SERUM PROFILING AND TISSUE EXPRESSION PATTERN OF SIALYLTRANSFERASE IN PIGS EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA BRUCEI BRUCEI*

BY

JAMILA ABDULHAMID ATATA

DEPARTMENT OF VETERINARY PATHOLOGY,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

MAY, 2021

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Jamila Abdulhamid ATATA,
DVM (ABU) 2013, MSc (ABU), 2017
P17VTPT9003

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD
OF DOCTOR OF PHILOSOPHY IN VETERINARY PATHOLOGY

DEPARTMENT OF VETERINARY PATHOLOGY,
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

MAY, 2021

DECLARATION

I declare that the work in this thesis entitled **"SERUM PROFILING AND TISSUE EXPRESSION PATTERN OF SIALYLTRANSFERASE IN PIGS EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA BRUCEI BRUCEI*."** has been carried out by me in the Department of Veterinary Pathology, Ahmadu Bello University, Zaria. The information derived from the 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 any other Institution.

Jamila Abdulhamid ATATA

_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This Thesis entitled **"SERUM PROFILING AND TISSUE EXPRESSION PATTERN OF SIALYLTRANSFERASE IN PIGS EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA BRUCEI BRUCEI.*"** by Jamila Abdulhamid ATATA meets the regulations governing the award of the degree of Doctor of Philosophy in Veterinary Pathology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. K.A.N. Esievo

_____ _____ _____

Chairman, Supervisory Committee Signature Date

Prof. S. Adamu

_____ _____ _____

Member, Supervisory Committee Signature Date

Dr. E.O. Balogun

_____ _____ _____

Member, Supervisory Committee Signature Date

Prof. S. Adamu

_____ _____ _____

HOD, Veterinary Pathology SignatureDate

Prof. Sani A. Abdullahi

_____ _____ _____

Dean, School of Postgraduate Studies Signature Date

DEDICATION

I dedicate this work to my beloved parents who were my source of inspiration. And also, my husband and children whose uncompromising love, support, and encouragement have enriched my soul and inspired me in many ways I couldn't have imagined.

ACKNOWLEDGMENT

It is a great pleasure to thank all those who made this thesis possible. First of all, I thank Almighty God for answering my prayer and blessing me with the strength, power of the mind, protection, skill, healthy life, and the ability to complete this thesis. May His peace and salutations be upon our beloved prophet, Muhammad (S.A.W), his family members, his companions, and all those who follow their path.

I want to thank my major supervisor and mentor, Prof. K.A.N. Esievo. It has been an honor to be his M.Sc. and Ph.D. student. I am very glad he accepted to mentor and supervise my MSc. when I got employed at the Department of Veterinary Pathology, University of Ilorin in 2015. This is a favour I would live to remember. Sir, you have taught me, both consciously and unconsciously, how to be a good researcher. I appreciate all your contributions of time and ideas to make my Ph.D. experience productive and stimulating. May you live long to reap the seed of goodness you have sown in all of us.

I would like to express my profound gratitude and appreciation to my second supervisor and the Head of Department of Veterinary Pathology, Prof. S. Adamu for his guidance, excellent supervision, and patience throughout my period of study. You taught me that humility is the best asset. I have gone through excellent training, and would forever remain indebted to you.

I would like to thank Dr, E.O. Balogun for introducing me to the basics of molecular biology and also for his unwavering support in my academic pursuit. I am grateful that despite his tight schedule, he magnanimously accepted to be part of this work in a supervisory capacity.

I thank Prof (Mrs). Stella Smith, Director of Research, Molecular Biologyand Biotechnology at the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos state for

accepting to be part of this research despite the short notice and assisting me in all capacities on the molecular aspect of this research. Dr. Olumide Adegbesin, Dr (Mrs) Muinah Fowora, Dr. Isaac Abraham,and Mr. Afolabi Olumide were all wonderful people I met and worked with all through the days of my stay in NIMR laboratories, thanks for all your support.

I would like to express my sincere gratitude to Dr. Annas Salleh of the Faculty of Veterinary Pathology, Universiti Putra Malaysia (UPM) for his continuous guidance, advice, invertible support throughout my stay in Malaysia.My housemates at UPM International Guesthouse; Fatma, Raihanatullah, Ummu Nasitah, Aslamiyah, Shifa, Damia, and Shaba are all wonderful people and I say thank you to you all.

I am also grateful to my mentor Prof. S. F. Ambali for always been supportive and for his valuable suggestion towards the success of my research. My utmost gratitude goes to the Dean, Faculty of Veterinary Medicine; Prof. S.O. Salami,The Director of the Veterinary Teaching Hospital; Dr. O. B. Akanbi, my HOD; Dr Adam Muhammed and my colleague; Dr Abdullateef Abiodun Ajadi, and other staff of the Department of Veterinary Pathology, all of University of Ilorin whom without their continuous support this study would not have been possible.

I also appreciate the helpful and meticulous support of Dr. Samson JamesEnam, whom our path crossed at the beginning of this research and who jokingly referred to me as his IT student.His family has become mine and I thank my stars for knowing a wonderful person like you. I think we did great as a team. Dr. Idris Sheriff was a down to heart person that assisted me in many ways. Both of you are great people, thanks for allowing me to use your office and making me feel at home in it.

Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria has become my second home and all the faculty staff members are family to me. It would be difficult to mention names, I seek to single out Mr. Dennis Otie and Mallam Lawal Iliya for the immense help they offered in their laboratories. I would like to thank Mal. Muazzu Ahmad Abubakar, Mrs. Christiana Alaba Ajanusi and Mrs.Suberu A. Bosede for always being there and helping me navigate the usually tortuous official issues. Hajia MarioAhmad of the blessed memory was such a great friend to me. May your gentle soul rest perfectly (Ameen). Thanks for all your support and encouragement. It has always been fun working with you.

This acknowledgment will be incomplete without special thanks to all the senior members of the Faculty, for the knowledge imparted and the mentoring during my study years. Additional thanks also go to all colleaguesand postgraduate students in the Faculty for their invaluable inputs to this work during the usual seminars. I would like to appreciate all the respected teachers that taught me a few things that had changed my life. Thank you all for your time and commitment. And to anyone that I might have forgotten; I apologize and say thank you to you as well. Finally, I would like to acknowledge my country Nigeriathrough the TETFUND for their financial support, without which this work would have been difficult to achieve.

My beautiful family made sure that I always felt loved throughout this journey even though, they suffered a lack of adequate attention through my frequent travels. I hope a successful completion puts smiles on your faces. Thanks for your support and understanding. I also got support from numerous people who were kind enough to extend their help at various phases whenever I approached them. My profound gratitude to Drs Abdulsalam Hassan, Ochuko Orapkogenor, S.T. Muhammad, Joseph Elijah, Rukkayyah Nasiru, Felix Samuel, Sunny Idoko, Edith Esievo, Olatunji Aishat and Kareemat Hussein.

ABSTRACT

African trypanosomiasis (AT) is a devastating disease that causes mortality in both man and animals with anaemia being the most consistent complicating factor. The role of increased activity of sialyltransferase (ST) has been advanced in the recovery from anaemia or stabilization of red blood cell mass in trypanosome-infected animals. This study was therefore aimed at determiningthe serum profiles and tissue expressionpattern of sialyltransferase in serum and tissues of pigs experimentally infected with *Trypanosoma bruceibrucei* (*T. b. brucei*) with the view to unraveling the role of sialyltransferase activity and tissue expression of ST3Gal1 and ST6Gal1 in the recovery from anaemia of porcine trypanosomiasis. Ten (10) apparently healthy pigs assigned to *T. b. brucei*-infected (n = 5) and control non-infected (n = 5) groups were used for this experiment. Parasitaemia, body temperature, haematological values, the concentrations of erythrocyte surface sialic acid (ESSA) and free serum sialic acid (FSSA) as well as activities of neuraminidase and sialyltransferase (ST) were determined on a daily basis for a period of 21 days. Body weights were also determined on weekly basis. The mean thyroid gland ST activities and the expression levels of ST3Gal1 and ST6Gal1 in the liver and kidneys were determined at the termination of the experiment. Clinical trypanosomiasis was established in the infected pigs as early as day 3 post-infection (PI); with all pigs becoming parasitaemic by day 5 PI. The parasitemia observed was associated with a gradual and progressive drop in the mean PCV valuesfrom 41.40±1.33 % to a minimum value of 19.40 ± 1.66 % recorded on day 13

PI. The mean ESSA concentrationin *T. b. brucei* infected pigs droppedsignificantly ($P < 0.05$) below normal values from day 6 PI, 3 days after parasites were first detected in their blood and this was then followed by progressive risefrom day 14 to stabilize with occasional surges in its value until the termination of the experiment. Conversely, the FSSA concentration increased significantly ($P < 0.05$) from day 7 to reach a peak level on day 11 PI. This was accompanied by an apparent but fluctuating decrease to stabilize on day 16 until the termination of the experiment. A significant ($P < 0.05$) rise in neuraminidase activity was observed as early as day 7 with peak level reached on day 11 which was followed by a fluctuating decrease to stabilize at a near pre-infection value on day 14 and remained so until termination of the experiment. Meanwhile, increase in mean serum sialyltransferase activity occurred from day 6 PI and attained a significant level ($P < 0.05$) on day 16 and this was then followed by non-significant ($P > 0.05$) fluctuations which were sustained until the termination of the experiment. Similarly, thyroid tissue sialyltransferase activity of the infected pigs was significantly increased. There was a strong relationship between sialyltransferase activity and parasitemia ($r = 0.5$); packed cell volume ($r = -0.8$); decrease in free serum sialic acid concentration ($r = -0.7$); increase in erythrocyte surface sialic acid concentration ($r = 0.8$) and decrease in neuraminidase activity ($r = 0.4$). The mRNA expression of *Trypanosoma brucei brucei*-infectedand (non-infected) control-derived kidney and liver genes showed that hepatic ST6Gal 1, Renal ST6Gal 1, Hepatic ST3Gal 1 and Renal ST3Gal 1 gene expression were significantly ($P < 0.0001$) upregulated (41.87-fold; 19.17-fold; 15.16-fold; and 5.00-fold respectively) in the infected pig compared to the control. It was concluded from the findings in this study that serum sialyltransferase activity and increased tissue expression of ST3Gal1 and ST6Gal1 played a pivotal role in the stabilization of erythrocyte mass by the process of resialylation of

desialylated red blood cells thereby promoting recovery of the red blood cells in trypanosome-infected animals. The tissue expression of ST3Gal1 and ST6Gal1 in *T. b. brucei*-infected pigs reported for the first time in this study could serve as a novel drug development tool that could be explored for enhancing recovery from anaemia of trypanosomiasis in both animals and man. It was therefore recommended that the expression of serum ST3Gal1 and ST6Gal1 be investigated, further in trypano-tolerant breeds of animals to determine the role of these genes in pathogenesis of trypanosomiasis.

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LIST OF ABBREVIATIONS

AAT	Animal African Trypanosomiasis
ACD	Acid citrate dextrose
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
CATT	Card agglutination test
cDNA	Complementary DNA
Ct	Threshold cycle
DG	Dark ground
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
EPO	Erythropoietin
ESSA	Erythrocyte surface sialic acid
FAM	Fluorescein amidites
FFA	Free fatty acid
FSSA	Free serum sialic acid
HAT	Human African trypanosomiasis
Hb	Haemoglobin
HCT	Hematocrit centrifuge technique
HSPCs	Hematopoietic stem and progenitor cells
IFA	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMF	Infant milk formula
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MHC	Major histocompatibility complex
mRNA	Messenger RNA
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NO	Nitric oxide
OD	Optical density
PCR	Polymerase chain reaction
PCV	Packed cell volume
PP	Prepatent period
qRT – PCR	Quantitative reverse transcription polymerase chain reaction
RBC	Red blood cell
RLT	RNA lysis
RNA	Ribonucleic acid
SD	Sialidase
Sia	Sialic acid

SIT	Sterile insect technique
ST	Sialyltransferase
T. b.	*Trypanosoma brucei*
TBA	Thiobarbituric acid
TNF	Tumor necrosis factor
VSG	Variant surface glycoprotein
WBC	White blood cell

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Tsetse-transmitted African trypanosomiasisaffects humans, cattle, small ruminants, and pigs, as well as other species including wildlife. The causative agents are flagellated extra-cellular protozoan parasites that cause fatal diseases commonly called sleeping sickness in humans (Human African Trypanosomiasis, HAT) or nagana (Animal African Trypanosomiasis, AAT) in domestic livestock (Barrett *et al*., 2003). In animals, the disease was reported to be characterized by anemia, weight loss, reduced milk yield, impairment of immune function, reproductive disorders, and death when affected animals were not treated (Omotainse *et al*., 2004). Ruminants were reported to be mostly affected by *Trypanosoma congolense, T. vivax, and T. brucei*, with *T. simiae*being the most economically important trypanosome in pigs (Mare, 2008 as cited by Maina *et al*., 2015).

