos,Nigeria showing 618bp long PCR products, 100 bp DNA size marker (Lane M) and a negative control (–Ve).

4.20 Morphological and Molecular Identification of *Lymnaea natalensis*

Five representative samples in pools of 15-20 were identified as *Lymnaea natalensis* (Plate 4.6) using taxonomic keys. The 18S rDNA of *Lymnaea natalensis* was determined in five (5) Irepresentative samples of the identified *Lymnaea natalensis* grouped in pools of 15-20, by the use of conventional PCR. All the samples (lanes 16, 31-34) gave a 450bp band for *Lymnaea* spp (Figure 4.17).

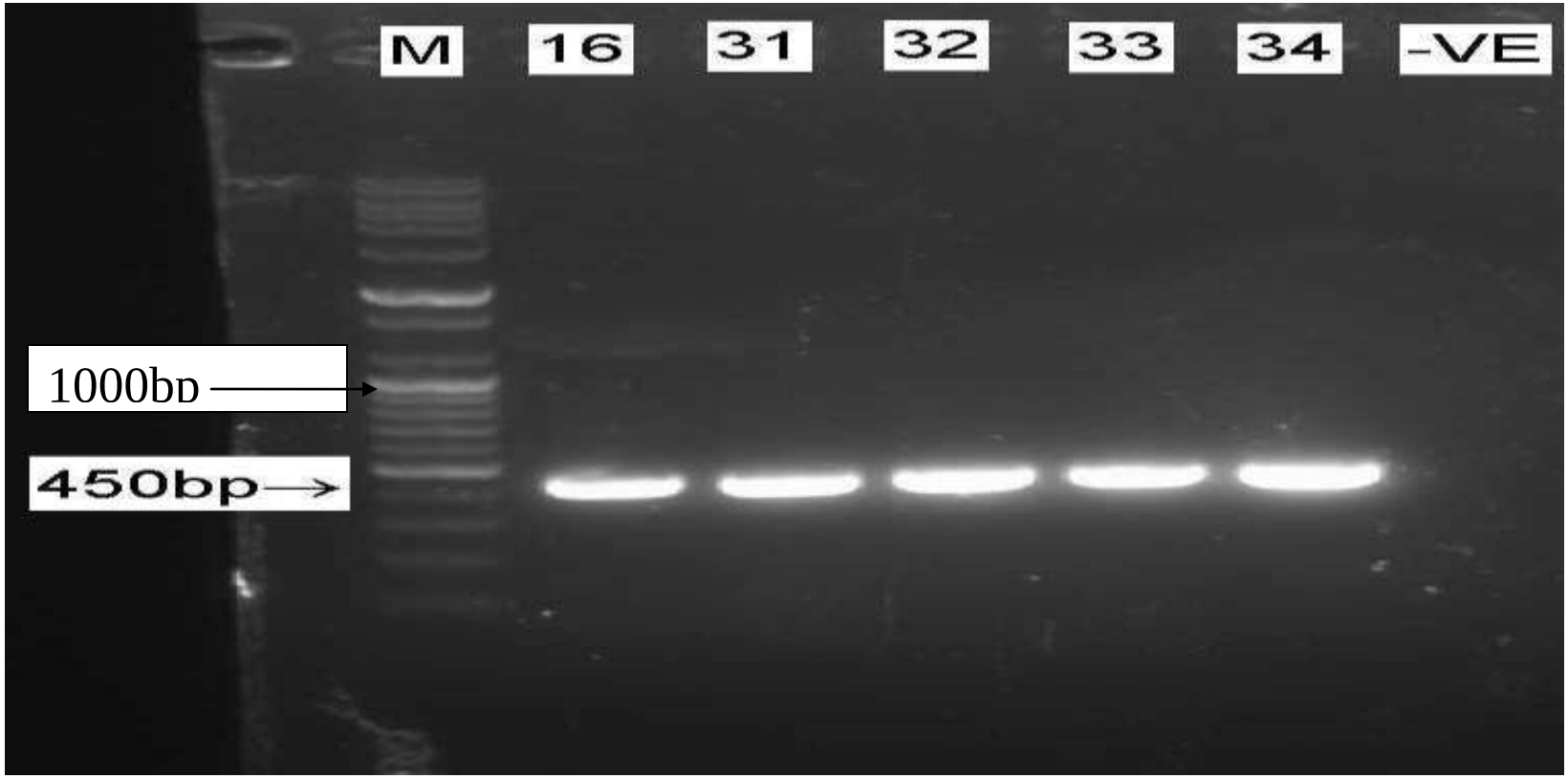

Figure 4.17: Gel electrophoresis of PCR products of 18S rDNA of *Lymnaea natalensis* from Gombe (Lanes 16) and Jos (lanes 31-34) showing 450bp long PCR products, 100 bp DNA size marker(Lane M) and a negative control (–Ve)

4.21 Results of DNA Sequencing and Alignment of *Radix natalensis*

4.21. 1 Single sequence alignment (SSA) of *Radix natalensis*

The chromatograph of the 18S rRNA gene of *Radix natalensis* is presented in Figure 4.18, while the aligned sequences from the NCBI Blastn search and as aligned in MEGA 6.06 in (Figure 4.19). Area in between the arrows (Figure 4.18) with prominent bars at the background represents good quality sequencing areas. This was obtained after areas of low quality sequencing and ambiguities were trimmed. The query sequence was 100% identical to a subject sequence, with 0% gaps in the 380 nucleotides compared (Figure 4.19). There was a low expect value (0.0) and no mismatch was also recorded.

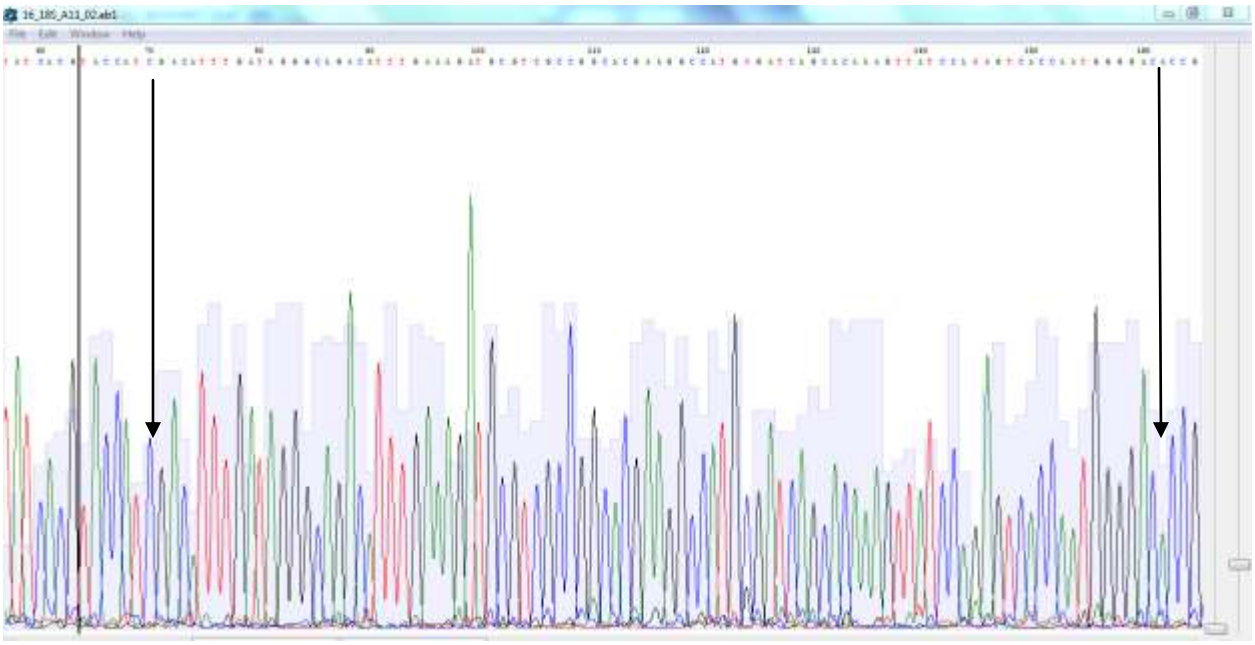

Figure 4.18: Chromatograph sequence of *Radix natalensis* (MF348191) as seen in ApE

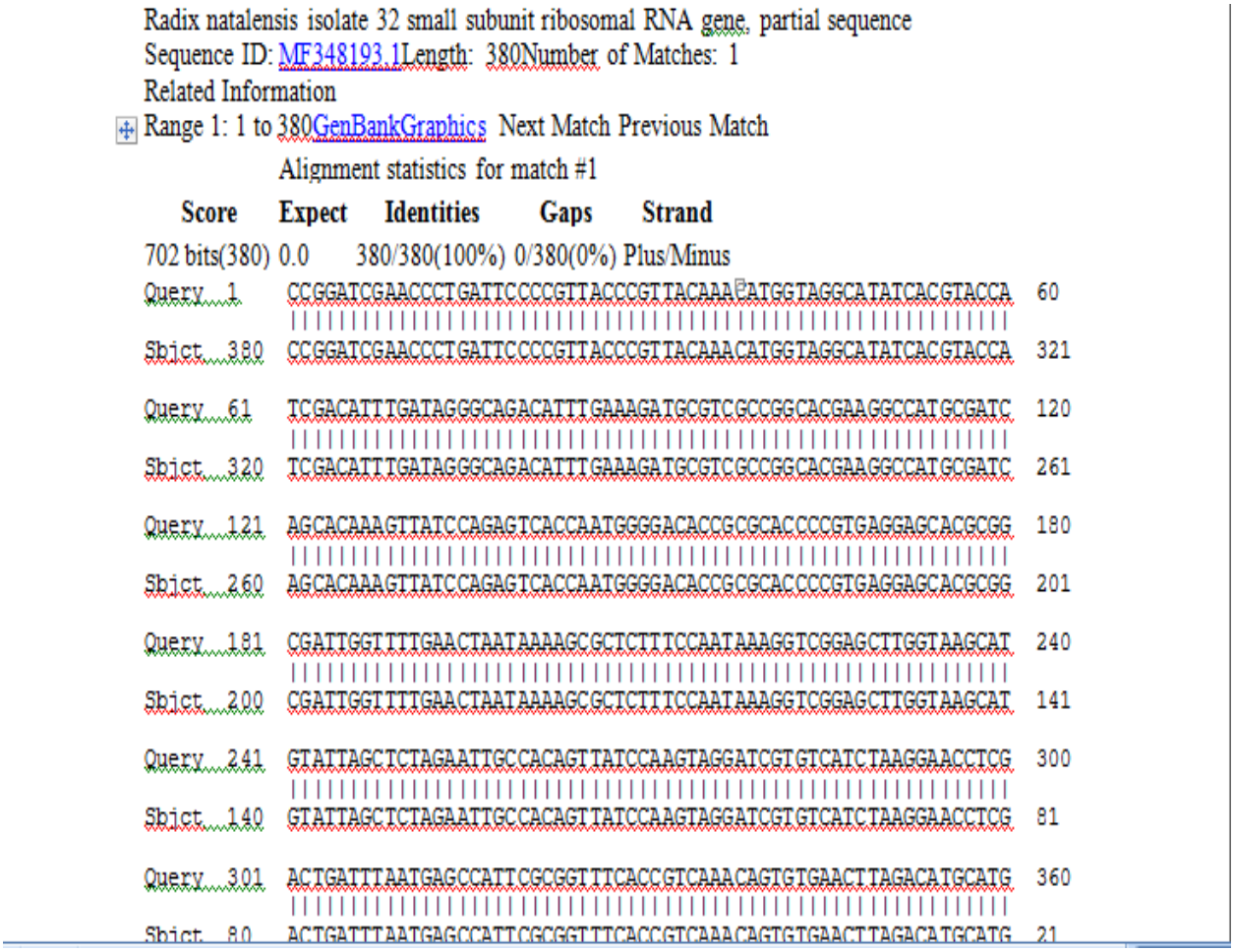

Figure 4.19: Aligned *Radix natalensis* (MF348191) from the NCBI Blastn search

4.21.2 Multiple sequence alignment (MSA) of *Lymnaea natalensis*

The result of the multiple sequence alignment of *Lymnaea (Radix) natalensis* is presented in Figure 4.20. The gaps represented by arrows, was common to subject sequences and that differentiated them other imported sequences from GenBank.

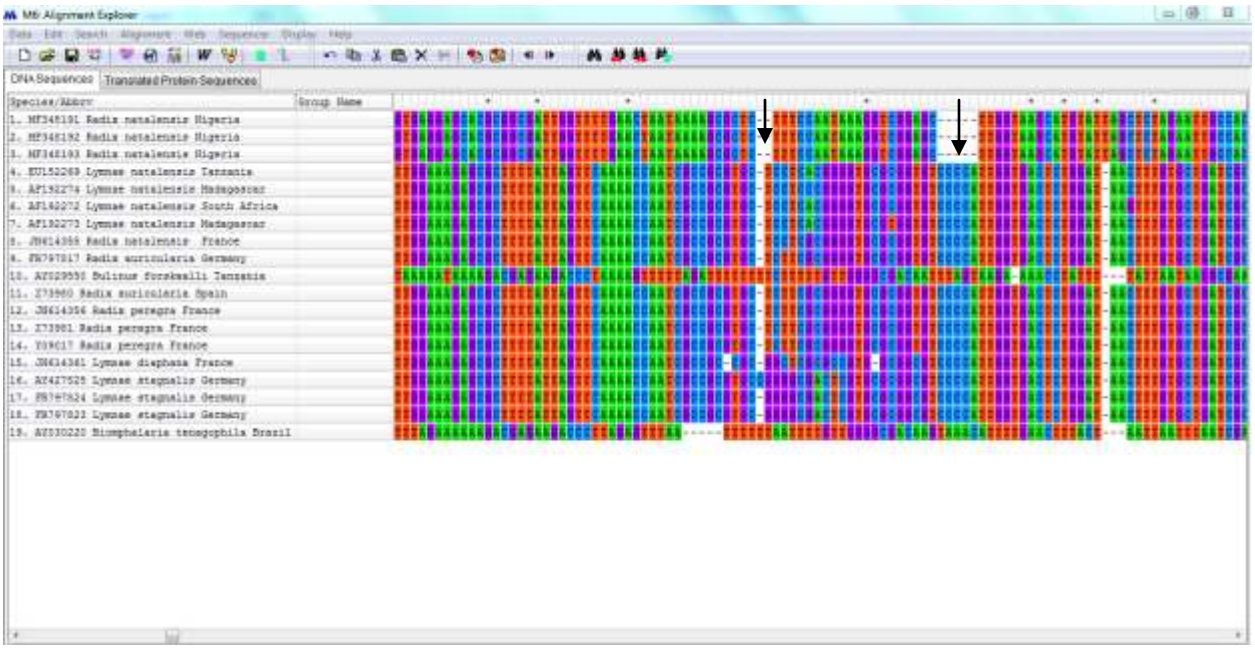

Figure 4.20: Result of multiple sequence alignment (MSA) of *Radix natalensis* (MF348191-348193) sequences along with similar sequences from GenBank in MEGA 6.06

4.22 GenBank Accession Numbers for *Radix natalensis* Sequences

The three (3) sequences submitted to GenBank for assignment of accession number were assigned the following numbers:

Seq1 [*Radix natalensis*) Isolate number 16 = MF348191

Seq2 [*Radix natalensis)* Isolate number 31= MF348192

Seq3 [*Radix natalensis*) Isolate number 32= MF348193

4.21 Results of Phylogenetic Analysis of *Lymnaea (Radix) natalensis* Sequences

The evolutionary relationship of *Radix natalensi*s sequences with other sequences is depicted in the phylogenetic tree below (Figure 4.21). Two different clades; one formed by our sequences and another by sequences from Africa and Europe are presented in the tree.

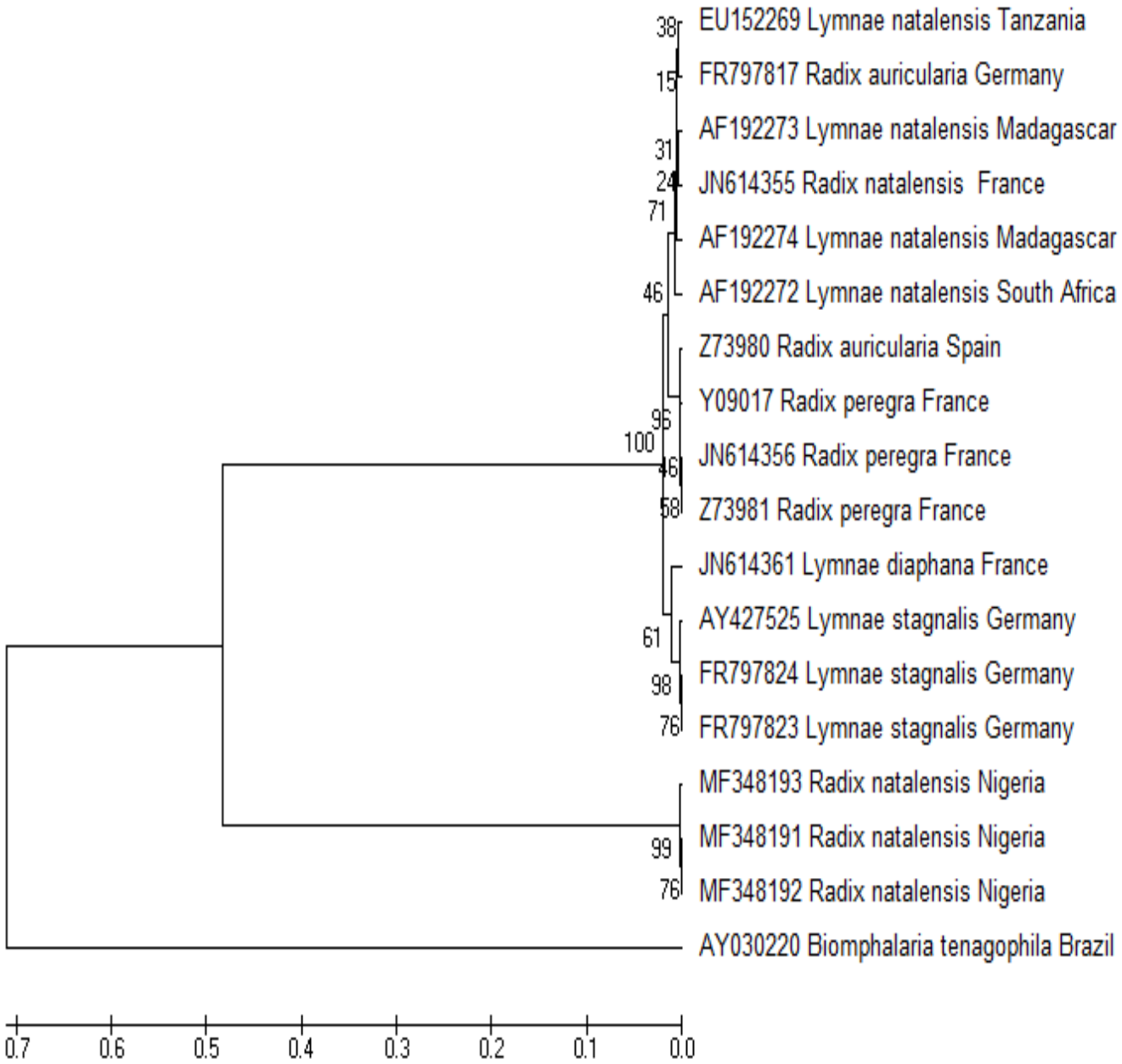

Figure 4.21: Phylogenetic relationship of *Radix natalensis* collected from Jos and Gombe and other similar isolates from different localities based on 18S rRNA gene construncted using UPGMA; *Biomphalaria tenagophila* served as outgroup

CHAPTER FIVE

DISCUSSION

The prevalence rate of *Fasciola* infection detected in cattle in this study is an indication that bovine fasciolosis is endemic in the study area. According to Gupta (2014), prevalence similar to our finding in cattle in this study is to be expected in regions endemic for *Fasciola* infection. However, the lower prevalence observed in sheep does not fit into the range. The overall prevalence in cattle is lower than reported by Karim *et al*. (2015) in Bangladesh, Greter *et al*. (2016) from eastern shores of Lake Chad and by Gordon *et al*. (2015) from the Phillippines, but similar to previous results obtained by Hussein and Khalifa (2010a) from Egypt, Magaji *et al.* (2014) from Sokoto, Nigeria, Adedokun *et al*. (2008b) in Ibadan, Nigeria and Tasawar *et al.* (2007) among goats in Pakistan. On the contrary, the previous results obtained by Aliyu *et al*. (2014) in Zaria, Nigeria, Omawaye *et al*. (2012) in Jos, Nigeria, Cringoli *et al*. (2002) in Southern Italian Apennines, Chakraborty and Prodhan (2015) in Bangladesh and Khan and Magbool (2012) from Pakistan fall below the values obtained in this study. Similarly, in sheep, the overall prevalence from the three locations (Maiduguri, Gombe and Jos) is far less than the previous report of Martinez-Valladares *et al*. (2013) in Spain, Hussein and Khalifa (2010a) in Egypt, Esonu (2014) in Zaria, Nigeria, Abdulhakim and Addis (2012) in Ethiopia, Mungube *et al*. (2012) in Kenya and Kanyari *et al*. (2009) in Kenya. The result of the present study in sheep is however similar to the report of Akhtar *et al*. (2012) in Dera Khan, Pakistan, Haleem *et al*. (2016) in Pakistan, Ahmed *et al*. (2007) in Ethiopia and Adediran *et al*. (2014) in Ibadan, Nigeria, but higher than the result of Bansal *et al*. (2015) in Mhow, Indore, India, Tramboo *et al*. (2015) in Kashmir valley, Pakistan and Nwosu and Srivastava (1993) in Maidugiri, Nigeria.

Kusiluka and Kambarage (1996) asserted that climate, management system, parasite, definitive and intermediate hosts of trematodes, each play an important role in the epidemiology of the diseases caused by trematodes, fasciolosis inclusive. The results obtained in cattle may be a reflection of the influence of these factors in the three agro-climatic regions, representative of the study locations. Jos, located in the northern Guinea Savannah, with higher rainfall and abundant water bodies had the highest infection rate, followed by Gombe, which is located in the Sudan Savannah. Maiduguri located in the Sahel with the least rainfall pattern, few water bodies and prolonged drier period, had the least infection rate.

The same variations in climatic conditions were demonstrated by Martinez-Valladares *et al*. (2013) to influence prevalence of *Fasciola* infection in sheep. Thus, factors such as vegetation cover, temperature, rainfall and soil moisture which influence the activity and abundance of the intermediate hosts of *Fasciola* (Khanjari *et al.,* 2014) may have varied degree of occurrence in the three agro-climatic zones, which in turn influence the development of the parasites from egg to miracidium, and may also be responsible for regional and annual variations in prevalence (Relf *et al.,* 2011). The recent report by Yatswako and Alhaji (2017) on fascioliosis burden in abattoirs of North-central, Nigeria agrees with the result obtained in this study, particularly for animals investigated in Jos, which is also within the North-Central Nigeria. However, the results from sheep did not wholly represent these agroclimatic divides, as nearly the same rates were recorded in Gombe and Maiduguri.

Furthermore, the pattern of distribution of prevalence based on the agro-climatic settings in this study differs from previous investigations conducted in the study areas based on coprology

(Nwosu and Srivastava, 1993; Omawaye *et al*., 2012; Pam *et al*., 2013) as higher count was recorded in Maiduguri and lower counts in Jos, compared to other previous studies. Local environmental factors such as the rainfall and the vegetation cover determine pasture availability and ultimatey management practice. The management practices in turn determine susceptible animals' exposure to the infective form of the parasite. In addition, temperature and indirect sunlight are necessary factors for cercarial release and may thus affect local prevalence based on their intensities.

Mas-Coma *et al.* (2009b) affirmed that higher temperature may enhance cercarial production due to increased host metabolic activity and the greater energy available to the parasite, in addition to markedly increased physiological processes associated with increasing temperatures in ectothermic animal like snails. In addition, Jos had a lot of abandoned ponds, pits and water pools arising from previous tin excavation activities on the Plateau. These might have provided ecological niches for the snail intermediate hosts of *Fasciola*.

The role of plant and plant products on helminth prevalence especially in animals having unrestricted access to natural pastures and fodders under extensive system of management cannot be totally negated. Azadiractin, an active principle present in *Azadiracta indica*, was previously shown to produce significant mortality *in vivo* and *in vitro* on rediae and cercariae of *Fasciola* spp. (Sunita and Singh, 2011), while absence of larval development characterised anti-fasciolicide action of lyophilized crude extract (CE) of *Momordica charantia* leaves and its sub-fractions on *Fasciola hepatica* eggs (Pereira *et al*., 2016). These and many thousands of plants in the wild could be playing a silent role in the trend of prevalence in some of the study locations.

The higher prevalence found in cattle than sheep had been previously demonstrated in other studies (Cringoli *et al*. 2002; Hussein and Khalifa, 2010a; Mungube *et al*. 2012), and may be due to the occurrence mainly of acute fasciolosis in sheep, thereby limiting the possibility of being slaughtered in abattoirs, since death often ensues before any sign could be noticed (Bhatia *et al*., 2006; Radostits *et al*., 2006). The larger size of the cattle liver, capable of providing feed and predilection site for *Fasciola* spp, has been proposed as likely reason for the chronicity of the infection in the species as against the rather small liver of sheep. Additionally, the widely-practiced system in the study areas of rearing cattle mainly under extensive system of managment as against sheep which are kept under semi-intensive system, might also have been responsible for the higher prevalence in cattle, since extensively reared animals have increased possibility of coming in contact with the infective stages of *Fasciola* spp.on pastures. On the other hand, the finding of higher infection in sheep than cattle is sometimes not uncommon as shown previously (Taye *et al.,* 2016), and may be related to local climatic conditions and husbandry system widely practiced. The results obtained from cattle aptly represented these agro-climatic regions.

The overall prevalence of infection showed significant variation between the ages in sheep, with higher infection among adults, but not in cattle.Results documenting higher prevalence among adult than young animals have been reported among cattle, sheep and goats from Ethiopia (Abdulhakim and Addis, 2012), sheep in Ethiopia (Ahmed *et al*., 2007), Sheep and cattle in Zaria, Nigeria (Aliyu *et al*., 2014; Esonu, 2014). However, reports of higher prevalence among younger animals are equally abundant as reported in sheep in Kenya (Mungube *et al*., 2012), goats in Pakistan (Tasawar *et al*., 2007) and cattle in Turkey (Gebeyehu *et al.*, 2014). The

prevalence obtained in this study, similar to that for endemic regions (Gupta, 2014) can be explained by the continued exposure of adult animals to contaminated pastures, which invariably increases their chances of acquiring the metacercariae of *Fasciola* spp. Young animals, however, especially during pre-weaning times, are normally confined, thereby reducing the likelihood of acquiring infection.

The prevalence of infection in this study based on breeds of cattle (Rahaji, Bunaji, crosses, Sokoto Gudali, Wadara, Kuri and Ambala) and sheep (Yankasa, Balami and WAD) examined showed no statistical significant variation with the exception of cattle from Gombe. This contrasts the earlier reports of Ahmed *et al.* (2007) in sheep in Ethiopia, Chakiso *et al*. (2014) in cattle in Ethiopia and Yatswako and Alhaji (2017) in cattle in Nigeria, where breed disposition to *Fasciola* infection was demonstrated, and this may be explained by appreciable relatedness in the genetic, physiological and immunological dispositions of the host animals as earlier elucidated (Yatswako and Alhaji, 2017).