The disease can be acute or chronic and the severity of infection differs from species to species as well as the age and breed of the host. The poorbody condition observed and other related factors tended to increase the severity of the disease and to cause relapse in chronically infected animals (Sterverding, 2008; CFSPH, 2009). Methods of Diagnosisof the disease include direct examination of fresh blood or the buffy coat and polymerase chain reaction (Stear, 2005). Several drugs available for treatment tend to have narrow therapeutic indices, making correct dosage essential. In West Africa, resistance to trypanocidal drugs became a growing problem in cattle, particularly in the cotton zones (Clausen *et al*., 2010). The disease occurred throughout the tsetse belt of Africa between

latitudes 14°N and 29°S stretching right across the rain forests of Africa extending to the dry areas of the Sahara in the North and the more diffuse southern dry areas of Namibia, adjacent parts of South Africa, Botswana and Angola (Akanji *et al.,* 2009; Mbaya *et al.,* 2009).It was estimated that USD 4.75 billion worth of agricultural products and USD 2.75billions of milk were lost each year as a result of trypanosomiasis (Shaw, 2004). In Nigeria, trypanosomiasis became a disease of great economic importance when the mortality, loss in productivity, cost of treatment, and other control measures were comprehensively considered (Shaw, 2004; Omotainse *et al*., 2004). Trypanosomiasis was rated the most devastating and widespread disease of African livestock (Omotainse *et al*., 2004), especially where animal husbandry is carried out in vector infested areas of the continent.

Tsetse infestation was considered to be one of the most serious pest problems in the world; covering over36 countries and a total area of 10 million km^2 in Africa within which trypanosomiasis has limited the keeping of domestic livestock, thus restricting struggling rural populations the advantages of meat, milk, animal traction and manure to which was added its devastating effects on humans (FAO, 2008). The control of animal trypanosomiasis relied mainly on tsetse and trypanosome elimination and/or eradication, as the antigenic diversity of the trypanosome made the development of a vaccine against the disease difficult (Doyle, 1977; Cross, 1990; PATTEC, 2003, Grace *et al*., 2015). Bush clearing to destroy tsetse fly habitat, elimination of wild animal reservoir hosts and the use of insecticide sprays targeted at the tsetse fly were currently considered ecologically and environmentally unfriendly, leaving the options of use of trypanocidal drugs, promotion of trypanotolerant livestock, trapping of tsetse and possible use of the sterile insect technique

(SIT) as viable control measures (PATTEC, 2003).Vector control is an important component of any trypanosomiasis control program and includes a variety of strategies such as insecticides for animals and the environment, bush clearing, and tsetse screens and traps (Stear, 2005).

Human African Trypanosomiasis (HAT) also known as sleeping sickness, is the collective term for two distinct and fatal parasitic diseases, caused by two morphologically indistinguishable subspecies of *Trypanosoma brucei s.l.; Trypanosoma brucei gambiense*, the causative agent of chronic HAT and *Trypanosoma brucei rhodensiense*, the causative agent of acute HAT (Welburn and Maudlin, 2012). Domestic and wild animals had been shown to harbor both *T. b. gambiense and T. b. rhodensiense* but their significance in disease epidemiology was mainly unspecified (Njiokou *et al.*, 2010). *Trypanosoma b. rhodensiense* infection was reported as a true and well-established zoonotic disease on epidemiological grounds, infecting man, and a wide variety of wild and domestic animals (Kiminyo and Lucey, 2001; Premaalatha *et al*., 2014).

Trypanosomiasis causedaberrationsto normal physiological parameters due to wide range of clinicopathological changes (Esievo and Saror, 1983; Allam *et al.,* 2011; Anene *et al.,* 2011; Ajibola and Oyewale, 2014) resulting from complex interactions between the hosts' immune responses and the trypanosomes and these changes included anaemia, tissue inflammation, meningoencephalitis, splenomegaly, glomerulonephritis and cachexia(Leta *et al*., 2016). Although, the mechanism or pathophysiology of anemia in trypanosomiasis had remained complex and multifactorial in origin (Naessens, 2006), it was postulated that the anaemia might be attributable to the trypanosome neuraminidase which cleave off

erythrocytes surface sialic acid thereby exposing the desialylated erythrocytes to phagocytosis (Esievo *et al.*, 1982; Nok and Balogun, 2003; Adamu *et al.*, 2008) and hence the growing interests in this aspect of disease pathogenesis. Observations on the free serum and erythrocyte surface sialic acid profiles of trypanosome-infected animals suggesteda possible involvement of sialyltransferase (ST) in the recovery or stabilization of red blood cell mass in some of the trypanosome-infected animals and hence a justification for increasing interests in sialyltransferase research especially in the area of molecular biology (Adamu *et al.*, 2009).

1.2 Statement of Research Problems

Anemiaas a dominant clinicopathological feature of AAT account for majority of death during the acute phase of the disease (Nok and Balogun, 2003; Adamu *et al*., 2008). Several mechanisms had been implicated in the pathogenesis of the anaemia; these included immunological factors (Facer *et al*., 1982), macrophage hyperactivation (Anosa and Kaneko, 1983), erythrocyte injuries via hemolysins, proteases (Lonsdale-Eccles and Grab, 1987), phospholipases activities (Tizard *et al*., 1978) and erythrocyte surface sialic acids cleavage by trypanosoma sialidases (SD) (Esievo, 1979; Esievo *et al*., 1982; Ibrahim *et al*., 2005). Increased validations of the latter hypothesis of trypanosomes sialidase-mediated desialylation of erythrocytes which was thought to predispose them to erythrophagocytosis had been reported (Nok and Balogun, 2003; Ibrahim *et al*., 2005; Buratai *et al*., 2006; Fatihu *et al*., 2008; Coustou *et al*., 2012) in AAT.How some trypano-tolerant animals were able to surmount anemia by maintaining erythrocyte counts at relatively low level had

remained the subject of several investigations while the genetic and molecular basis of this phenomenon were not fully understood, it was believed that this trait is exhibited by animals with greater tendency to manage parasitemia and anemia during the infection (Naessens *et al*., 2003; Stjlemans *et al*., 2008). Bone marrow response playeda minimal role in the recovery of red blood cell mass in trypanosomiasis because of the reported trypanosome-induced dyserythropoiesis (Igbokwe, 1989; Buzza *et al*.,1995; Taylor and Authié, 2004). Downregulation of erythropoietin (EPO) receptor expression seemed to impact on the proliferation, differentiation, and maturation of the erythroid precursors and led to inadequate erythropoiesis in the infected animals (Igbokwe, 1989; McAllister, 2019).

Sialyltransferases (STs) were suggested to be involved in resialylation of desialylated erythrocytes thereby enhancing recovery in the course of an infection (Esievo *et al*., 1982; Esievo *et al*., 1986; Bratosin *et al*., 1998). STs are enzymes that transfer sialic acids to terminal portions of the desialylated glycolipids (gangliosides) or the N-or O-linked sugar chains of glycoproteins (Kaufmann *et al*., 1999; Ellies *et al*., 2002; Borman, 2004; Wang, 2005).In health, it was found to be expressed in various mammalian tissues (Ellies *et al*., 2002; Borman, 2004) in varying proportions. It has been reported that an increased sialyltransferase activity with decreasing concentration of erythrocyte surface sialic acid (ESSA) and increasing concentration of free serum sialic acid (FSSA)occurred in *T. congolense* infection in sheep (Adamu, 2009); a similar study also revealed differences in the biochemical activities of STs in *T. congolense* infected and non-infected sheep (Baraya, 2015). Despite rapid advances in expression technology and genetic engineering for producing therapeutic recombinant proteins (Khan and Sadroddiny 2016), hurdles remain to be overcomed. Interestingly, the molecular expression pattern of the ST genes in African

Animal trypanosomiasis is not yet elucidated or of probably scanty information, and therefore a better understanding of this process requires further research in the area of molecular biology. This work was conceived in view of this knowledge gap. Advances in the field of sialic acid and sialidase biology as well as the role of STs in the pathogenesis of many mammalian diseases including trypanosomiasis, diabetes mellitus and cancer have stimulated interest in these enzymes that are potential targets for therapeutic intervention.

1.3 Justification of the Study

Sixty million people and more than 70 million livestock in Africa are at risk of contracting trypanosomiasis (PATTEC, 2003). The Heads of member states of the African Union (AU) declared the year 2000 as the beginning of the Pan African Tsetse and Trypanosomiasis Eradication Campaign, to eradicate tsetse flies and the diseases they transmit from the continent (WHO, 2019). Trypanosomiasis and the tsetse related problem contributed adversely to the economic and social well-being of humans and their animals in sub-Saharan African, with consequential effects on the fragile economy ofTropical African countries (Bourn *et al.*, 2001; Swallow, 2002; Odoya *et al*., 2003), thus adding to the 'heart' of Africa's poverty. Estimates of gross national per capita income showed that 20 of the world's 25 poorest countries were affected by tsetse-transmitted trypanosomes (Cattand *et al*., 2010) and over 4.5 billion dollars annual agricultural losses were incurred due to AAT in sub-Saharan African (Holt *et al*., 2016; Leta *et al*., 2016). It was also estimated that 60 million people and 48 million cattle were at risk of contracting African trypanosomiasis transmitted by 22 species of tsetse flies across Africa (Seifert, 1996; Kristjason *et al*., 1999; Stear, 2005), which led to the death of more than 55,000 people and 3 million livestock

annually (ILRAD, 1990b; Abenga *et al*., 2002). It was therefore evident that this disease constituted an impediment to improved life stock production through reduced calving rate, drop in meat and milk production, reduced feed conversion and work efficiency of draft animals and an overall reduction in profitability in mixed farming (Hursey, 2001; Machila *et al.,* 2003). Efforts to control the fatal disease in man and livestock, based on the treatment of patients and livestock with trypanocidal drugs encountered drug resistance as a major impediment; also control measuresthat proved unsustainable due to the public attitude towards most control techniques such as aerial and ground spraying, traps created additional problems (ILRAD, 1990a).Statistics by WHO (2019) on trypanosomiasis revealed that the disease was widespread all over the continent with numerous outbreaks and reports of relatively high infection rates especially in the Jos Plateau that was regarded as tsetse-free and trypanosomiasis-free.

Development ofvaccines against the disease remains ineffective because of the parasite's ability to undergo antigenic variation (Naessens *et al*., 2003; Ezeokonkwo *et al*., 2012), thereby evading host's natural immune response. Unfortunately, this led to the re-emergence of both Human African and African animal trypanosomiasis (Seed, 2001; Waiswa *et al*., 2003; WHO, 2005), and hence the renewed interest in the control and prevention of trypanosomiasis as reflected by various regional and international measures (FAO, 2008; Cecchi *et al*., 2009).Despite the potential of being a reservoir host to human and animal infective trypanosomes as well as the resultant economic losses from infection, pigs received less priority in trypanosomiasis research because pig trypanosomiasis was believed to be mild in severity and progressed slowly (Omeke and Ugwu, 1991; Ademola and Onyiche, 2013; Biryomumaisho *et al*., 2013). As the reality of eliminating human

African trypanosomiasis (HAT) by 2030, becomes more compelling, it is pertinent to detect and identify the remaining areas of disease pathogenesis (Cunningham *et al*., 2016, Büscher*et al*., 2018) to be exploredusing in-depth knowledge of molecular biology in the evolution of diagnostic, curative and preventive approaches in the important reservoir hosts of HAT.

There is a wealth of data describing the serum activities of STs in different mammalian hosts from earlier reports (Adamu, 2009; Abenga, 2011; Baraya, 2015) as well as the activity of ST-like trans-sialidase in *Trypanosoma cruzi* infected hosts (Harrison *et al*., 2001; Paris *et al*., 2005; Cheng *et al*., 2010; Buschiazzo *et al*., 2012). Similarly, glycosylation has been linked to molecular and cellular function within the body system (Sato *et al*., 2009; Davicino *et al*., 2011; Varki, 2011; Khan *et al*., 2017; Benkoulouche*et al*., 2019; Park, 2019; Vajaria *et al*., 2019; Yehuda and Padler-Karavani, 2020). Despite their crucial role in body function, very little was known about ST sequence, function, and tissue-specific expression (Hardiun-Lepers, 2010). While, upregulation and downregulation of the different classes of STs were implicated in the pathogenesis of various types of cancers in humans (Hsieh *et al*., 2017; Roa-de La Cruz *et al*., 2018; Vajaria*et al*., 2019; Ou *et al*., 2020), there is a dearth of information on the molecular mechanism underlying the tissue expression and the role of STs in resialylation of RBCs in African Animal trypanosomiasis.Therefore, investigating sialyltransferase activity and the expression of ST3Gal1 and ST6Gal1 in *T. b. brucei* infected pigs is pertinent for the identification of a molecular target that could be exploited in developing efficient control measures against the disease. It is also obvious that more information is required in designing non-empirical therapy against both porcine and human trypanosomiasis.This is from the backdrop that

pigs are monogastric and also the fact that *T. b. rhodensiense* is zoonotic and could infect both humans and pigs (Nkinin *et al*., 2002; Hamill *et al*., 2013).

1.4 Aim of the study

The study was aimed at determining the serum profiles and tissue expressionpattern of sialyltransferase in serum and tissues of pigs experimentally infected with *Trypanosoma brucei brucei*.

1.5 Objectives of the study

The objectives of this study were to:

1. Determine the clinical, haematologicaland histopathological features of trypanosomiasis in pigs experimentally infected with *T. b. brucei*.
2. Determine the activity of neuraminidase in serum of T.*b.brucei*-infected and non-infected pigs.
3. Determine the profile of free serum sialic acid (FSSA) and erythrocyte surface sialic acid (ESSA) of *T. b. brucei*-infected and non-infected pigs.
4. Determine the profile of serum and tissue sialyltransferase (ST) of *T. b.brucei*-infected and non-infected pigs.
5. Amplify the gene of sialyltransferase from tissues of *T. b. brucei* infected and non-infected pigs.
6. Analyze the mRNA expression pattern of the ST3Gal1 and ST6Gal1 isolatedfrom liver and kidney of *T. b. brucei*-infected and non-infected pigs.

1.6 Research Questions

The research questions in this study are;

1. Does experimental *T. b. brucei* infection cause clinicopathological features of animal trypanosomiasis in pigs?
2. Does experimental *T. b. brucei* infection result in increased neuraminidase activity in pigs?
3. Does experimental *T. b. brucei* infection cause alteration in concentrations of FSSA and ESSA in infected and pigs?
4. Does experimental *T. b. brucei*infection result in increased activity of serumsialyltransferase in pigs?
5. Does experimental *T. b. brucei* infectionresult in increased sialyltransferase genes(ST3Gal1 and ST6Gal1) amplification of pigs?
6. Does experimental *T. b. brucei* infection result in increased mRNA expression of sialyltransferase (ST3Gal1 and ST6Gal1) genes in pigs?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Trypanosomiasis

Trypanosomiasis is an extraerythrocytic protozoan disease caused by infectionwith members of the genus *Trypanosoma*, which belongs to the phylum; Sarcomastigophora, order; Kinetoplastida and family; Trypanosomatidae. Trypanosomes multiply in the bloodstream, intercellular tissues, and body cavity fluid of their mammalian hosts. The disease is mainly transmitted by inoculation of the parasites by *Trypanosoma*-infected tsetse fly and the condition is usually not contagious except for dourine, a venereal trypanosomiasis of equines (Ugochukwu, 2008).

Trypanosomes are microscopic, unicellular, elongated, and motile parasites with a single flagellum located at their base around a structure called kinetoplast that houses the mitochondrial DNA of the parasite (Vickerman, 1985). The *Trypanosoma* genus is further subdivided into two sections namely; the stercoraria and salivaria, based on how the parasites are transmitted from the insect vector to the mammalian host as the parasite completes their cyclic development (Uilenberg,1998).

Many trypanosomes of veterinary and medical importance had been previously described (Abenga *et al.,* 2005). Most mammalian species are to some degree susceptible to trypanosomiasis transmitted by various hematophagous insects, mainly *Glossina* species commonly known as tsetse flies, which are considered to be the true vector of trypanosomes (Esievo and Saror, 1991; Ugochukwu, 2008). The Tsetse-transmitted AAT

(mainly caused by*T. b. brucei, T. vivax, T. congolense, and T. simiae*) has a greater impact on the fragile African economy with a resultant negative effect on vast livestock grazing areas as well as limiting the use of draught animals (Swallow, 2000) in arable agriculture. In humans, they cause human African trypanosomiasis (sleeping sickness). World Health Organization in 2006 estimated about 300,000 new cases of human African trypanosomiasis per annum while the economic cost of animal trypanosomiasis as at 2000 was estimated at US$4.75 billion per year (Swallow 2000). American trypanosomiasis (Chagas disease) caused by *Trypanosoma cruzi* was first reported, in the 1980s, to infect over 24 million people with another 100 million people at risk of the infection (Walsh, 1984). Although these estimates have reduced drastically, the disease was still considered the most important parasitic infection in Latin America with great socio-economic impact (WHO, 2016).

2.1.1 Transmission of the disease

Transmission of trypanosomes, from infected to susceptible hosts occurs cyclically through insect vector, tsetse flies, which are the sole vectors of several species of pathogenic trypanosomes in tropical Africa (Aksoy *et al*., 2003). The three main species of tsetse flies involved in the transmission of trypanosomes are *Glossina morsitans*, inhabiting the open woodland of the savanna; *Glossina palpalis*, of the shaded habitat immediately adjacent to rivers and lakes; and *Glossina fusca*, that prefersthe high, dense forest habitation.The pathogens' transmission occursthrough tsetse fly saliva during feeding (Wheeler *et al.*, 1989; Abenga, 2014). Mechanical transmission of trypanosomes species like *T. vivax, T. evansi*,and *T. cruzi* played a crucial role in the spread of the disease between infected and susceptible hosts (Desquesnes and Dia, 2003; Desquesnes, 2004). The primary mechanical

vectors are flies of the genus *Tabanus*and *Haematopota*, but fomites, surgical instruments, needles, and syringeare included. Several species of pathogenic trypanosomes had been reported to have a transplacental transmission in both man and animals (Okech *et al.*, 1996; Ijagbone and Agbede, 2000; Obaloto *et al*., 2015).

2.2 African Trypanosomiasis

The African trypanosomiasis is an infectious disease of humans and animals with similar etiology and epidemiology. The distribution of the disease corresponds to the tsetse fly belt, which covers an estimated area of about 8 million km^2 between 14^0 North and 20^0 South of the equator (Fig. 2.1) (Steverding, 2008). During the rainy season, the tsetse fly populations further encroached into more livestock herds and settlements, increasing the risk of *Trypanosoma* infection (Enwezor and Sackey, 2005; Odeyemi *et al.*, 2015). The disease is further subdivided into human African trypanosomiasis (HAT) and animal African trypanosomiasis (AAT) (Anene *et al.*, 2000; Steverding, 2008).

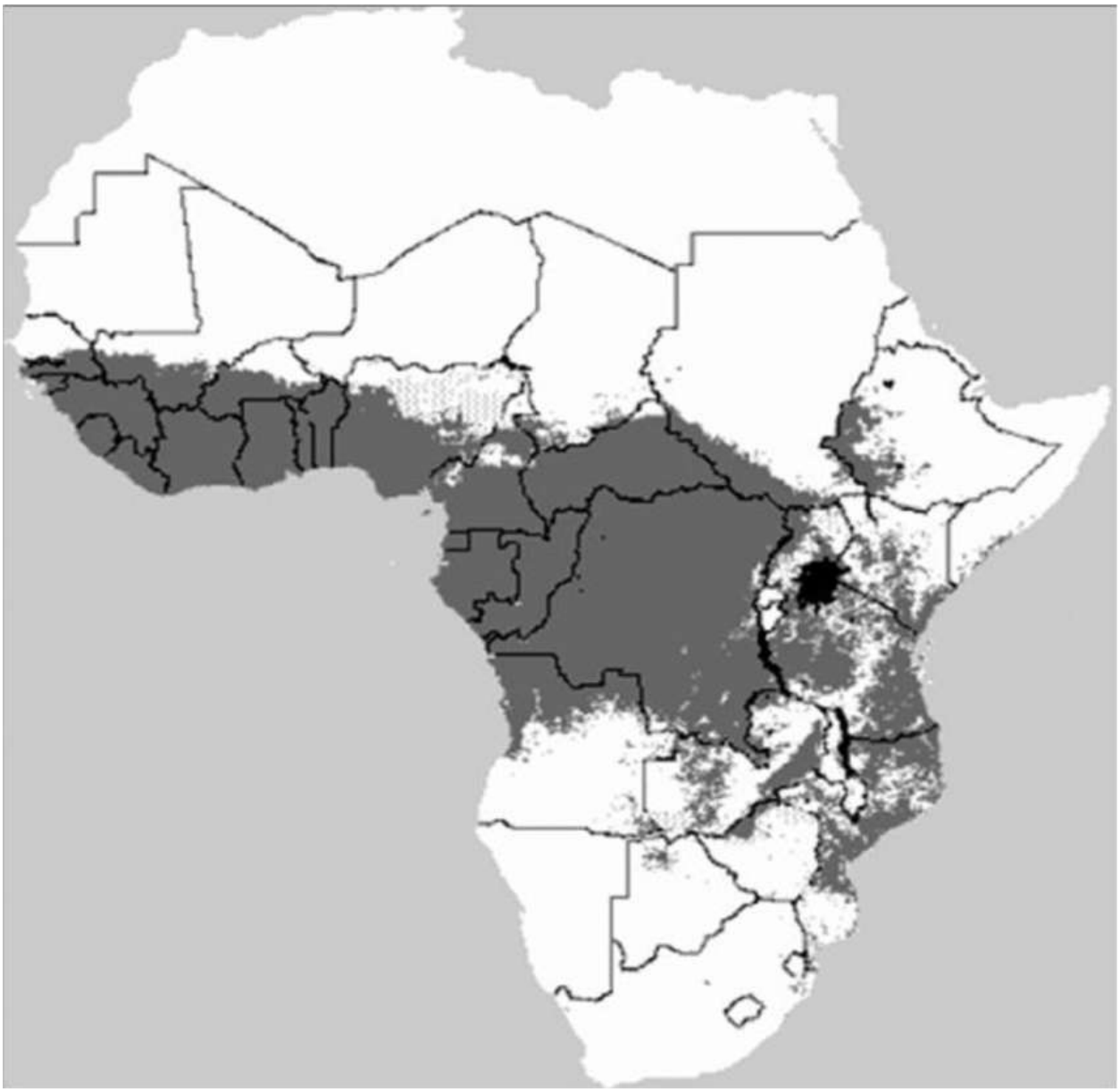

Figure 2.1: Tsetse fly belt in Africa – Grey shaded area indicates countries infested by tsetse flies (Aroko, 2014).

2.2.1 Animal African trypanosomiasis (AAT)

African animal trypanosomiasis is caused majorly by *T. brucei, T. congolense,* and *T. vivax*species. In domestic animals, these parasites cause a severe, often fatal disease called “nagana” butrather a mild infection in wild animals. The disease got its Zulu name "Nagana" meaning "powerless or useless" because of the associated-weakness (Steverding, 2008). The symptoms of the disease include; fever, listlessness, emaciation, alopecia, discharges from the eyes, edema, anemia, reproductive and CNS disturbances.The disease is a major cause of poverty and food shortages affecting the livelihood of about 500 million farmers in rural villages in Africa, consequent to over 3 million deaths in cattle and estimated economic losses in the production of about US$1-2 billion each year (Hendrickx *et al*., 2004; Hill *et al*., 2005).

2.2.2 Human African trypanosomiasis (HAT)

The pathogens responsible for HAT belong to the *T. brucei* complex which comprised morphologically indistinguishable species: *Trypanosoma brucei rhodesiense* the main pathogen of acute disease course in eastern and southern Africa, which accounts for 2% of all reported sleeping sickness cases;*Trypanosoma brucei gambiense* the major pathogen causing a chronic form of the disease in Western Africa which accounts for 98% of all reported cases of sleeping sickness (Fig. 2.2) (WHO, 1998; Franco *et al.,* 2014a). Although the two diseases occur in specific foci, they are also geographically separated by the Great Rift Valley (Fèvre *et al.*, 2006).There are two distinct stages in the development of sleeping sickness. The early-stage or haemolymphatic phase is characterized by the restriction of the trypanosomes within the circulation and the symptoms include development of chancre,

fever, headaches, joint pains, and itching. The late-stageof the neurological phase is characterized by the presence of the parasites in the cerebrospinal fluid and the signs at the stage include confusion, disturbed sleep pattern, sensory disturbances, extreme lethargy, poor condition, and infertility.If left untreated, the disease could be debilitating and fatal in the vast majority of cases (WHO, 2005). Molecular studies had distinguished the two HAT forms by the detection of the Serum Resistance Associated (SRA) gene and *T. b. gambiense* specific glycoprotein (TbgSG) gene for Rhodesian and Gambian sleeping sickness, respectively, and the presence of these genes enabled the trypanosomes to overcome the host immunity and subsequently lead to the disease process (Welburn *et al.*, 2001; Fèvre *et al.*, 2006). Absence of these genes in AAT pathogens was considered to be a reason for the unsuccessful establishment of the animal trypanosomes in human (Aksoy, 2011; Aksoy *et al.*, 2017). However, wild and domestic animals might play a major role as reservoirs of human infections (Njiokou *et al.*, 2006).