In a similar manner to the present study, the months of sampling showed very significant variations in both species, with increased likelihood of acquisition of infection in the month of February (cattle) and January (sheep). The observation of increased prevalence during the months of January, February and March in cattle and January in sheep were similarly demonstrated in the cool dry period among sheep in Ethiopia (Ahmed *et al*., 2007), winter and spring among cattle in Egypt (Abdella and Nossair, 2014) and dry season (November-April) in sheep in Yola, Nigeria (Ardo and Aliyara, 2014). On the contrary, observation of higher prevalence in the rainy season was not uncommon as previously shown by Khanjari *et al.* (2014)

among small ruminants in Iraq, Gboeloh (2012) among cattle in Port-Harcourt, Nigeria and Karim *et al*. (2015) among cattle in Bangladesh. This observation may be justified by the period of abundance of the snail intermediate hosts and the timing of release of cercariae. It was previously shown that increased temperature causes an increase in metabolic and physiologic activities of ectothermic animals like snails (Mas-Coma *et al*., 2009b), and this may coincide with the period of increased activity of the snail. The cool dry period of November to April coincides with a period of absence of rain, and may be responsible for pastoralist rearing their animals around available water bodies because of the presence of lush pastures, thereby increasing exposure to the infective form of *Fasciola*.

The number of cattle positive by the bile sedimentation method was twice higher than by the faecal sedimentation technique. The results obtained by the two methods in sheep did not show any definite correlation, although the number of animals detected by faecal sedimentation was 1½ times higher than by the bile sedimentation. The prevalence obtained in cattle using faecal sedimentation is similar to the results of Affroze *et al*. (2013) and Sothoeun *et al*.(2006), Magaji *et al*. (2014) using the same techniques in Bangladesh, Cambodia and Nigeria respectively. It is however lower than the previous result obtained by Ntonifor and Ndaleh (2012) and Oke (2016) in Cameroun and Nigeria respectively, using faecal examination, but higher than that obtained using coprology by Abunna *et al*. (2010) in Ethiopia. Similarly, the higher prevalence by the bile sedimentation method is consistent with a previous finding in sheep (Shahzad *et al.*, 2012) and cattle (Adedokun *et al*., 2008a). The obtained result can be explained by the fact that, bile from the gall bladder, discharges eggs intermittenly into the gastrointestional tract. The finding of a higher prevalence by the faecal examination than by bile examination method in this study had

similarly been shown among cattle in Nigeria (Adedokun *et al*., 2008b). Although, not common, but this type of results may be found in low-infection statuses, where egg per gram/milliliter of faeces/bile is low. Despite the superiority of the bile detection method in cattle in the current study, it has limitation in clinical application because of diffculities in assaying the bile in live animals.The increased count in the months of March and April, with a decrease observed in subsequent months of the year in this study agrees with the previous finding of Sothoeun *et al*. (2006).

The body length of *Fasciola* isolates from cattle and sheep sampled from Jos and Maiduguri were similar to results obtained among *Fasciola gigantica* gravid adults in cattle from Burkina Faso, *Fasciola gigantica*-like gravid adults in cattle from Iran and *Fasciola gigantica*-like gravid adults in buffaloes from Iran, while isolates from Gombe appeared similar to *Fasciola gigantica*-like slightly gravid adults obtained from cattle in Iran (Ashrafi *et al*., 2006). The ranges reported by Hussein and Khalifa. (2010b) among *Fasciola gigantica* isolates from sheep, cattle and buffaloes from Qena Governorate, Egypt and the earlier finding by Shaldoum *et al.* (2015) on the body length of *Fasciola gigantica* collected from sheep, cattle and buffaloes in Egypt are equally similar to our results in this study, but contrary to the lower values reported by Biu *et al*. (2013) from Maiduguri, Nigeria.

The lack of significant variations in the body length of *Fasciola* isolates from the two species in this study disagrees with the earlier demonstration of differences in the length of *Fasciola gigantica* from sheep and buffaloes in Iran (Yakhchali *et al*., 2015b). The differences may be linked to the variation in the microhabitat, the bile duct. Furthermore, the body width of isolates from the three locations, although slightly lower in sheep, is in tandem with that recorded among

Fasciola gigantica isolates from cattle and buffaloes (Ashrafi *et al*., 2006; Shafie *et al*., 2014), but lower than measurements for *Fasciola hepatica* obtained from cattle, sheep and goats (Shafie *et al*.,2014). Thus, the isolates examined in the current study appear to be more closely related with *Fasciola gigantica* than *F. hepatica*. The cone width of the isolates from cattle in Jos and Maiduguri were similar, and differed very significantly from those recorded in isolates from Gombe. Shaldoum *et al*. (2015) reported a cone width among *Fasciola gigantica*, that was closely related to our findings in *Fasciola* isolates from cattle slaughtered in Maiduguri abattoir. In sheep, however, the cone width recorded in isolates from Jos was similar to the previous reports of Shaldoum *et al*. (2015) and Shafie *et al*. (2014) among *Fasciola hepatica* isolates from cattle, sheep, goats and buffaloes from Egypt and Iran. Contrarily, *Fasciola* isolates from cattle in Gombe and in sheep from Maiduguri had significantly higher cone widths than isolates from cattle in Jos and Maiduguri and in sheep from Jos. The underlining factor responsible for this variation is not fully known. However, ecological adaptation of parasites may partly explain this, as found in *Haemonchus contortus* (Rahman and Hamid, 2007; Kumsa *et al*., 2008). The cone length of *Fasciola* isolates from cattle in the three study areas and in sheep from Maiduguri did not vary significantly from each other, and is comparable to those for *Fasciola gigantica* isolates from sheep, cattle and buffaloes from Egypt (Shaldoum *et al*., 2015), *Fasciola gigantica* isolates in cattle from Zimbabwe (Chauke *et al*., 2014) and from Southwest Iran (Shafiae *et al*., 2014), but longer than for *Fasciola hepatica* isolated from cattle, sheep and goats from Southwest Iran. The cone length may thus play a significant role in differentiating between *Fasciola* species.

The distance between the ventral sucker (VS) and union of vitelline gland (Vs-Vit) and the distance between the ventral sucker and the posterior end of the body (VS-P) for all isolates

irrespective of locations and species were within the range reported by Ashrafi *et al.* (2006) for *Fasciola gigantica* adult parasites from Burkina Faso, Bolivia, Iran, but higher than values for *Fasciola hepatica* isolates from cattle, sheep and goats from Southwest Iran (Shafiae *et al*., 2014). Similalrly, the (VS-P) from both species were similar to values obtained among isolates from cattle in Iran (Yakhchali *et al*., 2015b). In addition, the obtained results in this study were higher than the previous report for *Fasciola hepatica* in cattle, water buffaloes and sheep, and intermediate *Fasciola* in cattle, but lower than values reported for *Fasciola gigantica* and intermediate *Fasciola* isolates collected from sheep and water buffaloes from Iran (Yakhchali and Bahramnejad, 2015).These variations may be linked to the type of final host, the immunity of final host, the intermediate host and the varied genealogy factors (Dalton, 1999; as cited in Yakhchali and Bahramnejad, 2015).

The previously reported BL/BW ratio for *Fasciola gigantica* isolates from cattle, sheep and buffaloes in Egypt (Shaldoum *et al*., 2015) and for *Fasciola gigantica* isolated from cattle in Iran (Shafiae *et al*.,2014) closely resembled values obtained among isolates collected from cattle in Jos and Maiduguri, but not Gombe. In all, the BL/BW ratio can serve complementary role to other linear biometric parameters in the differentiation of the two common species of *Fasciola*. For all the linear biometric parameters studied, the body length, body width, the cone width, BL/BW ratio and CL/CW showed the greatest variations, particularly among isolates from cattle and to a lesser extent, sheep. This observation agrees with the earlier report where the body length, BL/BW ratio and the distance between the ventral sucker and the posterior end of the body (VS-P) successfully distinguished between the two main species among isolates from Egypt (Periago *et al*.,2008; as cited in Yakhchali *et al*.,2015b), and also with the previous

proposal of Mas-Coma *et al*. (2009a) on research activities involving *Fasciola* spp from Africa and Asia, where he recommended a minimum morphometrical study of at least body length, maximum BW, BL/BW ratio, VS-P distance and Vit-P distance of adult flukes should be adopted in genetic characterisation papers.

The results of amplification of ITS 1 in *Fasciola* isolates and snails appeared similar to the previous observation of Reaghi *et al*. (2016). However, longer fragment for the same ITS 1 was reported by Bozorgomid *et al*. (2016) among *Fasciola* isolates from sheep, cattle and goats in Iran. In addition, positive samples, for which 28S rDNA was amplified, showed a fragment similar to the previous observation of Marcilla *et al*. (2002) in *Fasciola hepatica* and *Fasciola gigantica* isolates.Thus, the *Fasciola* DNA had been successfully demonstrated in *Fasciola* isolates and snails using the two genetic markers. Similarly, the mitochondrial gene; NADH dehydrogenase subunit 4 (NAD4) was successfully amplified through the use of separate primers for each species. Apart from the genes employed in this study, others such as the nuclear marker, *Phosphoenolpyruvate carboxykinase (pepck), cytochrome C oxidase* and the mitochondrial genes; NADH dehydrogenase subunit 1 (*nad1*) and cytochrome c oxidase 1 (*cox1*) had been applied in the detection of *Fasciola gigantica* in the first report of molecular characterisation of *Fasciola* species from Nigeria (Ichikawa-Seki *et al*., 2017b). The employment of three genes for the detection of *Fasciola* DNA in this study agrees with the suggestion of Mas-Coma *et al*. (2009a) for researches on evolutionary genetics and molecular epidemiology of fascioliasis in Africa and Asia, where the two species of *Fasciola* have been known to overlap. Furthermore, to our knowledge, this study is the second attempt at molecular characterisation of *Fasciola* spp from Nigeria, apart from the previous study of Ichikawa-Seki *et al*. (2017b) and the first on

isolates from sheep and in snails. In this study, obtained sequences revealed the existence of *Fasciola gigantica* and *Fasciola hepatica*.

The molecular characterisation of *Fasciola gigantica* in Nigeria was first demonstrated by Ichikawa-Seki *et al*., (2017b). However, to the best of our knowledge, this is the first molecular evidence for the presence of *Fasciola hepatica* in Nigeria. Furthermore, results of the sequencing indicated that most of them are those of *Fasciola gigantica*. This observation is similar to the result obtained by Ali *et al*. (2008) in the first genetic characterisation of *Fasciola species* from Niger Republic. Blastn searches within the NCBI database revealed similarity of 86% for *Fasciola hepatica* and between 95-98% for *Fasciola gigantica*, with deposited sequences in the GenBank database from African countries of Burkina Faso, Egypt and from China, Iran and India. The relative similarities observed is a reflection of the genes investigated. The ITS 1 is a highly variable gene, while the 28S rRNA is conserved gene. However, the mitochondrial genes do not possess the needed properties for comparsion because of their relatively short length (Mas-Coma *et al*., 2009a).

The ancestral relationship of our sequences as shown on the phylogenetic tree presented three clades and four subclades. One *Fasciola gigantica* from Iran (JN811689) formed a distinct clade alone, almost similar to the out group, separate from others. The sequences of *Fasciola gigantica* obtained in this study belong to a separate clade, with the subclade clearly showing the clustering of sequences Number 25 and 26 into a distinct subclade, while sequence 37(*Fasciola hepatica*) formed a clade with a *Fasciola hepatica* (LC126261) sequence from Iran giving 99% homology. The second clade consisted of all *Fasciola gigantica* sequences from African countries of Egypt,

South Africa, and Burkina Faso and also China.The only ITS sequence (sequence 3) of *Fasciola gigantica* also belongs to a distinct subclade under this clade.

The clustering of *Fasciola gigantica* from Nigeria with those from other African countries like Egypt agrees with the findings of Ichikawa-Seki *et al*.(2017b) using *cox*1 and *nad* 1 genes.This may indicate the ancestral origin of the species, which has been traced to probably warm, eastern Africa, where the Lymnaeaid snail *Radix natalensis* assured the transmission, with the origin probably due to its adaptation to ancestors such as of Alcelaphinae, Reduncinae and Bovinae, during the second pecoran episode (Mas-Coma, 2015). Furthermore, the clade formed by the only *Fasciola hepatica* sequence determined using 28S rDNA, with another from Iran, probably indicate the Eurasian near-East origin of *Fasciola hepatica*. Results of the phylogenetic relationship study and of infection pattern by these two species reflects this origin elucidated (Mas-Coma, 2015).

The collected and morphologically identified snails consisted of *Limicolaria flammea, Limicolaria kambeul, Limicolaria turris, Biomphalaria pfeifferei, Pila ampullacea*, *Lymnaea* spp, *Melanoides tuberculata* and an unidentified spp. The earlier taxonomic keys of Mandahl-Barth (1962) and Brown (2005) identified the snails. While the ability of *Lymnaea* spp to transmit *Fasciola* is known, that cannot be said of the other snails identified in this study. However, parasites known to be transmitted by the identified species consisted of *Limicolaria flammea* and *Limicolaria aurora* serving as intermediate hosts of *Dicrocoelium dendriticum* and *Alaria* spp (Igbinosa *et al*., 2016), while *Biomphalaria* spp transmits *Schistosoma mansoni* (Rowel *et al*., 2015). Additionally, *Centrocestus formosanus, Philophthalmus gralli* and other

parasite species belonging to the families Heterophyiidae, Echinostomatidae, Schistosomatidae; Plagiorchiidae and Philophtalmidae were demonstrated to be transmistted by *Melanoides tuberculata* under natural condition (Farahnak *et al*., 2006; Pinto and Melo, 2010; Paula-Andrade *et al*., 2012), while *Pila ampullacea* was reported to transmit *Gnathosoma spinigerum* and *Echinostoma ilocanum* as paratenic and definitive hosts respectively (Komalamisra *et al*.,2009; Sri-aroon and Richter, 2012). The fact that most of the identified snails serve to transmit one parasite or the other, means that they may transmit parasites other than the described if fully studied. Also, the recent demonstration of the sporocyts of *Fasciola gigantic*a in *Achatina fulica*, a snail not known to transmit the parasite (Igbinosa *et al*., 2016), may mean that other snails than *Lymnaea* spp may serve to transmit *Fasciola* spp.