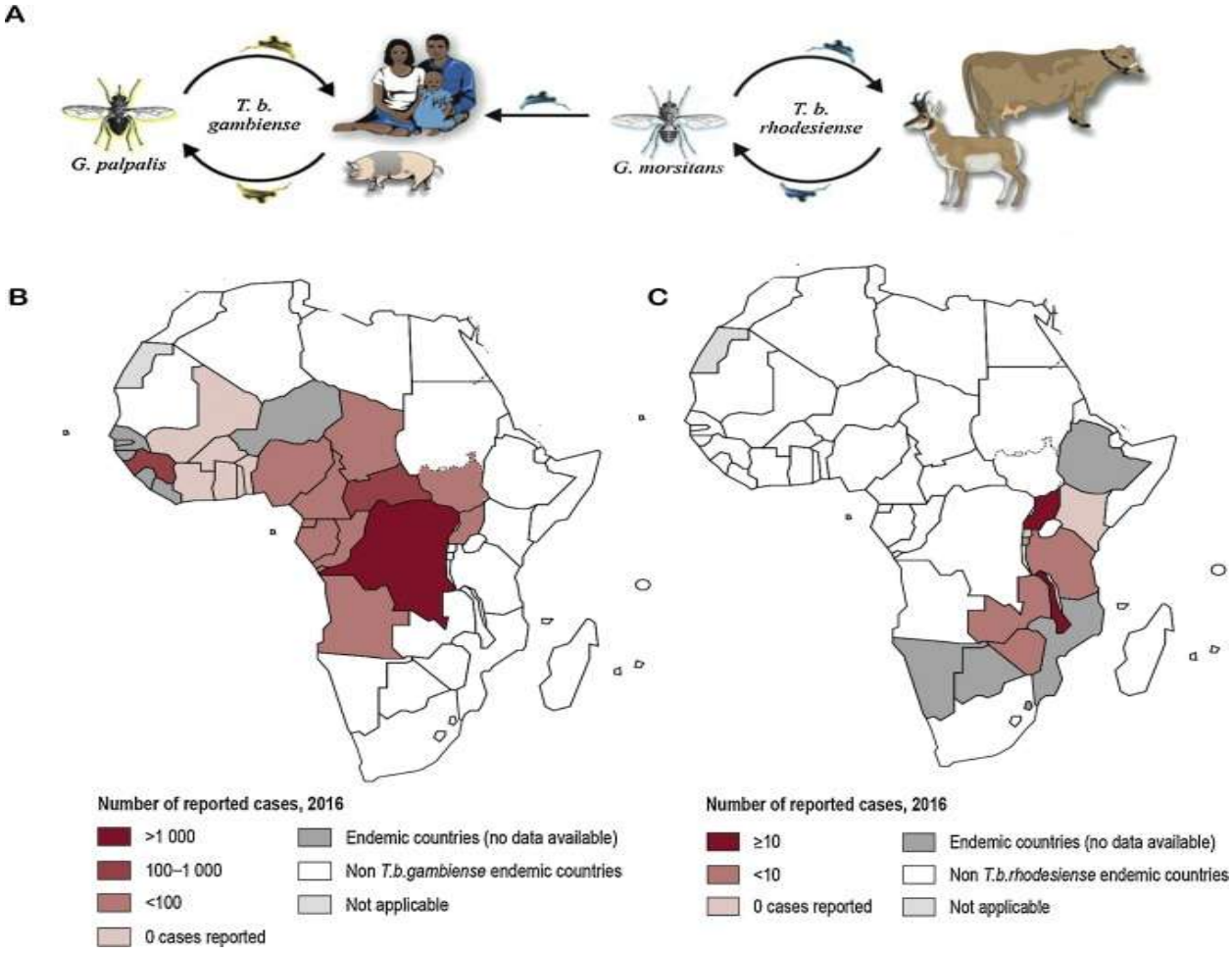

Figure 2.2: The transmission cycle and distribution of the two forms of human African trypanosomiasis (HAT)(Kristensson *et al*., 2010; http://www.who.int/en/).

2.3 Trypanosomes

Trypanosomes are eukaryotic haemo-flagellates that cause fatal diseases in man and animals. They were described as two hosts obligate parasites that multiplied in body fluids (especially blood) of their vertebrate hosts but habited the digestive tract of the invertebrate hosts, generally biting insects (Itard, 1981; Mbaya *et al*., 2010). The existence of several variations in the strains of trypanosomes had been acknowledged to influence the disease process and disease severity in the affected hosts (Garcia *et al*., 2006; Sternberg and MacLean, 2010). Research showed that the virulence of trypanosome was selective and some selected traits in their genes, such as virulence factors, which become determinant factors of the disease expression and outcome had been identified (Garside and Brewer, 2008; Morrison, 2011).

2.3.1 Geographical distribution

Due to the biological association, the geographical distribution of trypanosomes was found to be closely related to that of tsetse flies which are endemic in Africa between latitude 15^0N and 20^0S, from the southern edge of Sahara-desert to Zimbabwe, Angola, and Mozambique. However, *T. vivax* has spread beyond the "tsetse fly belt" into the south and Central America and the Caribbean areas by transmission through mechanical vectors (Shereni *et al*., 2016).

2.4 Classification of African Trypanosomes

Trypanosomes are classified under kingdom Protista, sub-kingdom protozoa, phylum Sarcomastigophora, class Mastigophora (although there were some differences of opinion among taxonomists on the exact application of these terms), order Kinetoplastida, family Trypanosomatidaeand genus *Trypanosoma* (Hoare, 1972). Genus *Trypanosoma*was further subdivided into two sections, salivaria and stercoraria (Fig. 2.3). Salivarian trypanosomes mature in the salivary medium of the "anterior station" and got transmitted by inoculation into susceptible vertebrate hosts (Hoare 1972), with the exact location of this maturation in the salivary glands (e.g.,*T. brucei* spp.) or the proboscis (*T. congolense* and *T. vivax*) (Vickerman, 1985; Peacock *et al*, 2012). Section salivaria consisted of four subgenera, including *Trypanozoon* (*T. brucei brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. evansi*,and *T.equiperdum*, *T. equinum), Duttonella* (*T. vivax* and *T. uniforme)*; *Nannomonas* (*T. congolense* and *T. simiae)* and *Pycnomonas* (*T. suis*) (Hoare 1972; Radwanska *et al*., 2018). Apart from *T. b. rhodesiense* and *T. b.gambiense* which causes disease in humans, other members of section salivaria cause disease in livestock and wildlife. Domestic and wild animals were further identified as reservoirs of the zoonotic *T. b. rhodesiense* (Simarro *et al*., 2008) and less commonly of *T. b. gambiense* (Cordon–Obros *et al.*, 2009); *T. b. gambiense* was mainly assumed to be anthroponotic (Simarro *et al.*, 2011). Section stercoraria had only one pathogenic trypanosome, *T. cruzi*, which causes Chagas disease (American trypanosomiasis). *T. cruzi* matures in the fecal medium of the "posterior chamber" of the Triatomine insect, also called reduviid or kissing bug, and was therefore transmitted through contamination of bite wounds with the insect's feces (Hoare, 1972; OIE, 2012).

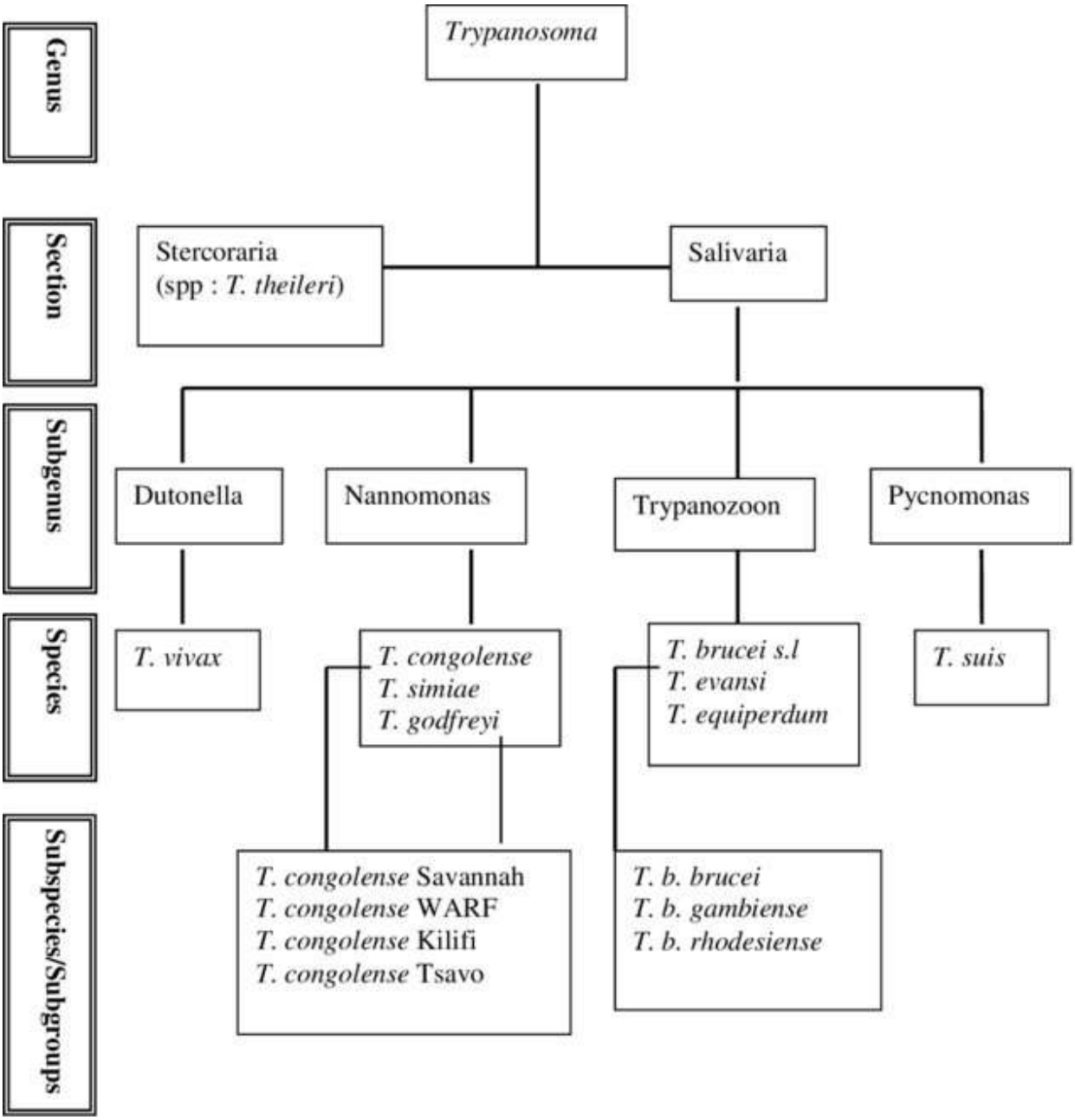

Figure 2.3: Classification of pathogenic human and animal trypanosome species (Masumu Mulumbu, 2006).

2.5 Transmission of African Animal Trypanosomiasis

Arthropod are the intermediate hosts responsible for both cyclical and non-cyclical transmission of trypanosomes. In cyclical transmission, the trypanosomes multiply in the proboscis of the arthropod, where they undergo a series of morphological transformations into the infective forms of the parasite (anterior station development) and the infection is transmitted during feeding (Fig. 2.4) (Urquhart *et al*., 2002). However, with posterior station development, multiplication and transformation occurred in the gut and the infective forms migrate to the rectum and are passed in the feces. All trypanosomes (except *T. equiperdium*, and *T. evansi*) are transmitted cyclically by the tsetse fly (Soulsby, 1982; Sterverding, 2008). In non-cyclical transmission, trypanosomes are transmitted from one mammalian host to another by the interrupted feeding of biting insects, notably Tabanids and Stomoxys (Maxie *et al*., 1979; Diall *et al*., 2017) and this mode of transmission is responsible for the persistence of *T. vivax* in tsetse-free zones (Maxie *et al*, 1979; Sterverding, 2008).

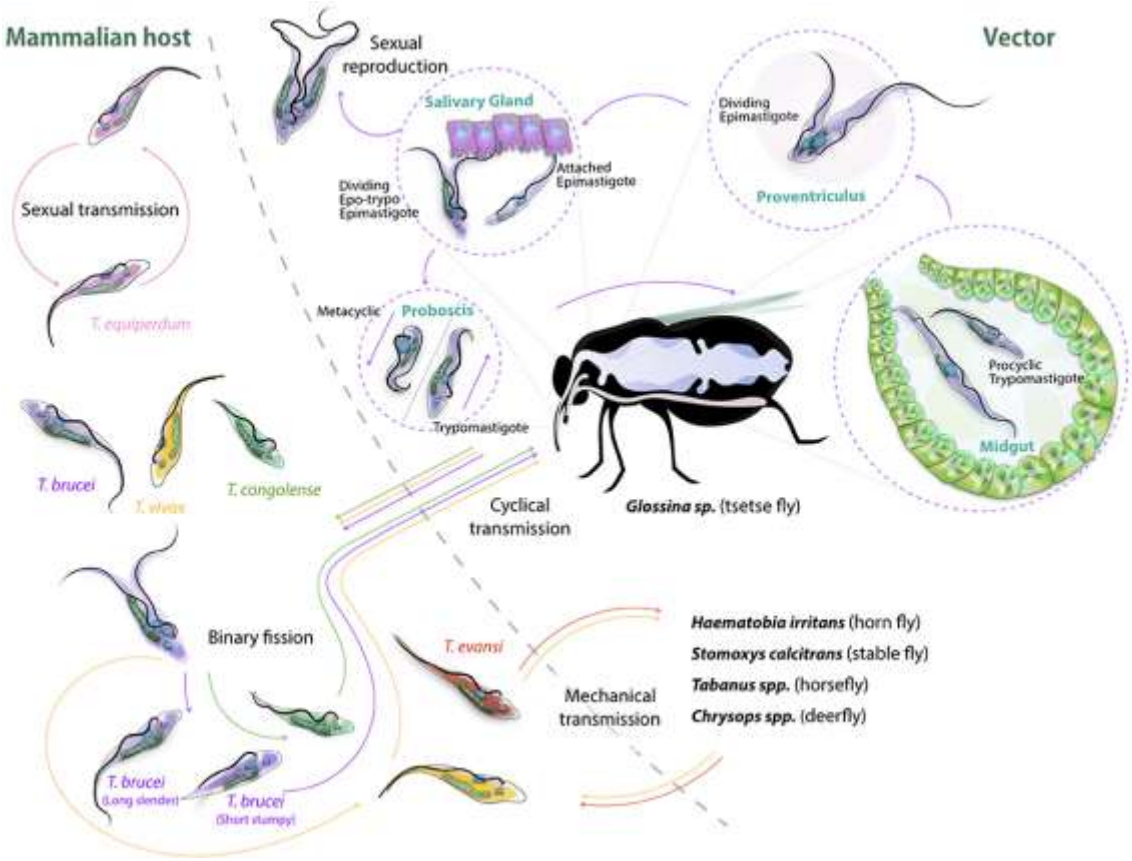

Figure 2.4: Cyclic transmission of salivarian trypanosomes mediated by tsetse and other biting flies (Kristensson *et al*., 2010).

2.5.1 Role of reservoir hosts in the disease transmission

Wild and domestic animals are reservoir hosts of Africa trypanosomiasis and play an important role in disease transmission. Reservoir hosts can carry pathogens indefinitely without displaying any clinical symptoms. This phenomenon complicated the epizootiology of AAT greatly, as elimination of the disease required consideration of and control measures for the reservoir hosts as well (Clair, 1988; Sterverding, 2008).

2.5.2 Life cycle

Most tsetse-transmissionsare cyclical,which begins with the ingestion of blood by a tsetse fly from a trypanosome infected animal (Fig. 2.5); *Trypanosoma brucei* species were reported to migrate from the gut to the proventriculus, the pharynx and eventually to the salivary glands; the cycle for *T. congolense* stopped at the hypopharynx and the salivary glands were not invaded; the entire cycle for *T. vivax* occurred in the proboscis(Urquhart *et al*., 2002). The vectors ingest trypanosomes during their feeding on an infected host and the trypanosomes subsequently, undergo a transformation losing their typical coat to form trypomastigote, the bloodstream trypomastigotes transform into procyclic trypomastigotes in the tsetse midgut where they proliferate by binary fission. The procyclic trypanosomes migrate to the salivary glands of the tsetse where they first transform into epimastigotes and then undergo further binary fission before transforming into metacyclic trypomastigotes (Fig. 2.6). The period from ingestion of bloodstream trypomastigotes to the establishment of an infection within the tsetse fly and transformation to metacyclic trypomastigotes lasts approximately 3 weeks (CDC, 2019), after which the tsetse fly becomes infective. The trypanosomeloses its surface coat, multiplies in the fly, then re-acquired a surface coat and

becomes infective. The animal-infective form in the tsetse salivary gland is referred to as the metacyclic form. The life cycle in the tsetse might be as short as one week with *T. vivax* or extend to a few weeks for *T. brucei* species (Murray and Gray, 1984; Pays *et al*., 2006).

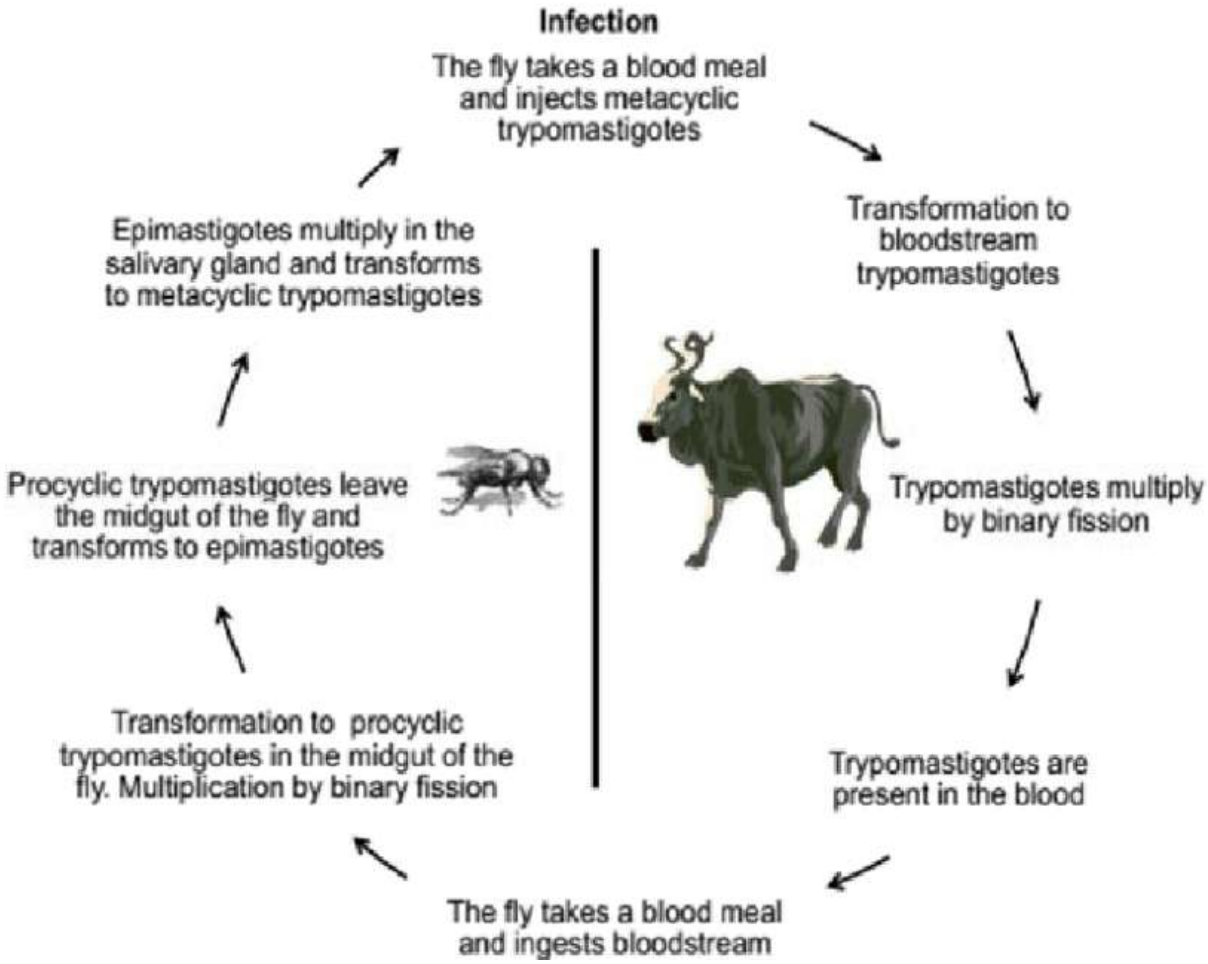

Figure 2.5: The main phases in the life cycle of the trypanosome, both in the intermediate host (tsetse fly) and in the mammalian host (Dwinger and Hall, 2000).

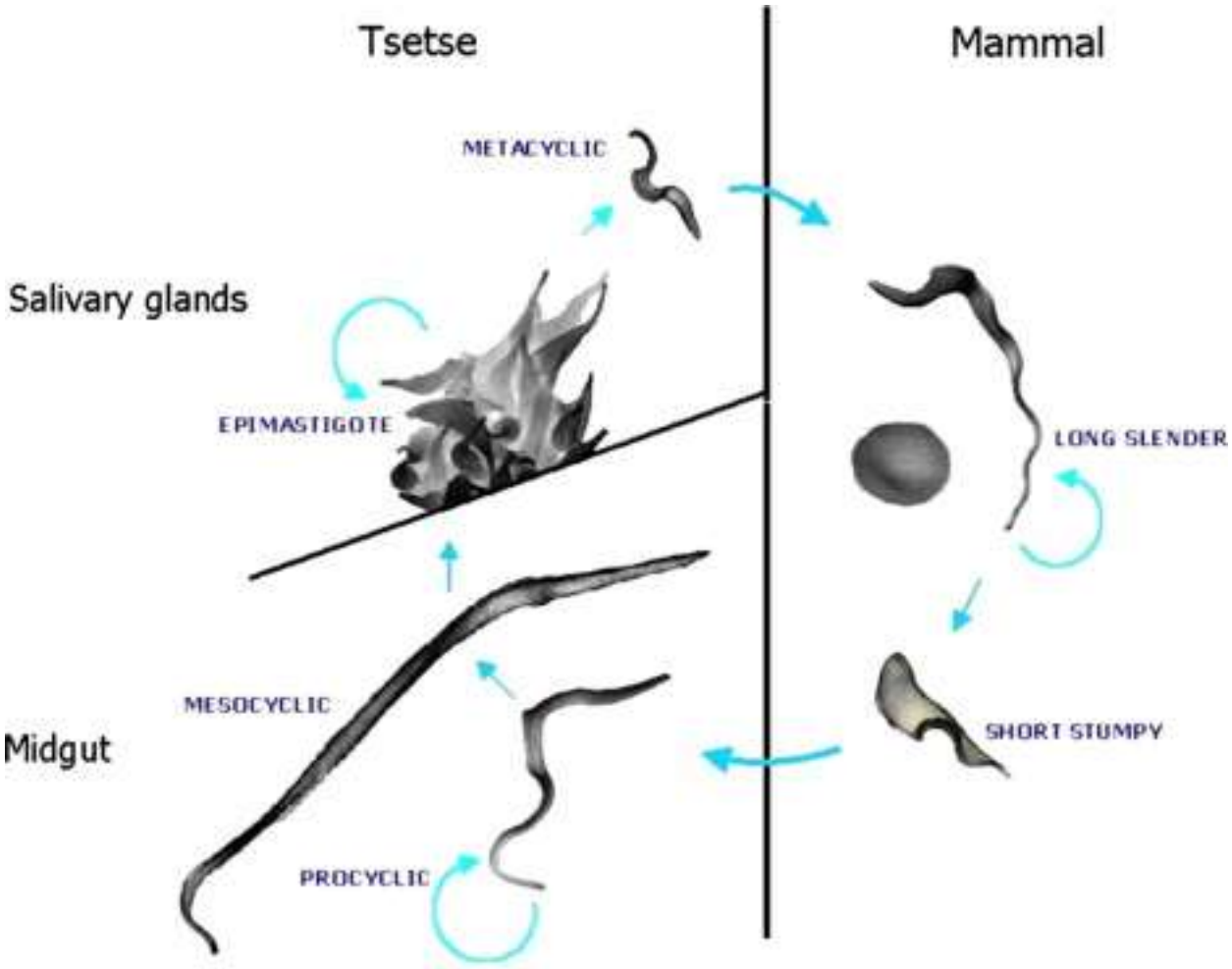

Figure 2.6: The life cycle of *Trypanosoma brucei* (courtesy of Laurence Tetley and Dave Barry of Glasgow University).

2.5.3 Pathogenesis

Animals become infected with trypanosomes through an infected tsetse fly bites, during the process of taking a blood meal, and the infected tsetse fly deposits saliva laden with trypanosomes in the connective tissue of the host's skin. The metacyclic forms multiply locally at the site of inoculation and differentiate to the bloodstream form, which had specially adapted to live in mammalian blood, producing within a few days, an indurated raised cutaneous inflammatory swelling on the host skin called chancre in few days (Urquhart *et al*., 2002). Thereafter, they enter the blood circulation via lymph vessels and can survive in the blood circulation throughout the infection of the host (Akol and Murray, 1983; Esievo and Saror, 1991). The*T. brucei* species can penetrate the walls of capillaries, invade interstitial tissues and remain extracellular, while *T. congolense* tends to bind to walls of capillaries and small vessels of infected cattle and mice, the mechanism of which has been unknown (Banks, 1978; Taylor and Authié, 2004). Within the bloodstream, the parasites multiply by binary fission and become disseminated into other tissues through the hematogenous route. Parasitemia characterized by intermittent fever and anemia is evident within 1-3 weekspost-infection and may persist for many months, although its levels may wax and wane due to the immune response of the host (ILRAD, 1990b). Fever is highest at the first peak of parasitemia and fluctuates thereafter with level of parasitemia (Taylor and Authié, 2004).

The pathogenesis of trypanosomiasis can best be described under the following headings: chancre, lymphadenopathy, anemia, and tissue damage.