The molecular analysis of the 18S rDNA of *Lymnaea natalensis* resulted in a PCR fragment, similar to the observations made by Stothard *et al*. (2000) and Howell *et al*. (2012). The obtained result also agrees with the previous report of Bargues and Mas-Coma (1997), where 18S rDNA was described as an excellent marker for species determination and differentiation among Lymnaeaidae. Other genes such as the ITS-2, ITS-1, mitochondrial DNA 16S and Cox1 and inter simple sequence repeat (ISSR) have successfully identified members of the family Lymnaeaidae (Bargues *et al*., 2012; El-Khayat *et al*., 2015). Our study, the first molecular characterisation of *Lymnaea (Radix)* in Nigeria, to the best of our knowledge, has successfully identified the three sequences as *Radix natalenisis*. This further confirmed the existence of the species within the study location. Blastn search in NCBI revealed that the three sequences had between 99-100% similarities with database sequences from African countries of South Africa, Madagascar and Tanzania and varied significant similarities with other sequences from *Lymnaea species* from

Europe and other parts of Africa deposited in the GenBank.The 18S ribosomal gene is highly conserved and may be responsible for the high level of similarity seen in the obtained sequences. The identity of our sequences based on Blastn search and the clades and sub-clades formation, following phylogenetic analysis lends credence to the earlier report by Brown (2005) to the fact that *Lymnaea natalensis* may be the major, if not the only vector host for the transmission of *Fasciola spp* in large areas of Africa, especially West Africa. However, reports of other *Lymnaea species* such as *Lymnaea truncatula,* a snail host known to transmit *Fasciola hepatica* from Uganda and probably other parts of Eastern and Southern Africa abound (Howell *et al*., 2012). All the other sequences imported from GenBank clustered into a distinct clade, similar to the previously decribed *Radix (Lymnaea)* clade (Correa *et al*., 2010), although only species belonging to the *Radix (Lymnaea)* were used in this study. From the observed clade and with respect to geographic origin of *Radix spp*, the earlier suggestion of Correa *et al*. (2010) for transfer of *Radix natalensis* from the Indopacific clade to the European and North America clades is hereby sustained. This is in view of the clade formed by most European *Radix spp* with the African species (*Radix natalensis*). The separate clade formation by our sequences in this study possibly reflects the effect of local geographic factors on the snails over a considerable period of time.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The study had shown variations in the prevalence of *Fasciola* spp infection in the study locations and such prevalence can be of great challenge to economic output of the production animals.

2. The study showed a direct correlation between prevalence and egg counts in bile and faeces from the study area. That cannot be said of sheep, where higher prevalence and lower egg count was detected by faecal sedimentation and lower prevalence and higher egg count detected by bile sedimentation.

3. *Fasciola* spp is been characterised for the second time from Nigeria and molecular evidences supports the existence of *Fasciola hepatica,* the first to be reported from the study areas.

4. Molecular evidences from our study support the existence of *Lymnaea (Radix) natalensis* from the locations. This is the first molecular characterisation of this species from Nigeria. Similarly, there are evidences that snails such as *Melanoides tuberculata* and *Biomphaaria* spp, which are not known intermediate hosts of *Fasciola* spp, may be important in the transmission, with the demonstration of the DNA of *Fasciola* within the snails in this study.

6.2 Recommedations

We recommend as follows from the results of this study;

1. More studies from different locations should be done in order to determine the exact relationship between egg counts and prevalence especially in sheep. Factors determining the release of bile from the duct into the intestine should be studied.

2. Experimental infection using different breeds should be done to further ascertain variation in susceptibility of the various breeds of cattle and sheep in Nigeria.

3. Further mophometry of *Fasciola* spp should be conducted in order to generate data that can serve complimentary role in the genetic characterisation of the species.

4. Complete sequence of the respective genes should be done with *Fasciola* and snail samples from the study locations and beyond.

5. Snails sharing similar ecological sites with *Radix natalensis*, such as *Melanoides tuberculata* and *Biomphalaria pfeifferei* should be investigated for possible role in transmission of *Fasciola* spp through detection of *Fasciola* DNA. Such snails may need to be collected from sites not shared with *Radix natalensis.*

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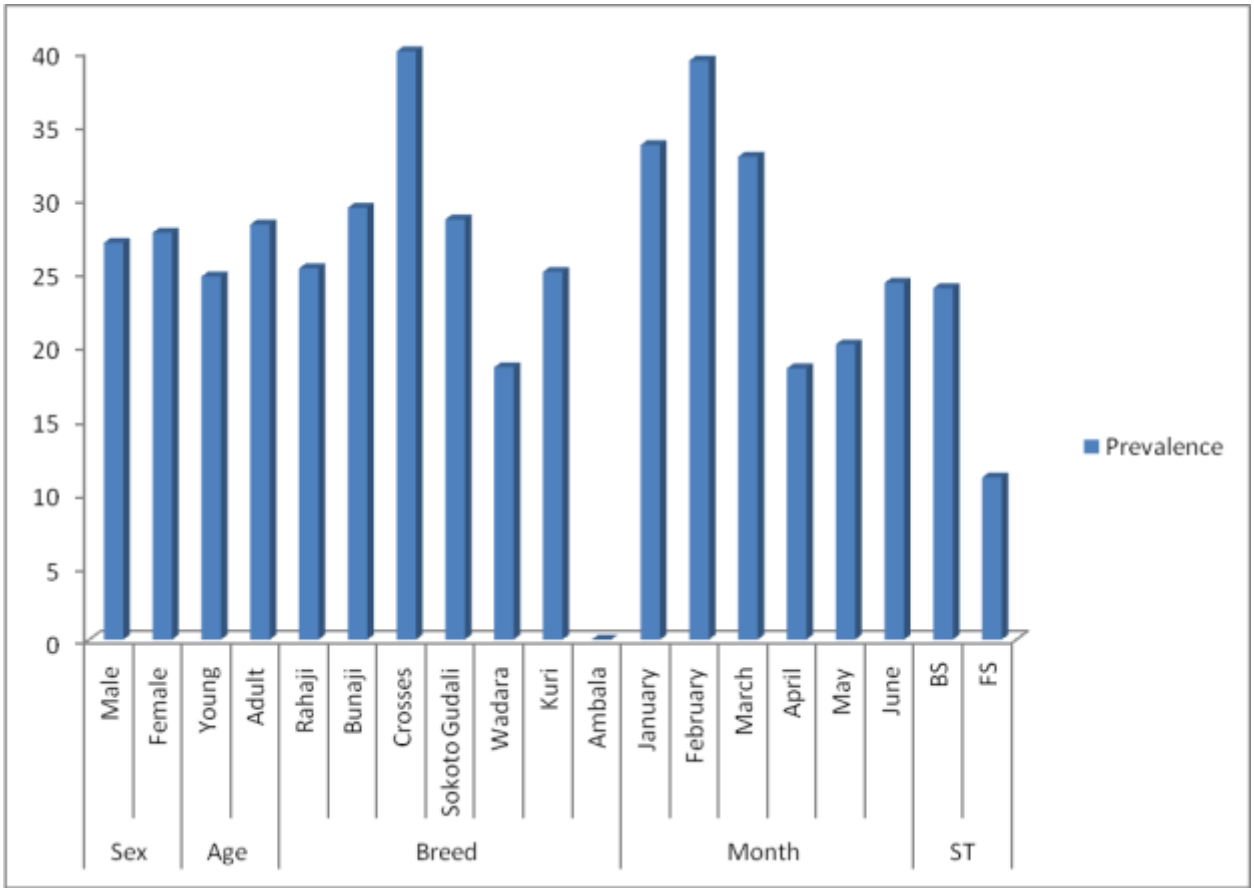
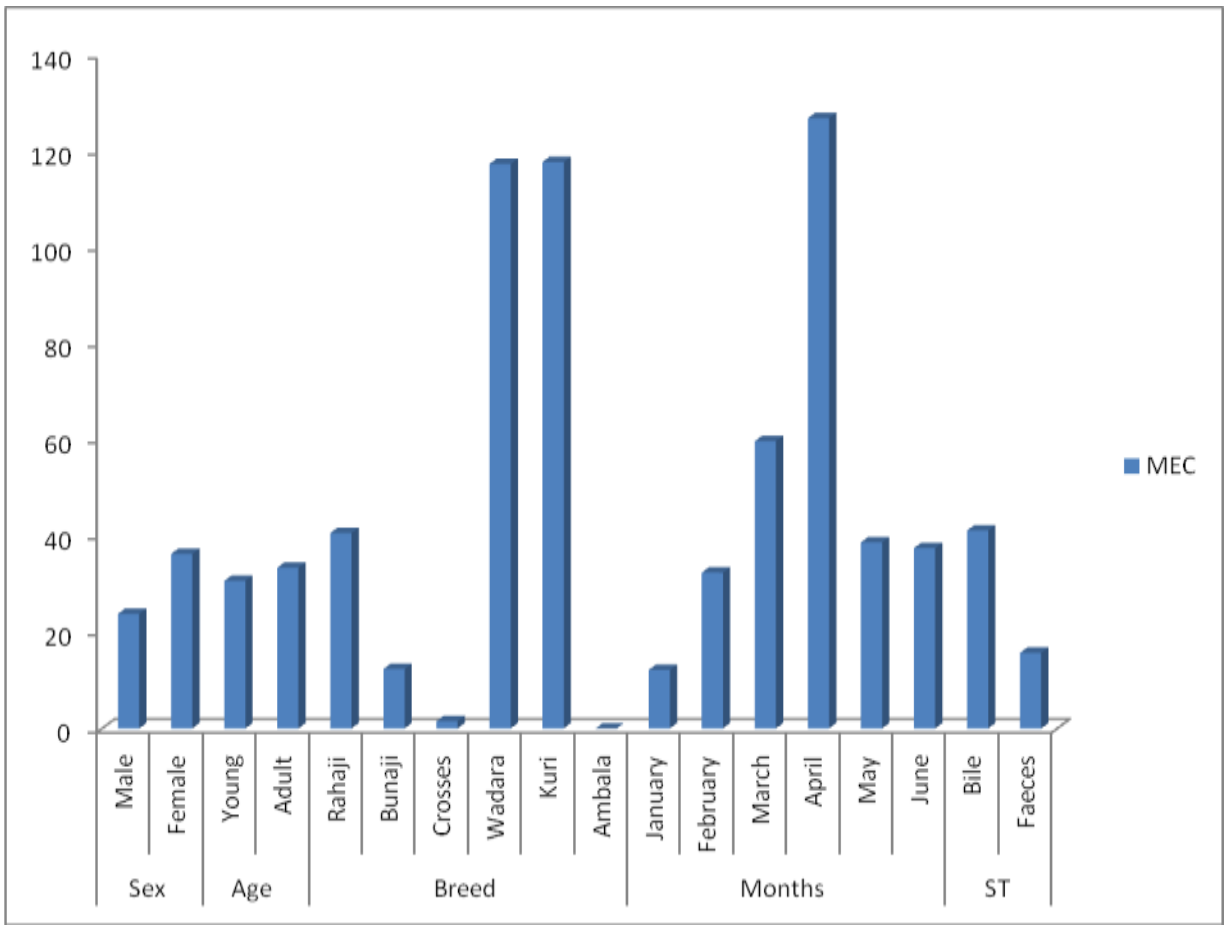
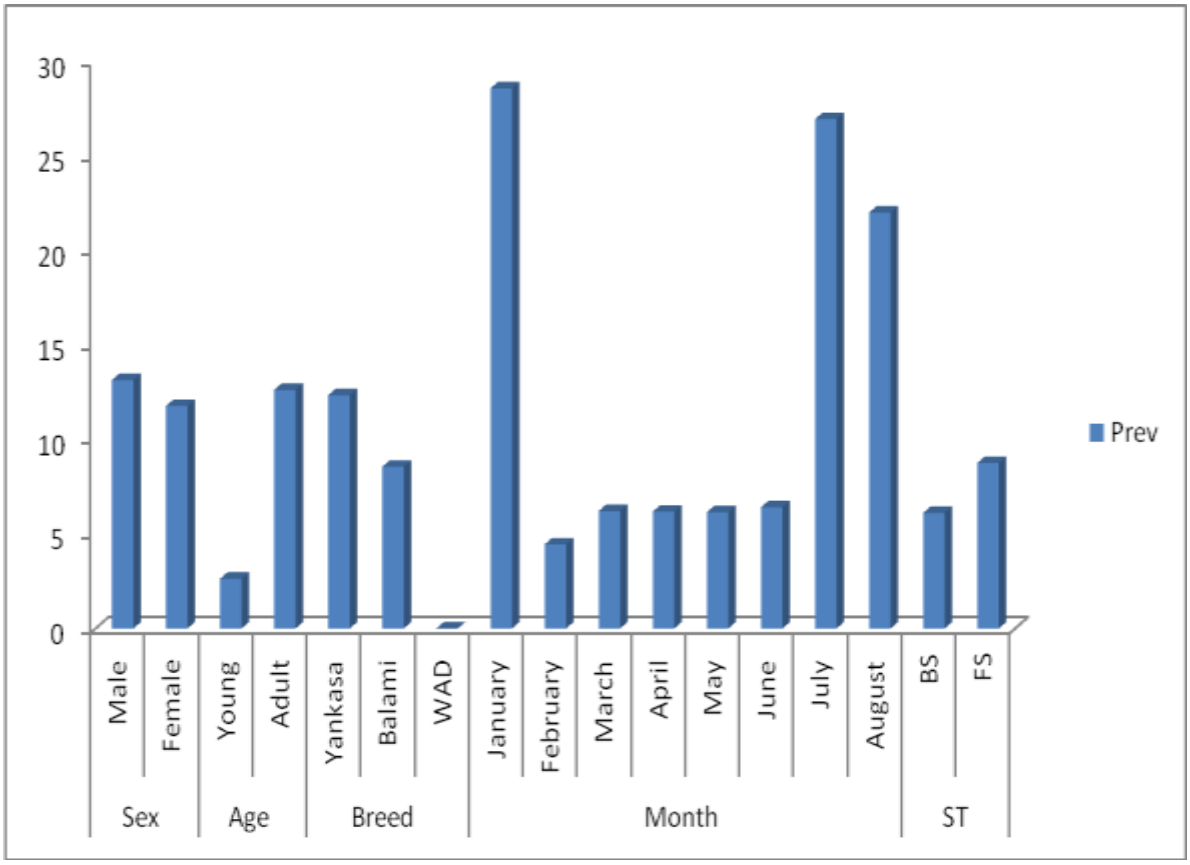
APPENDICES