2.5.2.1Chancre

A Chancre is the formation of an indurated cutaneous inflammatory swelling produced within few days after insect “bite” at the site of inoculation of the metacyclic forms of the trypanosomes which multiply locally.This local response in the skin corresponds to the first protection developed by the host (Taylor and Authié, 2004). Trypanosomes were detected in efferent lymphatic vessels 1-2 days before chancre formation and their numbers declined during the development of the chancre (6 days) followed with an increase later on. In *T. congolense* infected sheep, neutrophils were in abundance in the early days and then T and B lymphocytes infiltrated the chancre. Later, T lymphocytes predominated, especially CD8+ T cells (Vincendeau and Bouteille, 2006). An early response due to an increase in CD4+ and CD8+ T cells was revealed by flow cytometry in the afferent lymph that drains the chancre and as the chancres regressed there was an increase in lymphoblasts and surface immunoglobulin bearing cells (Urquhart *et al*., 2002). During this first stage, trypanosomes expressed variable antigen types (VATs) found characteristically in the tsetse fly, which changed after a few days. An antibody response specific to these VATs appeared in the lymph and then in the plasma (Vincendeau and Bouteille, 2006). Generally, all the three cyclically transmitted species eventually induced chancres (Vickerman and Barry, 1982); although the local skin reaction was less severe with *T. vivax* infections (Taylor and Authié, 2004)

2.5.2.2Lymphadenopathy

Lymphadenopathy is characterized by generalized enlargement of lymph nodes and subsequent splenomegaly. This is associated with a marked proliferation of lymphoid cells

in the organs. In the medullar cords of lymph nodes and splenic red pulp, there are increases in plasma cells and numerous large active germinal centers andan increase in the number of activated macrophages (engaged in erythrophagocytosis) in the splenic red pulp (Hendrickx *et al*., 2004; Taylor and Authié, 2004).

2.5.2.3Anaemia

The onset and severity of anemia are directly related to the appearance of the parasites in the blood and the level of the parasitemia. According to Saror (1980), the development of anemia was a well-recognized and inevitable consequence of trypanosome infection in domestic animals. Anaemia isthe principal factor involved in the pathology of African trypanosomiasis in humans and animals; although the mechanisms through which the anaemia occurred remained debatable (Luckins, 1999; Mbaya *et al*., 2011, Esievo, 2017). However, hemolysis was involved at the early stage of the infection as a result of erythrophagocytosis by the mononuclear phagocyte system (Saror, 1980) which accounted for an extravascular haemolysis. Immunologic mechanisms also played a role in the trypanosomiasis induced anemia, which rendered the erythrocytes more susceptible to phagocytosis (Fatihu *et al*., 2008).

Trypanosomes produce sialidase, a potent enzyme that cleaved off the membrane surface sialic acid of erythrocytes which resulted in a shortened life-span of erythrocytes by exposing the galactose of the cell membrane which getsreadily bound to lectin on the surface of Kupffer cells and other macrophages, leading to erythrophagocytosis (Nok and Balogun, 2003; Adamu *et al*., 2008; Fatihu *et al*., 2008; Esievo, 2017). The initial fall in packed cell volume was associated with the first wave of parasitemia in the blood, due to

anemia from extravascular haemolysis as a result of increased red blood cell destruction by erythrophagocytosis in the spleen, lungs, hemal nodes and bone marrow by direct traumatic effect on red cells thereby increasing red cell fragility (ILRAD, 1990b; Mbaya *et al*., 2012). According to (Morrison *et al*., 2009), packed cell volume (PCV) progressively decreased by about 40-50% over the first 4-6 weeks in Trypanosome-infected cattle subjected to a single fly challenge.

2.5.2.4 Tissue damage

Many factors were believed to act in concert to cause cellular injury and ultimately tissue damage in animal trypanosomiasis. These factors might have emanated from the physical and metabolic activities of the *Trypanosoma species*, trypanosome autolysates, and oxidative lipoperoxidation, among others (Igbokwe, 1994). The extent of tissue invasion varied with the Trypanosome species involved, with the *T. brucei* group being the most tissue invasive, followed by the *T. vivax* and *T. congolense* which were rather more restricted to the blood circulation (Igbokwe, 1994). Trypanosomes might cause direct mechanical injury to erythrocytes and other cells by the lashing action of their locomotory flagella and their microtubule reinforced bodies (Vickerman and Tetley, 1978; Igbokwe, 1994). The trypanosomes also produced numerous biochemical substances that were considered toxic which further worsened the severity of the tissue damage suffered by the affected host.This included among others protease, neuraminidase, phospholipase, and some other toxic metabolites (Igbokwe, 1994).

i. Proteases: These are circulating proteolytic enzymes were reported in trypanosomes infected hosts (Vickerman and Tetley, 1978), which probably caused tissue

inflammation and ultimately tissue damage (Cook *et al.*, 1966; Seed and Hall, 1985).

ii. Neuraminidases: It has been suggested that the trypanosomesproduced neuraminidase *in vivo* during the infection, which cleaved off the surface sialic acids of blood cell and rendered them more prone to phagocytosis (Esievo, 1979; Esievo, 1983).

iii. Phospholipases and free fatty acids: The trypanosomes which generated phospholipases caused cell destruction or haemolysis (Tizard *et al.*, 1978). They also produced free fatty acids (FFA) which were considered relatively safe at minute quantity since they merely bound to plasma, However, other studies revealed that bound FFA might also be cytotoxic due to the activities of its oxidized product (Assoku *et al.*, 1977; Assoku and Tizard, 1978).

iv. Pyruvate: Pyruvate, the end product of glucose metabolism by the trypanosomes, is readily metabolized by the host for energy. However, pyruvate was found to accumulate in the blood of trypanosome infected animals in an amount directly proportional to their level of parasitaemia (Tizard *et al*., 1978). It was thought that the pyruvate in the blood might cause acidosis and probably lower the affinity of haemoglobin for oxygen, hence contributing to the development of tissue pathologies, in the infected hosts (Newton, 1978).

v. Aromatic by-products: Trypanosomes are capable of metabolizing aromatic amino acids to form toxic by-products (Seed and Hall, 1985). Phenylpyruvate possesses proteolytic activity and may inhibit glycogenesis and mitochondrial function, while indole-ethanol (tryptophol), a by-product of tryptophan catabolism acted on the cell membranes causing osmotic fragility and lysis (Igbokwe, 1994).

These listed factors and mechanisms of cellular injury in trypanosomiasis varied in their capacity to cause tissue damage and were dependent on the phases of the disease (Igbokwe, 1994).

2.5.4 Trypanotolerance

Trypanotolerance was defined as the ability of some breeds of animals to survive and be productive in tsetse infested areas without the aid of treatment, where other breeds succumbed to the disease (Murray *et al*.,1990; Murray *et al*., 1998). Certain breeds of cattle such as the N'Dama and the West African Shorthorn breeds showed more level of resistance to trypanosomiasis than breeds like Zebu and European cattle. Even though this feature only applied to about one-third of cattle in tsetse infested areas in Africa and 10% of cattle south of the Sahara, the production potential of these breeds was great, and breeding of these cattle would result in a solution to the AAT problem (Murray *et al*., 1982; Murray *et al*., 1998). Comparative studies between trypanotolerant and trypanosusceptible animals showed some differences in their antibody immune responses (antibody production, complement levels and cytokine resistance) and sialic acid contents which showed differences in chains and their electromagnetic charges (Esievo *et al*., 1986; Naessens *et al*., 2002).

2.6 Development of Immunity against Trypanosome infection

2.6.1 Host-parasite interaction

Interaction refers to the interdependent operation of factors to produce effect. The outcome of disease occurrence depends on the interplay of the host, the agent (the parasite), and environmental factors. By adding or modifying the factors the frequency of the occurrence of the disease can be changed (Thrusfield, 2018).

2.6.2 Trypanosome antigens and exposure to the host's immune system

African trypanosomes persist for a long period within the mammalian blood and tissues, where they are surrounded by a dense immunogenic surface coat (12–15 nm thick) of a single polypeptide species referred to as the variant surface glycoprotein (VSG) (Maxie and Losos, 1977; Vickerman *et al*., 1988; Cross, 1990; Turner *et al*., 1995) that shields invariant surface antigens from immune recognition. Moreover, trypanosomes constantly modified their VSG by the process of antigenic variation, thus, they evade the host's response which results in fluctuating waves of parasitemia that characterized African trypanosomiasis (Borst, 2002; Baral *et al*., 2006; McGovern and Wilson, 2013).

During African trypanosomiasis, a complex interaction between the host immune responses and parasite survival strategies occurs. As such, natural selection had enabled African trypanosomes to develop very sophisticated mechanisms to evade immune killing and survive in the chronically infected host and hence allow for the transmission to the next host, via the tsetse vector. Well-documented evasion mechanisms includedan antigenic

variation of the VSG (Morrison *et al*., 2009; Morrison, 2011) and the induction of alterations in the host's defense system (Stijlemans *et al*., 2007).

2.6.2.1Antigenic variation

Antigenic variation remains one of the most spectacular adaptive mechanisms exhibited by African trypanosomes and is the central most important immune escape mechanism by these parasites (Barry and McCulloch, 2001; Borst, 2002). Trypanosomes contain up to 1000 different genes in their genome which afford them extensive opportunities to escape host immune responses by displaying new coat antigens (Morrison *et al*., 2009). The parasites have intrinsic mechanisms that ensure that only one VSG gene is transcribed at any given time. By switching VSG genes and expressing a new variant antigenic type, trypanosomes evade B-cell and T-cell mediated immune responses. Furthermore, expression of VSG was reported to be central to the process of antigenic variation that eventually led to exhaustion of the host immune system for the benefit of the trypanosome (Borst, 2002; Gomez-Rodriquez *et al*., 2009).

2.6.2.2 Induction of alterations in the host's defense system

African trypanosomes developed several ways of evading immune killing through alteration of the host's immune responses because of the strong selection pressure from continuous contact with the host's immune system. For example,*T. congolense* and *T. b. brucei* induced a generalized state of immunosuppression following infection of cattle or mice, mediated by both MFs and T cells with suppressive phenotypes (Kodelja *et al*., 1998; Namangala *et al*., 2009).

2.6.3 Host immunologic responses to trypanosome infections

The inoculation of trypanosomes into their mammalian hosts triggers a series of events involving, at first, innate immunity and, secondarily, specific immunity. The latter requires an efficient presentation of parasitic antigens, activation of T and B cells implying specific antigen receptor recognition, and the development of effector cells and molecules:these mechanisms are highly regulated by multiple signals delivered through a large number of receptors transduced across the plasma membrane and processed(MacAskill *et al*., 1981; Pinder *et al*., 1986; Frevert and Reinwald, 1990; Kaushik *et al*., 2000; Billiau and Vandenbroeck, 2001; Taylor and Rudenko, 2006; Magez *et al*., 2008; Morrison *et al*., 2009).

2.6.4 Trypanosome-induced professional antigen-presenting cell defects

Macrophages and dendritic cells played a central role in the immune response as professional antigen-presenting cells. This function included internalization of trypanosomes and/or their VSG through phagocytosis, processing of parasite antigens in the acidic compartment of the endocytic pathway, co-stimulation and presentation of the immunogenic trypanosome peptides to antigen-specific T-helper cells (Th cells) in the context of MHC Class II molecules (Namangala *et al*., 2000; Shi *et al*., 2006).

2.6.5 Immunopathology

Infection by trypanosomes resulted in a rising parasitemia. Subsequently, a T-cell-independent antibody response to each VSG led to massive phagocytosis of trypanosomes by macrophages, especially in the liver and spleen. Most of the early product of anti-VSG

was of the IgM class. The phagocytosis of trypanosomes then led to rapid activation of the Kupffer cells in the liver with the release of monokines and enlargement of the Kupffer cells (Tabel *et al*., 2008).

2.7 ClinicopathologicalPresentation of Animal African Trypanosomiasis

2.7.1 Clinical signs of the disease

Animals suffering from trypanosomiasis might manifest syndromes ranging from subclinical, mild, or chronic infections to acute or fatal forms of the disease (Holmes *et al*., 2000).Like other infectious diseases, trypanosomiasis start with an increase in body temperature (hyperthermia), as a result of the contact between the trypanosomes multiplying in the host and the defense system of the host (FAO, 2008). The severity of the disease depended on the pathogenicity of the strain, the animal hosts, breed, genotype, age, sex, skin type, and most importantly, on the method by which the infection was induced that is natural or artificial. While other factors such as stress, poor nutrition, and concurrent diseases also played significant roles (Holmes *et al*., 2000). Dogs and cats are susceptible to *T. brucei* and *T. congolense* (Nfon *et al*., 2000). *Trypanosoma brucei* produced mild, chronic, or subclinical infection, except in horse, camel, dog, and cat where it caused severe to fatal infection which if untreated almost invariably caused mortality (Ezeokonkwo *et al*., 2012). The prepatent period (5-10 days) was characterized by intermittent fever, depression, lethargy, and weakness. The heartbeat and respiratory rate might be increased as well as progressive anemia, loss of body condition, and generalized enlargement of superficial lymph glands. Abortion was observed in females (Anene and Omamegbe, 1984;

Anene *et al*., 1991). At the terminal stage of the disease, animals became extremely weak and death was associated with congestive heart failure due to anemia and myocarditis or secondary bacterial or viral infections. The secondary infections were believed to develop because the immune defense mechanisms get compromised in trypanosome infected animals (Ikeme *et al*., 1984; Anene *et al*., 1989).