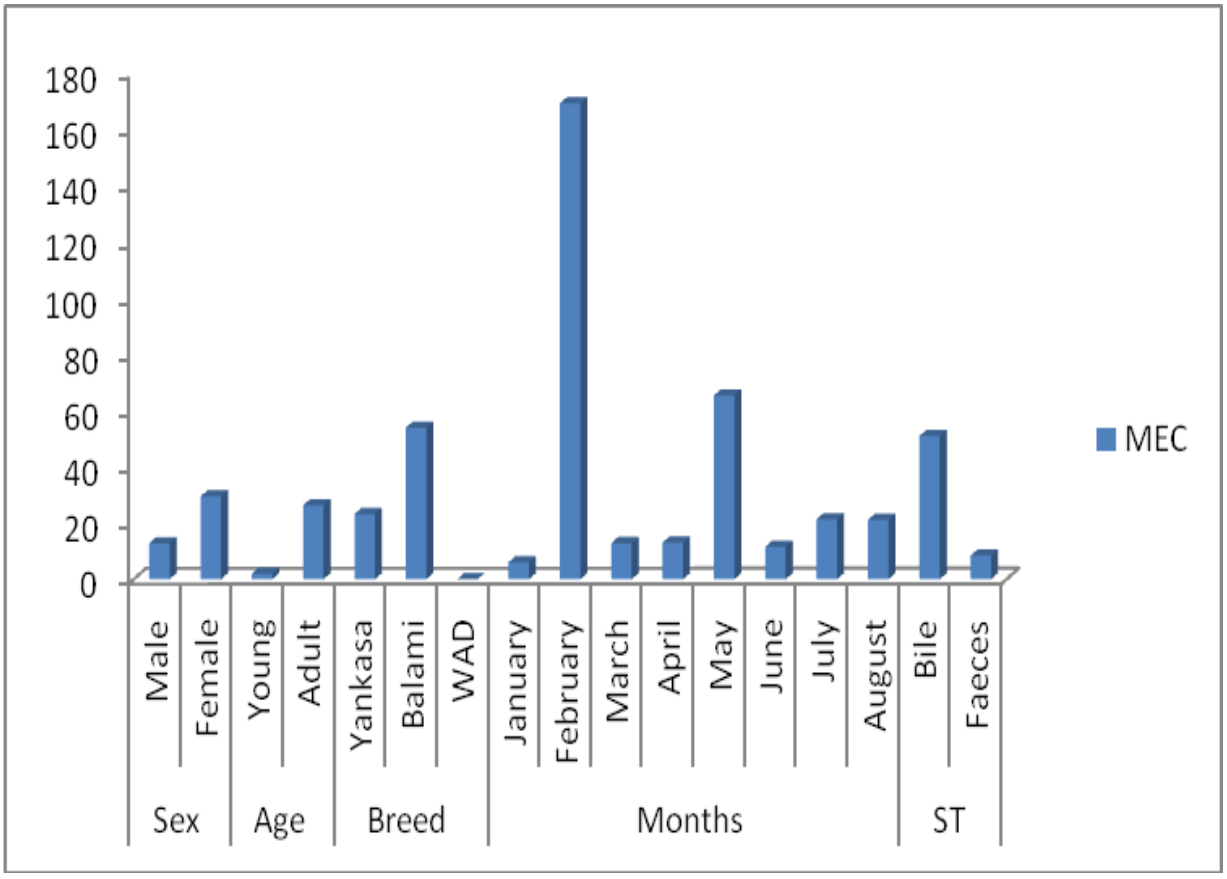
Appendix 1: Overall prevalence of *Fasciola* spp eggs in faeces and bile of slaughtered cattle from Maiduguri, Gombe and Jos abattoirs



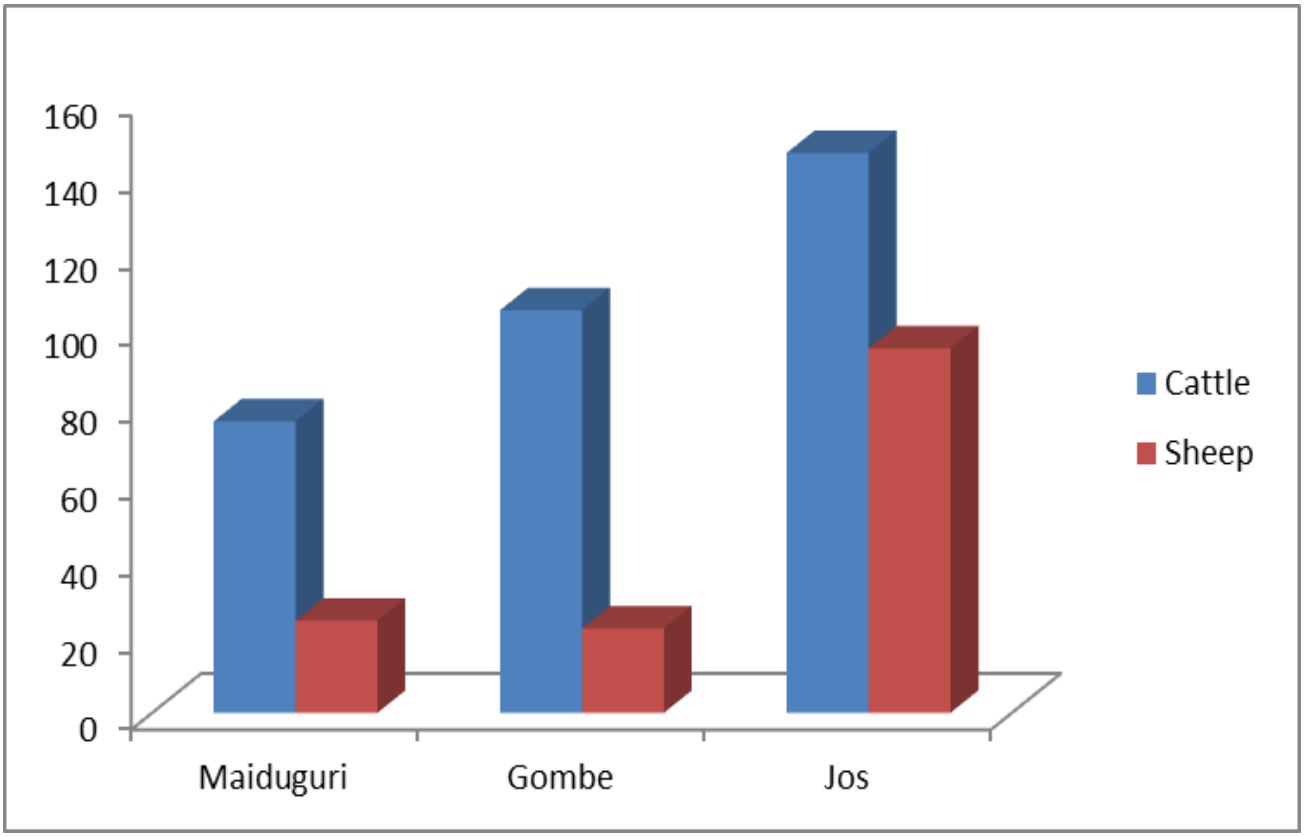
Appendix 2: Mean *Fasciola* egg count of slaughtered cattle from Maiduguri, Gombe and Jos abattoirs



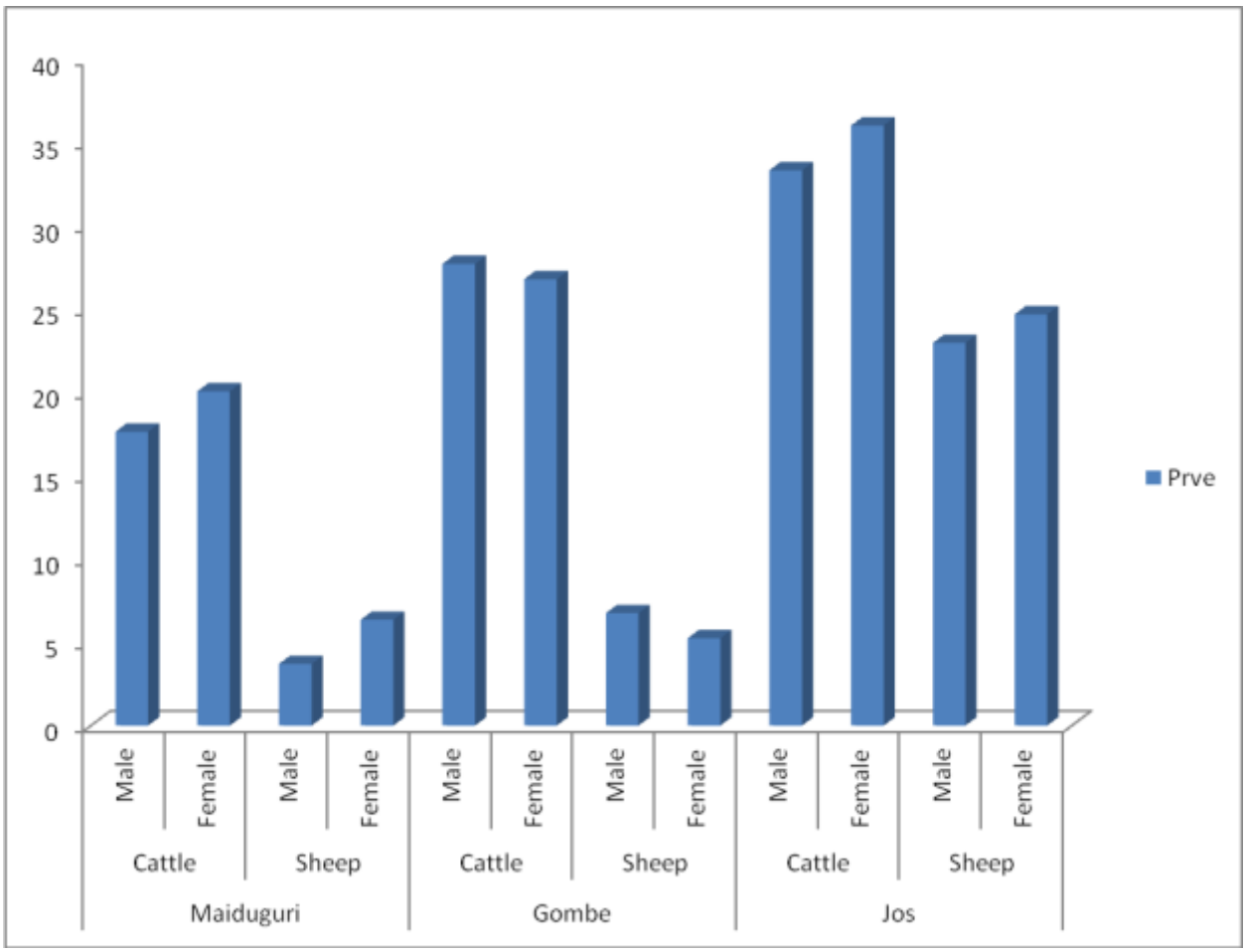
Appendix 3: Overall prevalence of *Fasciola* spp eggs in faeces and bile of slaughtered sheep from Maiduguri, Gombe and Jos abattoirs.



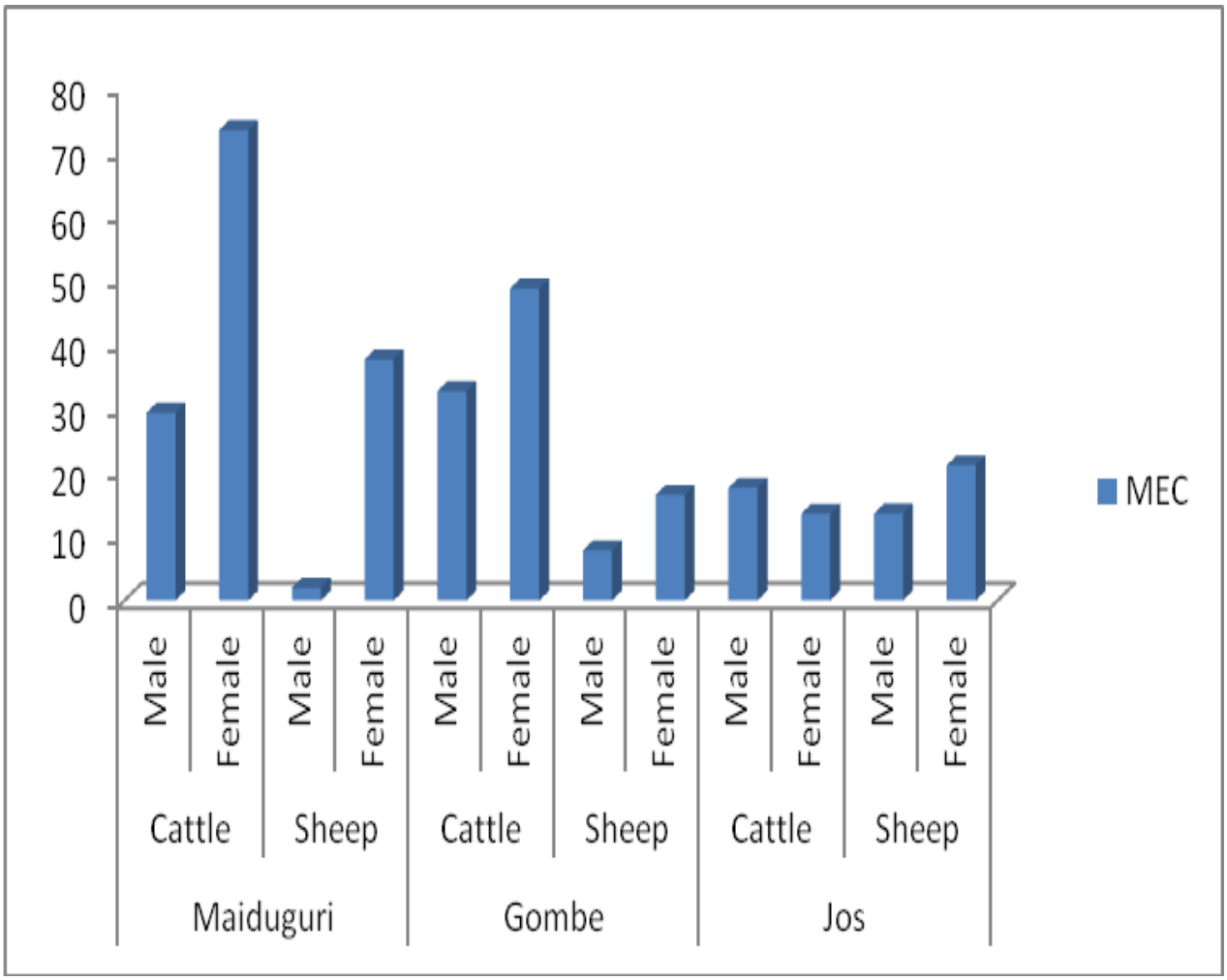
Appendix 4: Mean *Fasciola* egg count of slaughtered sheep from Maiduguri, Gombe and Jos abattoirs



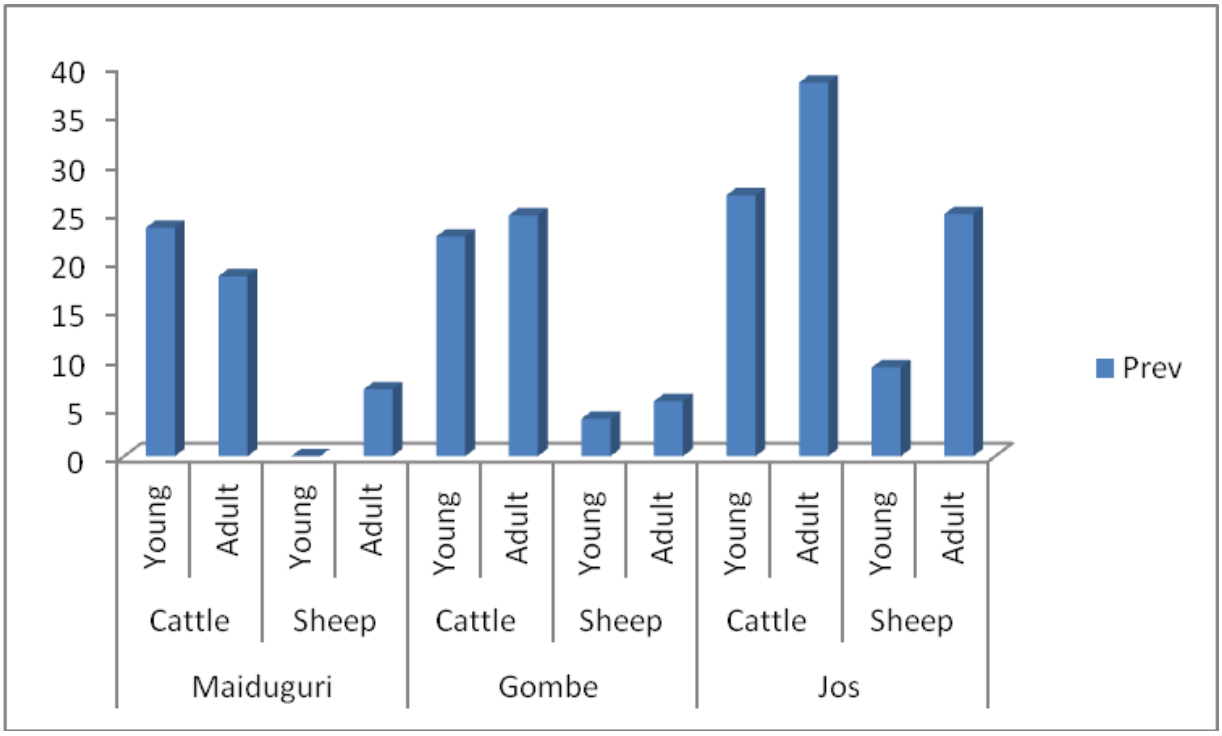
Appendix 5: Prevalence of *Fasciola* spp eggs in faeces and bile of slaughtered cattle and sheep based on location.



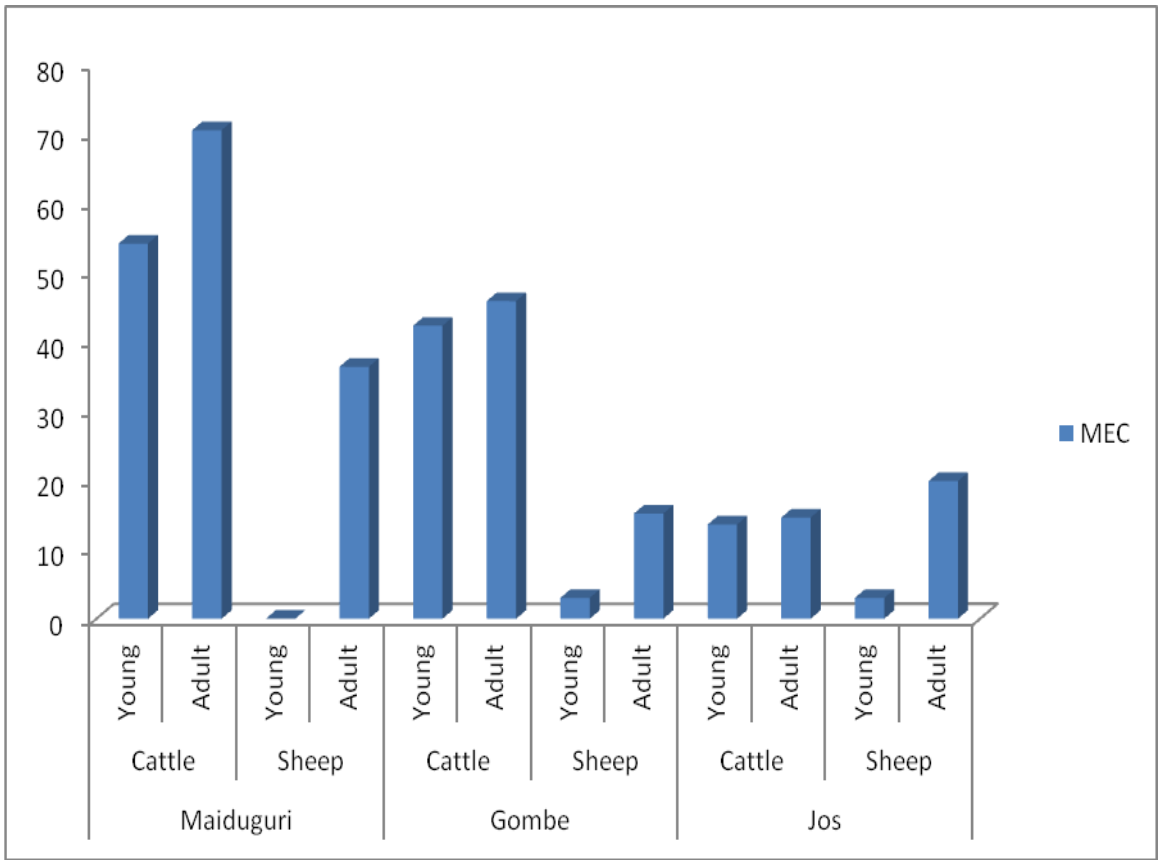
Appendix 6: Prevalence of *Fasciola* spp eggs in faeces and bile of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to sex.



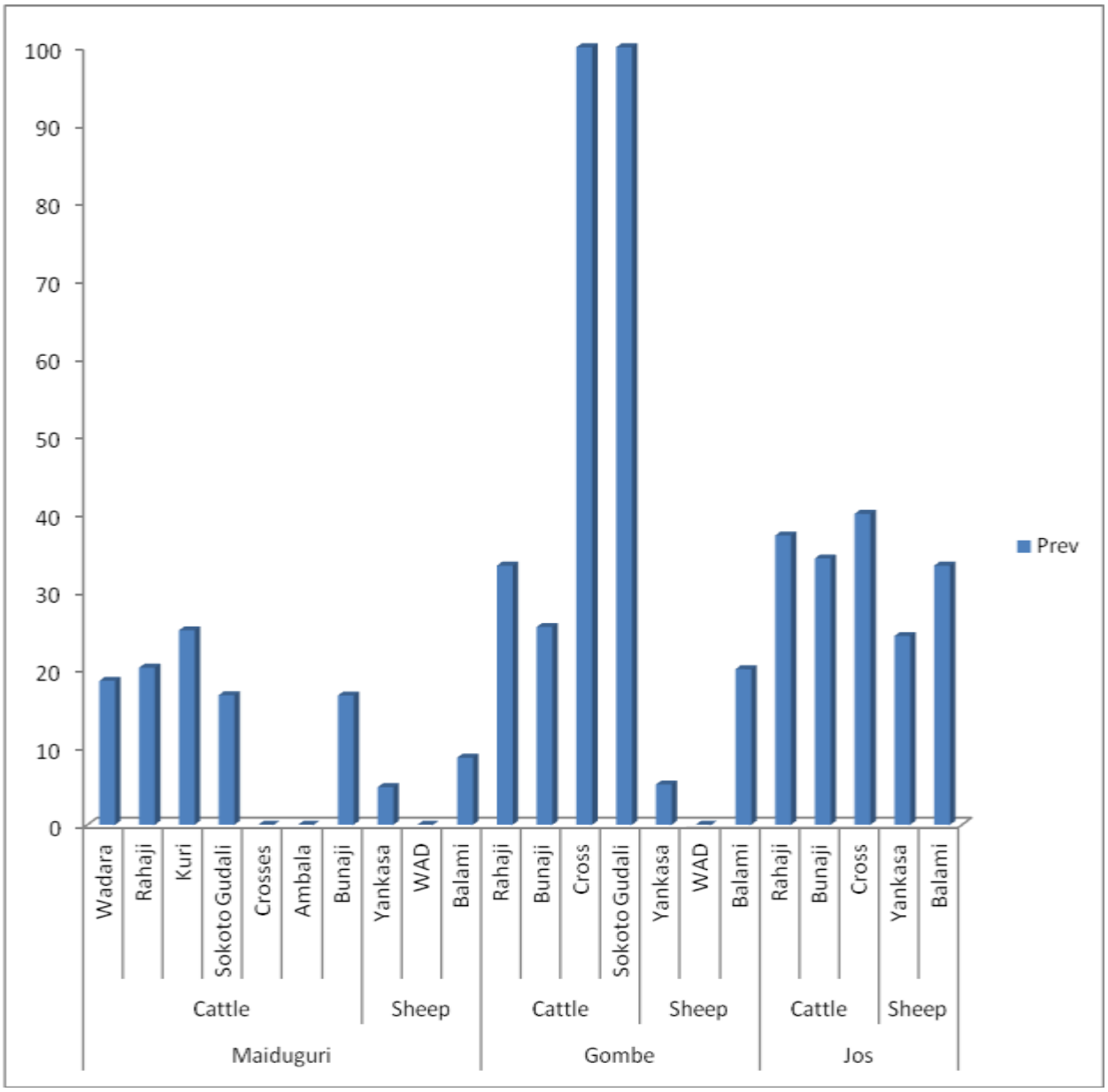
Appendix 7: Mean *Fasciola* egg count of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to sex.