2.7.1 Haematological changes in trypanosomiasis

The presence of trypanosomes in the blood is accompanied by numerous changes in the cellular and biochemical constituents of blood (Taiwo *et al*., 2003; Ngure *et al.,* 2008). These includeanemiawhich is associated with significantdecrease in packed cell volume (PCV), hemoglobin concentration and red blood cell (RBC) count in different animal species, as a result of massive erythrophagocytosis by an expanded and active mononuclear phagocytic system of the host (Igbokwe and Nwosu 1997; Lukins 1999).

The mechanism or pathophysiology of anemia in trypanosomiasis is complex and multifactorial in origin (Naessens *et al*., 2005). Anemia regarded as the most consistent finding in trypanosomiasis wasreported in *T. vivax*-infected cattle and goats and *T. congolense*-infected sheep (Bisalla *et al.,* 2007), *T. congolense*-infected dogs (Gow *et al*., 2007), *and T. brucei*-infected goats, sheep, and rabbits (Seed *et al.*,1969; Taiwo *et al.*, 2003). With *T. brucei* infection, the phenomenon of self-recovery from anaemia has been widely documented (Abenga, 2011). Significant increases were encountered in mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) values. No significant change was observed in mean corpuscular hemoglobin (MCH) (Abenga, 2011).

Thrombocytopenia has been reported in *T. congolense* infection (Wellde *et al*., 1978; Logan-Henfrey *et al*., 1999) particularly in the early phase of the infection due to a shortened platelet life span (Esien and Ikede, 1978). The WBC countdecreases during the early acute or sub-acute phase of trypanosome infections (Anosa, 1980; Anosa and Isoun 1980) but elevated leucocyte values were reported in *T. brucei* and *T. congolense* infected dogs at the later stage of the infection (Anene *et al*., 1989) and *T. brucei* infections in highly tolerant deer mice (Anosa and Kaneko, 1983). During the chronic phase of infections, the blood leucocyte values recovered gradually and sometimes attained pre-infection values (Anosa and Isoun, 1980). This may explain why Osman *et al*. (2012) observed that total leucocyte counts remained normal during infection.

The decrease in lymphocyte count was reported in the acute phase of trypanosome infection (Maxie *et al*., 1979; Anosa, 1980), although increased numbers were associated with *T. brucei* infection of highly tolerant deer mice (Anosa and Kaneko, 1983) and with human trypanosomiasis (Anosa, 1980). Lymphopenia occurred partly because of depletion of lymphocytes from lymphoid follicles which occurred in acute *T. vivax* infection (Anosa and Isoun, 1980) and partly because of the sequestration of many lymphocytes in the inflammatory reactions in *T. vivax* infections of ruminants and *T. brucei* infection in mice (Anosa and Kaneko, 1983; Esievo and Saror, 1983). Monocytosis is not an unusual in trypanosomiasis (Isoun, 1975; Anosa, 1980; Anosa and Isoun, 1980), usually coexisting with a marked proliferation of macrophages in the tissues of infected animals (Bezie *et al.*, 2014; Odeyemi *et al.*, 2015).

2.7.3 Biochemical changes in trypanosomiasis

Biochemical changes were observed in association with trypanosome infection in animals and several factors have been found to influence the nature and severity of these changes. These factors included the strain of the infecting trypanosome and host variability in susceptibility to infection. Biochemical evaluation of the body fluids indicated the functional state of the various body organs (Anosa, 1988). Varying such biochemical changes were also reported in studies of trypanosomiasis in animals by Awobode (2006) and Ezeokonkwo *et al*. (2012).

Significant increase in the serum activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) on single or mixed experimental infection in twenty mongrel dogs with *T. b. brucei* and *T. congolense* were observed in the past (Ezeokonkwo *et al*., 2012). These observations weresimilar to those of Obidike *et al*. (2005) and Akpa *et al*. (2008).

Such elevation in activities of ALP, ALT, and AST indicated that the integrity of the liver was compromised following infection of the dogs with the trypanosomes. In dogs, the ALP and ALT levels had relatively narrow range under normal circumstances and hence a rise in the level in serum was an indicator of hepatic malfunction (Lording and Friend, 1991; Enwezor and Sackey, 2005; Ezeokonkwo *et al*., 2012).

Serum total protein was reported as either increased, decreased, or within normal range by different researchers on African trypanosomiasis (Anosa, 1988; Abenga, 2011). Increase in serum globulin (chiefly gamma-globulin) has been consistent and implicated with the overall increase in total protein.The serum albumin however decreased leading to a fall in

albumin/globulin ratio (Abenga and Anosa, 2005). Adejinmi and Akinboade (2000) reported a decrease in serum total proteins with a lower level of albumin and albumin/globulin ratio in *T. brucei*-infected goats which had a mixed infection with *cowdria ruminantum*.

Elevation in serum creatinine during Trypanosoma infection was linked to either damage to host tissues or renal disorder as a result of the infection (Abenga and Anosa, 2005).This was corroborated with high levels of serum urea to confirm renal disorder and joint increases in the levels of serum creatinine and urea were observed in experimental trypanosomiasis (Abenga and Anosa, 2005; Abenga, 2011; Bakari *et al.*, 2017).

2.7.4 Gross pathology

The gross post-mortem lesions observed in sacrificed *T. congolense* infected rabbits were varying degrees of emaciation, dehydration, mucopurulent oculo-nasal discharges and pasted perineum. The lungs were congested and there wasserous atrophy of the perirenal, pericardia, and abdominal fats. In addition, splenomegaly and hepatomegaly were observed and the liver was greyish with depressed focal areas of necrosis. The skeletal muscles were pale (Takeet and Fagbemi, 2009). Similarly, a study on the comparative pathology of the lymph nodes, spleen, liver, and kidney in experimental ovine infection with *T. brucei*, *T. congolense,*and *T. vivax* revealed cachexia, serous atrophy of fat and enlargement of the prescapular and pre-femoral lymph nodes, spleen, liver, and kidneys which were more pronounced in chronic infections(Omotainse and Anosa, 2009).

2.7.5 Histopathology

The interstitial activities of trypanosomes in tissues have been shown to attract severe inflammatory reactions in various organs (Ormerod, 1970; Losos and Ikede, 1972; Poltera, 1985; Abdullahi *et al.*, 2018). According to Losos and Ikede (1972), the lesions of trypanosomiasis were characterized by interstitial and perivascular mononuclear cell infiltrations associated with extravascular localization of trypanosomes in connective tissues. In chronic experimental *T. brucei*-infected rabbits, there were severe granulomatous inflammation with the predominance of macrophages, epithelial giant cells, and polymorphonuclear leucocytes (Abdullahi *et al.*, 2018).

Hepatic lesions reported by Omotainse and Anosa (2009), were vascular congestion, perivascular cuffing by mononuclear cells, disorganization of the hepatic cords with hepatocellular degeneration and erythrophagocytosis. In donkeys infected with *T. b. brucei*, the liver was reported to show marked hemosiderosis, centrilobular congestion, and fatty change (Ikede, *et al.*, 1977).Omotainse and Anosa (2009), reported renal congestion, perivascular and interstitial mononuclear cell infiltration, thickening of glomerular capsules, desquamation of tubular cells and protein casts in the tubules. These observations were consistent with the glomerulonephritis earlier reported by Anosa and Kaneko (1983), apart from the thickened glomerular capsules, thickened intratubular and perivascular spaces due to infiltration by lymphocytes, plasma cells, and macrophages, in *T. brucei*-infected rodents.

Histopathological lesions on the spleen and lymph nodes reported in canine *T. brucei* infection were diffuse inflammatory reactions with hemorrhages, necrosis, and cellular

infiltrations with polymorphonuclear cells, lymphocyte, and plasma (Kaggwa *et al.*, 1984). The spleen, lymph nodes, and liver showed activated macrophages, erythrophagocytosis and hemosiderosis indicating massive destruction of red blood cells in these organs (liver with the lowest severity), and that is the reason why these organs were proposed as the sites of erythrocyte destructions in trypanosomiasis (Omotainse and Anosa, 2009).

2.8 Diagnosis of African Trypanosomiasis

Trypanosoma infections were often diagnosed based on clinical manifestations of the disease and the use of parasitological techniques (Enyaru *et al.*, 2010). The clinical signs of the animal trypanosomiasis are indicative but not pathognomonic, a reason for which further laboratory confirmation was required in its diagnosis (Elnasri, 2005). The classical direct parasitological methods for the diagnosis of trypanosomiasis, namely microscopic examination of blood or lymph node material, were not highly sensitive, but several techniques, including enrichment of the sample, rodent inoculation, and DNA methods might increase the sensitivity (Salih *et al.*, 2015). Diagnosis of trypanosomiasis could best be described under the following heading:

2.8.1 Clinical diagnosis

Although, the clinical signs of trypanosomiasis are not pathognomonic of the disease, their existence help raise strong suspicion about existence of the disease. The clinical diagnosis was found to have good sensitivity (78%) but low specificity (27%) when compared to parasitological tests and that treatment of cattle based on clinical examination may clear up

to 87.5% or 78% of the cases that would be positive by either molecular or parasitological diagnosis, respectively(Soulsby, 1982). Under field conditions, in the absence of simple and portable diagnostic tools or access to laboratory facilities, veterinarians could rely on clinical signs and direct parasitological diagnosis to screen and treat cases of bovine trypanosomiasis presented by farmers (Melaku and Birasa, 2013).

2.8.2 Parasitological methods of diagnosis

The basic techniques included the use of fresh blood samples (wet mount) or stained blood film (thin or thick smear). This method of diagnosis was used as reference method because of its usefulness in remote settings where the tsetse and trypanosomes were prevalent and laboratory infrastructures were limited.Although, these techniques were laborious requiring expertise in microscopy, other disadvantages included low sensitivity, failure to identify mixed and distinguish immature infections and hence, trypanosomes description beyond the subgenus level was not possible (Enyaru *et al.*, 2010).

Modification was to improve the diagnostic sensitivity of these techniques by concentrating the blood through centrifugation in a hematocrit tube by the hematocrit centrifuge technique (HCT) or the dark ground buffy coat technique (DG) (Woo, 1970). The HCT became one of the most widely used parasitological techniques. Motile trypanosomes were viewed between the leucocyte layer and plasma, enabling the detection of trypanosomes six to ten days before they could be detected in either thin or thick smear (Woo, 1970; Molyneux, 1975). In order of decreasing sensitivity, the results were as follows: DG>HCT> thick smear > thin smear > wet mount (Paris *et al.,* 1982). Suggested modification although not routinely used, involved the separation or removal of blood cells before centrifugation

by miniature anion exchange chromatography or hypotonic lysis (Nantulya, 1990). Freshly collected blood could also be inoculated into laboratory rodents which could then be examined for periods of 30 to 60 days for the development of trypanosome infection (Paris *et al.,* 1982; Enyaru *et al.*, 2010).

2.8.3 Serological techniques of diagnosis

A good number of serological tests such as immunofluorescence (IFA) and enzyme-linked immunosorbent assay (ELISA) for direct and indirect diagnosis of trypanosomes in the infected host became available, for the detection of Trypanosoma antibodies but they had rather poor specificity, because of cross-reaction from the different species of the genus *Trypanosoma*. The species specificity of the assay was improved following the development of monoclonal antibodies as captured antibodies that recognized the specific circulating antigens in the infected host (Nantulya and Lindqvist, 1989; Nantulya, 1990). Serological assays such as CATT (Card Agglutination Test for Trypanosomes) had been used for detecting *T. b. gambiense* (Magnus *et al.*, 1978*)* identify trypanosomes indirectly by detecting antibodies in mammalian hosts whereas others like dot-ELISA could be used in detecting antigens in tsetse by the use of known antibodies (Ouma *et al.*, 2000).

2.8.4 Isolation and purification of blood trypanosomes

For isolation of blood trypanosomes, different biochemical, biological, and physical methods had been used. Like the ion-exchange chromatography which is a biochemically based procedure, described by Lanham and Godfrey (1970), with a principle based on differences in electrical charges between erythrocytes and trypanosome cell membranes.

This technique was widely used in parasite separation and antigen preparation for ELISA antibody detection tests (Desquesnes *et al*., 2001).