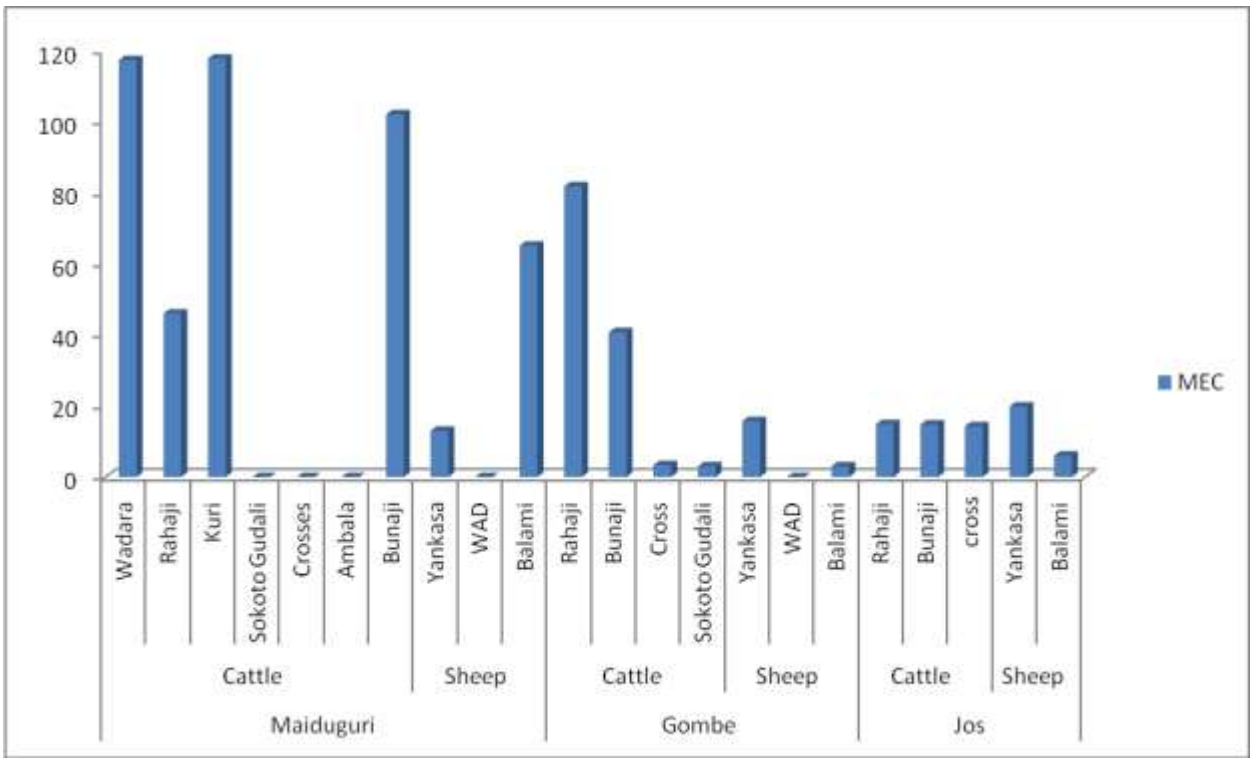
Appendix 8: Prevalence of *Fasciola* spp eggs in faeces and bile of slaughtered cattle and sheep from Maiduguri, Gombe and Jos in relation to age.



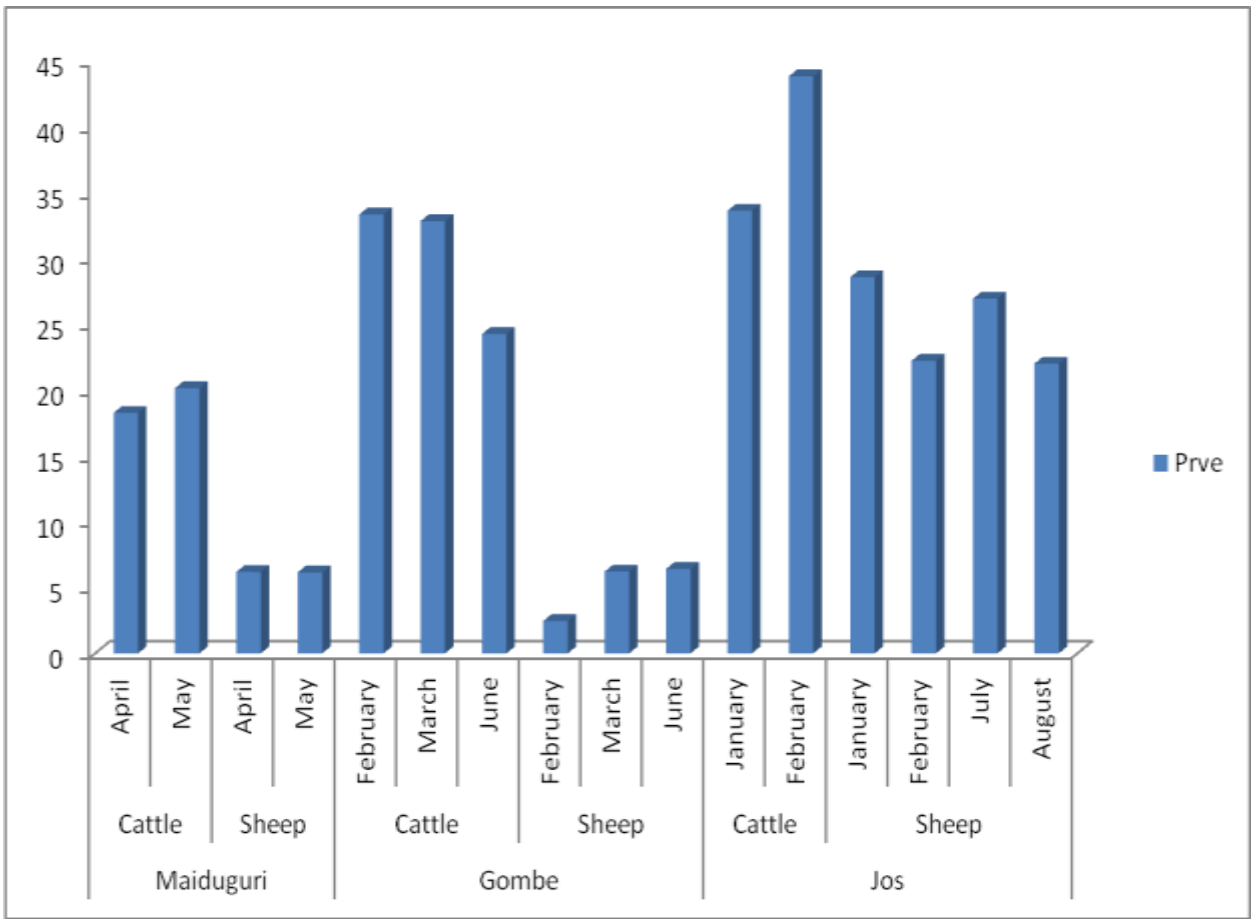
Appendix 9: Mean *Fasciola* egg count of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to age.



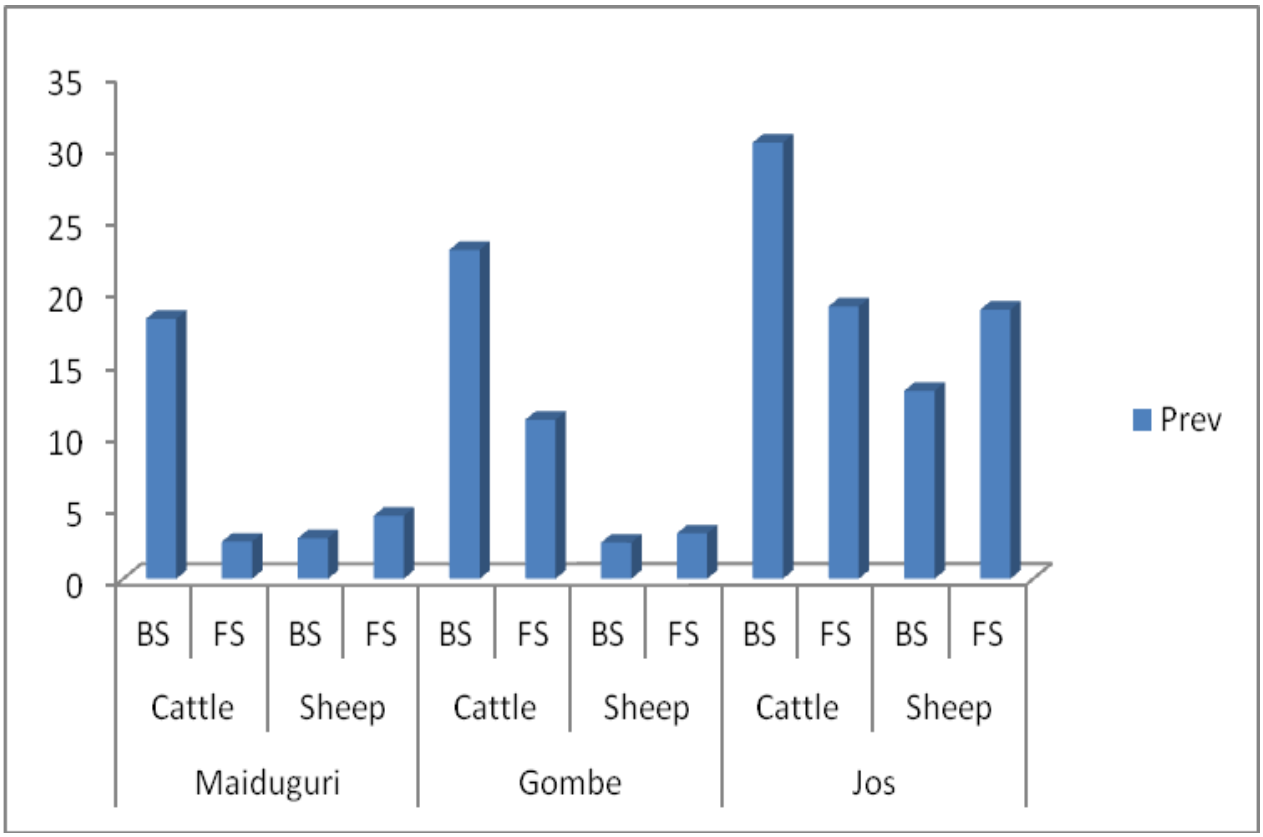
Appenidix 10: Prevalence of *Fasciola* spp eggs in faeces and bile of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to breed.



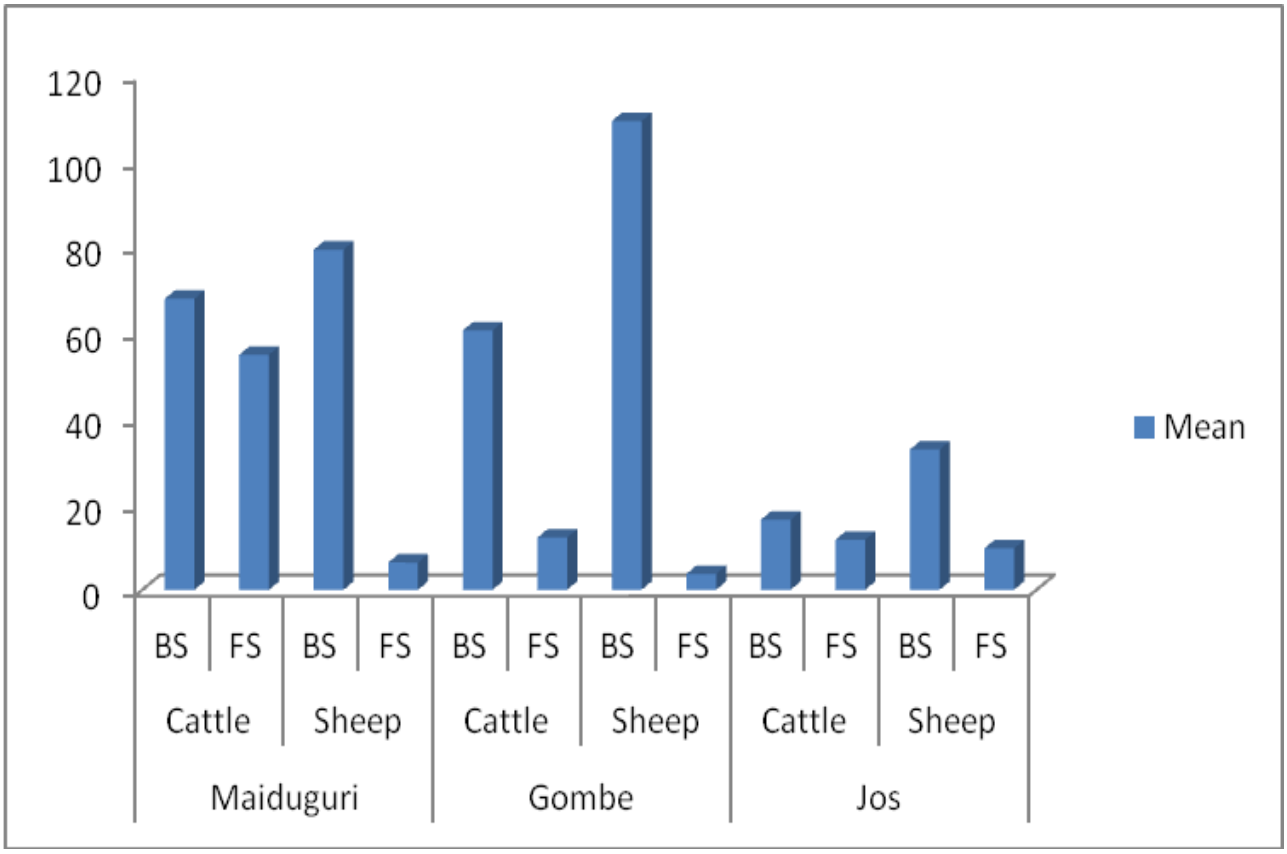
Appendix 11: Mean *Fasciola* egg count of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to breed.



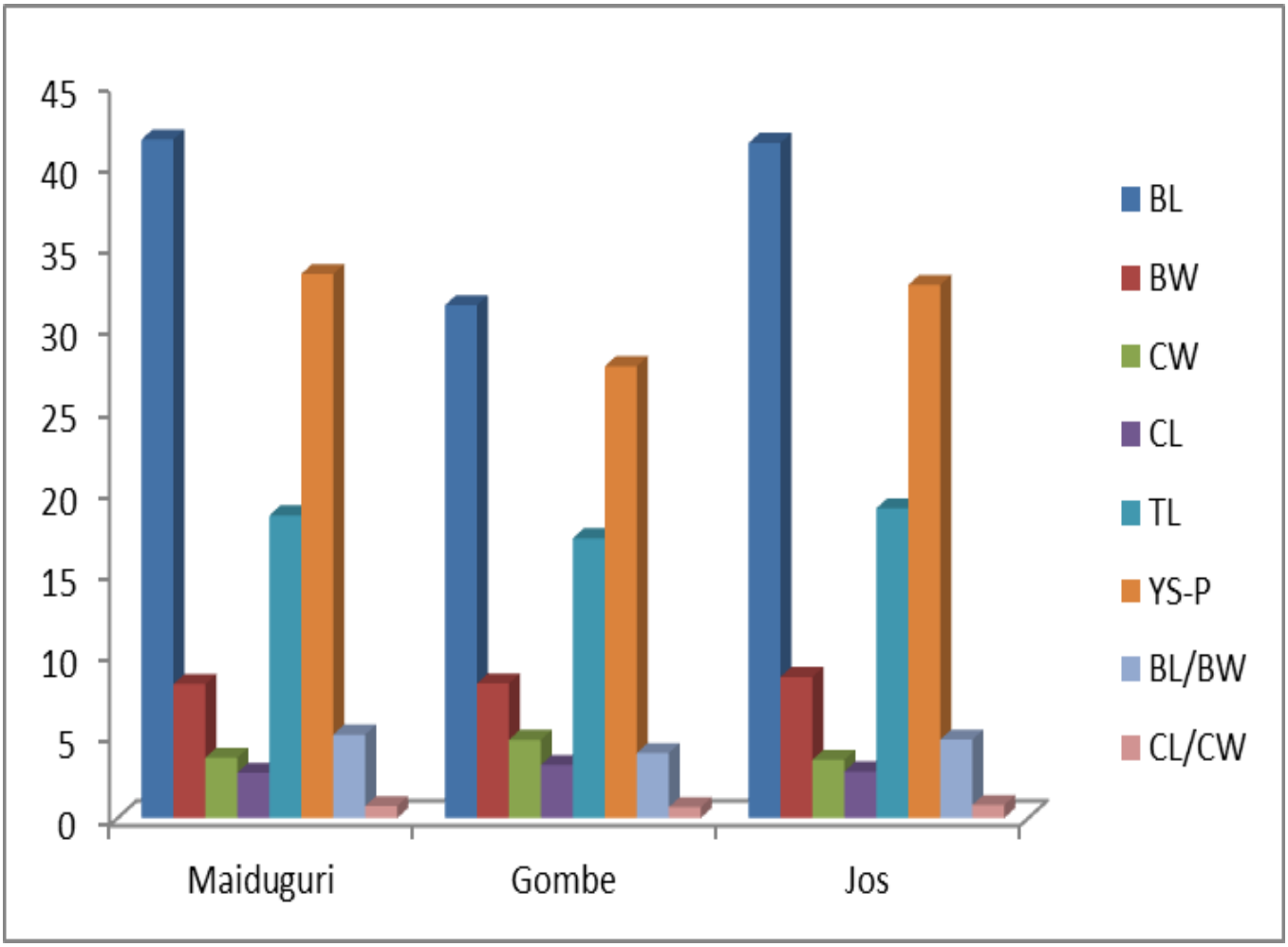
Appendix 12: Prevalence of *Fasciola* spp eggs in faeces and bile of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to months of sampling.



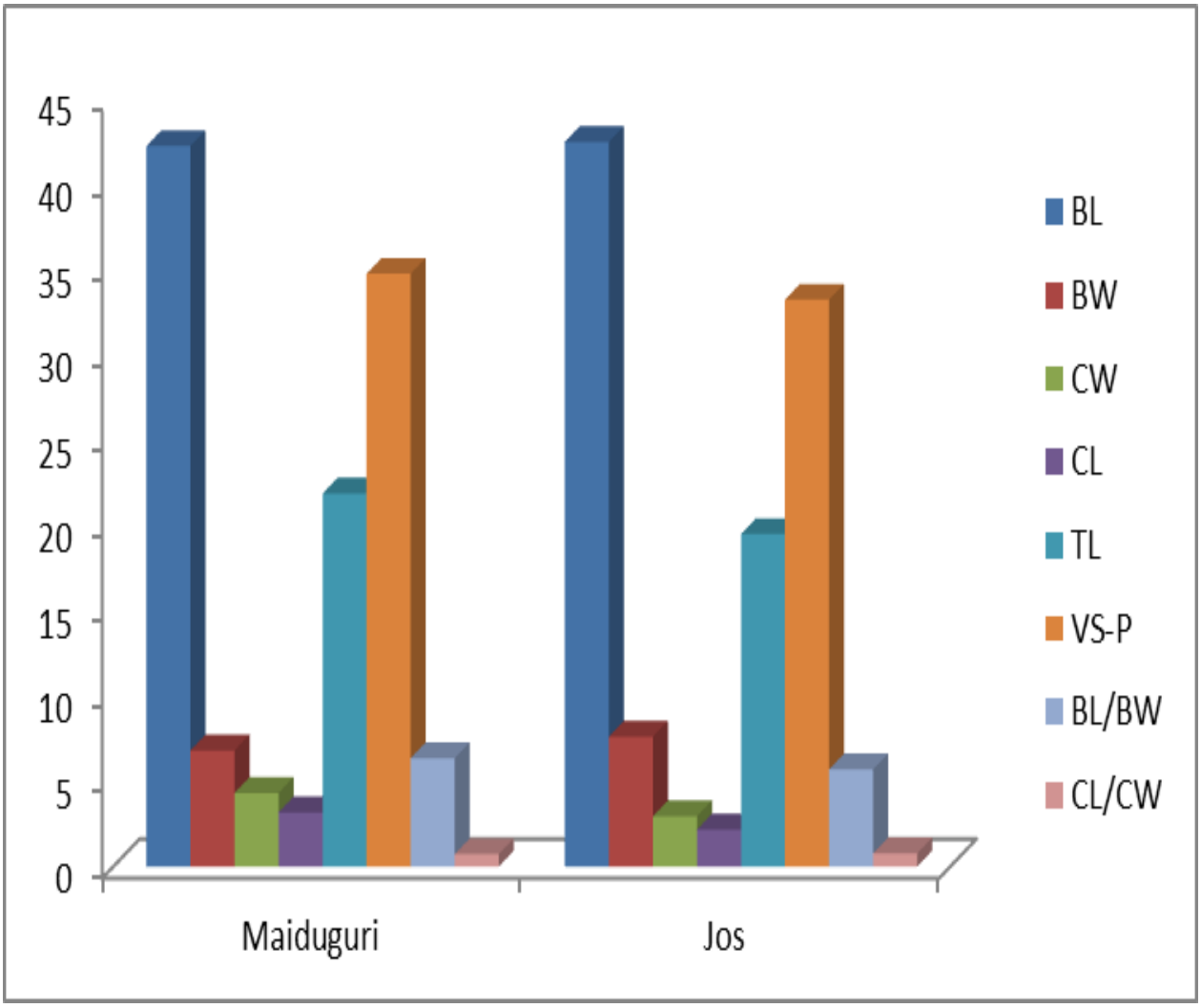
Appendix 13: Prevalence of *Fasciola* spp eggs in faeces and bile of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to sample type



Appendix 14: Mean *Fasciola* egg count of slaughtered cattle and sheep from Maiduguri, Gombe and Jos abattoirs in relation to sample type.



Appendix 15: Mean linear biometric parameters/ratios in (mm) of *Fasciola species* in slaughtered cattle from Maiduguri, Gombe and Jos abattoirs



Appendix 16: Mean linear biometric parameters/ratios in (mm) of *Fasciola* species in slaughtered sheep from Maiduguri, Gombe and Jos abattoirs.

Appendix 17: Nucleotide sequences of *Fasciola gigantica* and *Fasciola hepatica* from Maidugiuri, Gombe and Jos in FASTA format

>Seq No-3(ITS) [*Fasciola gigantica* Jos-Nigeria]

CAGTCCAACCCGGGATAGGCACCGCCGGGCGACTGTACGTGCAGTCCAGCCACAAG
GACCGGGTGCATGCGAGGGCAATGGCAAAGAATGGCAAGCCATTCAATACCACTTT
AACAGTGTAATTGTAGTAATGAAACAGTTCATAGGGCGGGAGCAGGGCCGTAGCCC
AAATCTCCTCTTTAAGCCTAGCACTACCAATCATGGCAGTACAACCCGTTCTCCT

>seq No-25(28S) [*Fasciola gigantica* Maiduguri-Nigeria]

ACACCTGATGAGTGATTCGCGCATCCCCACCGAAGCGGAGACCTGCAGCTAAGCAG
ACCCGAGCACACAACCAATATGACCAAGCTCATGCCATACTCACCAGCTGAATCGG
CAGAGCTTGCAGTTCAGTTCCATGTGTTCCGACACATCCGCCCTCAATCCAAAGCTC
TCCTTCAGGATAAGGACGGAATTGCCATCACTCCGGCCCCCTTGGGCAGGCAAGGG
TACCAGCTTAGACAGGTAAAGAAAAGTATATATTTTAAGATGGCTTTATATAC

>seq No-26(28s) [*Fasciola gigantica* Maiduguri-Nigeria]

GCCACACCTGATGAGTGATTCGCGCATCCCCACCGAAGCGGAGACCTGCAGCTAAG
CAGACCCGAGCACACAACCAATATGACCAAGCTCATGCCATACTCACCAGCTGAAT
CCTCAGAGCTTGCAGTTCAGCTCCACCTGTTTAGCTCCGAACGGCTTCACGCCCAAN
CTCCTCCTGCGGATAACGGACTTTCTGGCTTTCCTTCAGGACCTTGGGGGCAAGGAG
GGTACCCACTTCCCCTTGGGAATAAAAGGTTTTTTTTTTAGAATGGCTTATAAAANCT
CCCCATTGTNTGCTTGTGTTACCNGCCCCCAACCTTCTTTGTCTTGGGGGCANTCAAA
NAATTTACGATCGACACGGCGCCCCGGACATCNAGGCTCGTGTAG

>seq No-37(28S) [*Fasciola hepatica* Gombe-Nigeria]

CGCGTCGTAGCAACGATTCACACATACCCACCAAAAAGGAAGGCACCTGCGGTTAA
ACAGACACTGAAGGCGGCCAGTATGCCCAAGTACAAATCACACTCATCAGCTGAAT
TCCCAGAGCTTGCAGTTCAACTCCACCCGTTTACCTTTGAGCGGTTTCACGCACTGTT
TACTCTCTCTTCAAAGTACTTTTCAACTTTCCCTCACGGTACTTGTTTGCTATCGGACT
CGTGTAAGTATTTAGCCTTGGATGGAGTTTACCACCCACTTTGGGCTGCATTCACAA
ACAACCCGACTCCAGGGTAGCTYAGAGCAAAACTGTCACACTTGATCTCTGCCCCCA
CGGGCCTTTCACCCTCTTTGGGCCAGAATGGGAAGCCGTACTCATTGCTGGACTTGG
GACAGAGCAGTAATGCCTGAAGCCACCCTAAACACCACATTGCCTTACGATCAAAT
AACCGCAGGCTTCGGTGTTGGGCTAATCCCTGTTCACTCGCAGTTACTAGGGGAATC
CTTGTTAGTTTCTTTTCCTCCGCTTAGTGATATGCTTAAGTTCAGCGTA

Appendix 18: Nucleotide sequences of *Lymnaea* (*Radix) natalensis* in FASTA format

>Seq1 [*Radix natalensis*][isolate=16] [18S rRNA][Partial]

GATCGAACCCTGATTCCCCGTTACCCGTTACAAACATGGTAGGCATATCACGTACCA
TCGACATTTGATAGGGCAGACATTTGAAAGATGCGTCGCCGGCACGAAGGCCATGS
GATCAGCACAAAGTTATCCARAGTCACCAATGGGGACACCGCGCACCCCGTGAGGA
GCACGCGGCGATTGGTTTTGAACTAATAAAAGCGCTCTTTCCAATAAAGGTCGGAGC
TTGGTAAGCATGTATTAGCTCTAGAATTGCCACAGTTATCCAAGTAGGATCGTGTCA
TCTAAGGAACCTCGACTGATTTAATGAGCCATTCGCGGTTTCACCGTCAAACAGTGT
GAACTTAGACATGCATGGCTTAATCTTTGAGACAA

>Seq2 [*Radix natalensis*][isolate=31][18S rRNA][Partial]

GATCGAACCCTGATTCCCCGTTACCCGTTACAAACATGGTAGGCATATCACGTACCA
TCGACATTTGATAGGGCAGACATTTGAAAGATGCGTCGCCGGCACGAAGGCCATGC
GATCAGCACAAAGTTATCCAGAGTCACCAATGGGGACACCGCGCACCCCGTGAGGA
GCACGCGGCGATTGGTTTTGAACTAATAAAAGCGCTCTTTCCAATAAAGGTCGGAGC
TTGGTAAGCATGTATTAGCTCTAGAATTGCCACAGTTATCCAAGTAGGATCGTGTCA
TCTAAGGAACCTCGACTGATTTAATGAGCCATTCGCGGTTTCACCGTCAAACAGTGT
GAACTTAGACATGCATGGCTTAATCTTTGAG

>Seq3 [*Radix natalensis*][isolate=32][18S rRNA][Partial]

CCGGATCGAACCCTGATTCCCCGTTACCCGTTACAAACATGGTAGGCATATCACGTA
CCATCGACATTTGATAGGGCAGACATTTGAAAGATGCGTCGCCGGCACGAAGGCCA
TGCGATCAGCACAAAGTTATCCAGAGTCACCAATGGGGACACCGCGCACCCCGTGA
GGAGCACGCGGCGATTGGTTTTGAACTAATAAAAGCGCTCTTTCCAATAAAGGTCGG
AGCTTGGTAAGCATGTATTAGCTCTAGAATTGCCACAGTTATCCAAGTAGGATCGTG
TCATCTAAGGAACCTCGACTGATTTAATGAGCCATTCGCGGTTTCACCGTCAAACAG
TGTGAACTTAGACATGCATGGCTTAATCTTTGAGACAAGC