2.8.5 Molecular techniques

The development of molecular techniques provided a solution to the low sensitivity of parasitological and serological techniques. Their uses had increased upon the discovery of Polymerase Chain Reaction (PCR). Molecular techniques such as DNA probes and PCRs, though more sensitive than serological and parasitological techniques are expensive and required advanced infrastructure (Marcotty *et al*., 2008). These tests are used for the demonstration of nucleotides sequences, which are specific for all trypanosome subgenus, species, or even type or strain. Nucleotides are the constituents of DNA, the molecules which constitute the genes on the chromosomes in the cell nucleus. A positive result indicates active infection with the trypanosome for which the sequences are specific, as parasite DNA will not persist for long in the host after all live parasites have been eliminated. These tests were not only suitable for detecting parasites in the mammalian host, but also in the insect vector (Masake *et al.*, 2002; Marcotty *et al*., 2008).

2.9 Treatment of Trypanosomiasis

Trypanocidal drugs were widely available, affordable for farmers, and were often the first drugs tried by farmers when their animals developed (any) symptoms of the disease. Most often, treatment given by livestock owners produced serious drawbacks because most farmers did not have adequate knowledge on diagnosis and the appropriate drug to use even

in areas of a high prevalence of trypanosomiasis; and also, because trypanocides were frequently used in the absence of diagnosis or used to treat conditions for which they were not effective (Geerts *et al*., 2001). However, if properly used, these drugs allowed for higher levels of production, improve animal welfare and safeguard the livelihood and assets on which 700 million poor farmers in developing countries relied on;but improper use of these drugs led to waste of scarce resources, poor production and promoted drug resistance leading to exacerbated disease in animals and humans (Grace, 2015) and even death. Despite livestock keepers' dependence on trypanocides only three compounds namely isometamidium chloride, homidium (bromide and chloride), and diminazene aceturate, are currently available for treating cattle. All these drugs had been in the market for over 40 years and several generic forms of them from a wide range of companies became available on the African market (Holmes *et al*., 2004). Diminazene aceturate has shown increasing levels of resistances over the last few decades. Isometamidium is principally used as a prophylactic drug and can provide up to 6 months of protection against trypanosomiasis. Whilst homidium has limited prophylactic properties, it is primarily used as a therapeutic agent (Giordani *et al.*, 2016). Manufacturers of drugs did not consider treatment of trypanosomiasis to offer profitable potential (Veeken and Pecoul, 2000) and thus investment in drugs against these diseases was low. Therefore, the control of trypanosomiasis in livestock continued to depend on the use of currently available compounds, because it was unlikely that new trypanocides would be developed and be released shortly. The challenge, therefore, remained to make optimal use of the three relatively old compounds until new methods of treatments emerged (Holmes, 2013).

2.9.1 Drug resistance in treatment of trypanosomiasis

The exposure of trypanosomes to subtherapeutic concentrations of trypanocidal drugs, the treatment frequency and the degree of drug exposure of the parasite population were important factors that influenced the development of drug resistance. Furthermore, some trypanocidal drugs such as ethidium were well-known mutagenic compounds and might induce mutations, the most resistant of which might be selected under drug pressure (Holmes, 2013). The phenomenon of cross-resistance was demonstrated by quinapyramine induced resistance to isometamidium, homidium, and diminazene (Ndoutamia *et al*., 1993; Giordani *et al*., 2016).

2.10 Control Methods of Trypanosomiasis

In Africa, enormous efforts towards control of human African trypanosomiasis (HAT) over the past three decades drastically reduced the prevalence of the disease and elimination had yielded positive results especially with the advent of “one world, one health” approach towards HAT and AAT (Simo and Rayaisse, 2015; WHO, 2016). However, successful intervention and control programs were achieved from the knowledge of the transmission dynamics of the disease (Franco *et al*., 2014b; N’Djetchi *et al*., 2017). Control of the zoonotic *T. b. rhodesiense* disease, with a domestic and wild animal reservoir, required a multidisciplinary approach that included veterinary health services and vector control (Welburn *et al*., 2016; Mehlitz and Molyneux, 2019). *Trypanosoma b. brucei* exhibited a high genome identity with the more restrictivespecies responsible for human African trypanosomiasis, *T. b. gambiense* and *T. b. rhodensiense* and pigs were reported as the most

important reservoirs of these parasites (Nkinin *et al*., 2002; Hamill *et al*., 2013; Cordon-Obras *et al*., 2015). Tsetse blood-meal analysis identified *T. b. gambiense* in the mid-gut of flies that had fed on human and porcine hosts (Simo *et al.*, 2012). A recent study in Cote d'Ivoire showed that *T. brucei s.l.* as the most prevalent trypanosome species and that pigs were the most infected animals (N'Djetchi et al., 2017). Therefore, increased efforts in studying the pathogenesis of *T. brucei* in the most important animal reservoir (pigs) could provide useful information in the control and elimination of HAT.

In Nigeria, AAT remains an endemic disease due to lack of proper control measures that led to re-infestation into areas already cleared of the emergence of drug-resistant strains of the trypanosome parasites and lack of adequate control measures on mechanical transmitters and reservoir hosts (pigs and small ruminants) as well as poor government funding for research and disease control (Magona *et al*., 2005; Ng'ayo *et al*., 2005; Sinshaw *et al.,* 2006; Majekodunmi *et al*., 2013; Santirso-Margaretto, 2016; Isaac *et al*., 2017; N'Djetchi *et al.,* 2017; Tchamdja *et al*., 2017; Mulandane *et al*., 2018; Odeniran *et al*., 2018). Prophylactic trypanocidal drugs could be administered to protect the animal for several months, or as in the case of cattle, trypanotolerant breeds could be reared. Trypanotolerant cattle kept their productivity under tsetse and trypanosomiasis infected areas as evidenced in West Africa (Murray *et al*., 1982; Elnasri, 2005; Sachs, 2010). Insecticides, applied from the ground or the air, were used to control or eradicate the flies. Alternatively, the use of sterile insect technique (SIT) where males were made sterile through gamma irradiation often effectively reducedthe tsetse fly population (Ntantiso, 2012).The phenomenon of antigenic variation displayed by mammalian stages of trypanosomes made it very difficult, if not impossible, to develop vaccines based on their

VSG's(Vickerman, 1985). However, there were better prospects of success in developing transmission-blocking vaccines that interfered with the maturation of the insect stages of the trypanosome (Aksoy *et al*., 2001; Macleod *et al*., 2007; Diall *et al*., 2017).

2.11 Porcine Trypanosomiasis

Although pigs may be readily infected by human infective *T. brucei gambiense* and *T. brucei rhodensiense,* clinical trypanosomiasis in pigs was only associated with *T. simiae, T. congolense, T. b. brucei* and sometimes *T. suis*. Porcine trypanosomiasis due to *T. simiae*has described as a very acute disease, probably the most fulminating form of trypanosomiasis with death occurring few hours after first wave of parasitemia (Losos, 1986; Onah, 1991). Other associated features included fever with the increasing wave of parasitemia, increased respiration, dullness, stiff and unsteady gait, and hyperemic skin lesions. Infection with *T. b. brucei* occurred more commonly and more virulent than *T. congolense* infection and the signs and severity were similar to those of *T. simiae* including nervous signs characterized by circling and wobbling of hind legs, meningoencephalitis and the presence of trypanosomes in the brain parenchyma (Waiswa, 2005; Allam *et al*., 2011).

2.11.1 Socio-economic importance

2.11.1.1 Impact on agriculture

Porcine trypanosomiasis was prevalent in sub-Saharan Africa with increased morbidity and mortality resulting in about 50% reduction in herd numbers (FAO, 2000). Even though, the true status of porcine trypanosomiasis in Nigeria was not well documented, results of spot

surveys suggested that the disease was more prevalent in the southern part of the country and the Middle Belt with an infection rate of at least 30% (Omeke, 1994; Ogunsanmi *et al*., 2000; Omotainse *et al*., 2000). Economic losses from high mortalities, retarded growth, and weight losscoupled with the high cost of trypanocides and drug resistance limited the viability of commercial pig farming in tsetse endemic areas (Swallow, 2000; Waiswa, 2005).

2.11.1.2 Role of pigs as reservoir hosts for trypanosome infections

For many years, pigs were identified as one of the most important hosts for African trypanosomes, especially *T. brucei gambiense* and *T. brucei rhodensiense*, the causative agents of human sleeping sickness (Abenga and Lawal, 2005; Waiswa, 2005). The recent resurgence in the human disease in parts of Uganda, Equatorial Guinea and Cameroun was traced to pigs (Nkinin *et al*., 2002; Simarro *et al*., 2011). In addition, pigs were associated with the maintenance of old sleeping sickness endemic foci in Nigeria and signs of an impending outbreak of the disease in other parts of the country (Onah and Ebenebe, 2003). Molecular techniques suggested that human infection arose from pigs– tsetse – human transmission cycle in endemic areas (Waiswa *et al*., 2003).

2.12 Sialidases

Sialidases (neuraminidases) are glycoside hydrolase enzymesthat cleave the glycosidic linkages of neuraminic acids. They are responsible for the cleavage of sialic acids from glycoconjugates for catabolic purposes or post-synthetic remodeling of glycan chains.

Strictly speaking, they do not belong to the de novo biosynthetic pathway of sialic acids, but their functions can significantly contribute to the regulation of cellular sialylation. Mammalian sialidases fell into four general categories, based on their subcellular localization, enzymatic properties, and substrate, thus implicating them in the modulation of many important biological processes (Miyagi and Yamaguchi, 2012).

2.13 Transialidases

Transialidase is a class of sialidases found in some Trypanosoma species; their mechanism of action is similar to those of sialidase but they work in the presence of water and preferentially transfer sialyl residues (α2,3-carbohydrate linked sialic acids) from one glycan chain to the terminal galactose residue of another non-sialylated oligosaccharide or glycoconjugate. Thus, they were well suited for glycan sialylation (Engstler *et al*., 1995Schauer and Kamerling, 2011; Freire-de-Lima *et al*., 2015).

2.14 Sialic Acids

Sialic acids are a generic term for a wide family of related nine-carbon sugar acids that featured prominently at terminal positions of many eukaryotic surface-exposed glycoconjugates, where they conferred important properties upon the resulting cell surface (Severi *et al*., 2007) and when subjected to numerous modifications, more than 50 structurally distinct molecules were generated (Angata and Varki, 2002). Such a wide diversity was an outcome of evolutionary selection by host-pathogen interactions acting along with pressure to conserve critical endogenous processes (Freire-de-Lima *et al.,* 2012;

Giacopuzzi *et al*., 2012). The name sialic acid was coined from the Greek word "sialos" meaning saliva because the mucins of the saliva have high sialic acid content. It was also referred to as neuraminic acid because of the discovery of a sialic acid-containing glycolipid fraction (ganglioside) in the brain (Varki, 1992; Varki and Varki, 2007). There were about 40 naturally occurring sialic acids with *N*-acetylneuraminic acid [Neu5Ac] and *N*-glycolylneuraminic acid [Neu5Gc] being the two major ones found in mammals (Kelm *et al*., 1998, Freire-de-Lima *et al.,* 2012). They are terminal acidic sugars of many glycoproteins and glycolipids, especially of higher animals. In this exposed position they contribute significantly to the structural properties of these molecules, both in solution and on cell surfaces. Therefore, it is not surprising that sialic acid (Sia)isan important regulator of cellular and molecular interactions, where they play important roles in a myriad of biological processes. They can either mask recognition sites like subterminal carbohydrate structures or proteins or can serve as recognition determinants. They play roles in masking and in the binding of pathogens to host cells and also in non-pathological cellular interaction (Kelm and Schauer, 1997, Tiralongo *et al*., 2003). Trypanosomes lack sialic acids but expressed an enzyme that protected them from the immune system of the host (Engstler *et al*., 1995; Schenkman *et al*., 1994, Montagna *et al.*, 2002; Tiralongo *et al*., 2003).

2.14.1 Structural diversity of sialic acids

Sialic acid is the metabolic precursor of a family of more than fifty 9-carbon acid sugars;the structural diversity was generated by various substitutions at the 4, 5, 7, 8, and 9-carbon positions (Fig. 2.7) (Varki, 1997). The nomenclature that permitted easy abbreviation of the names of substituted sialic acids was made available (Schauer, 1991; Schauer *et al*., 1995;

Reuter and Gabius, 1996) and found general acceptance (the term “Sia” was used as a generic abbreviation for all the member of the family). Further diversity in the presentation of these molecules was generated by several different linkages from the 2-carbon of sialic acids to a variety of underlying sugar chains (Schauer, 1991; Varki, 1992; Reuter and Gabius, 1996; Tsuji*et al*.,1996). Combinations of the different substitutions and the variety of linkages afforded many ways in which sialic acids presented themselves. Further complexity arose from the fact that 0-acetyl esters could migrate along the side chain under physiological conditions (Schauer, 1991; Varki, 1992; Reuter and Gabius, 1996). This diversity was found in a cell type-specific and developmentally regulated manner, implying important roles in intercellular recognition phenomena (Chen and Varki, 2010). Sialic acid biology, with huge diversity in forms, structures, expression, metabolism, and functions in the diverse living world, became a fascinating science, and there are growing knowledge in this domain. While the pathogens primarily used sialic acid to mimic the host and escape immune responses, it exhibited different functions in mediating cell-cell interaction, signaling, immune reactions, and development in animals but it’s completely absent in plants (Ghosh, 2020).

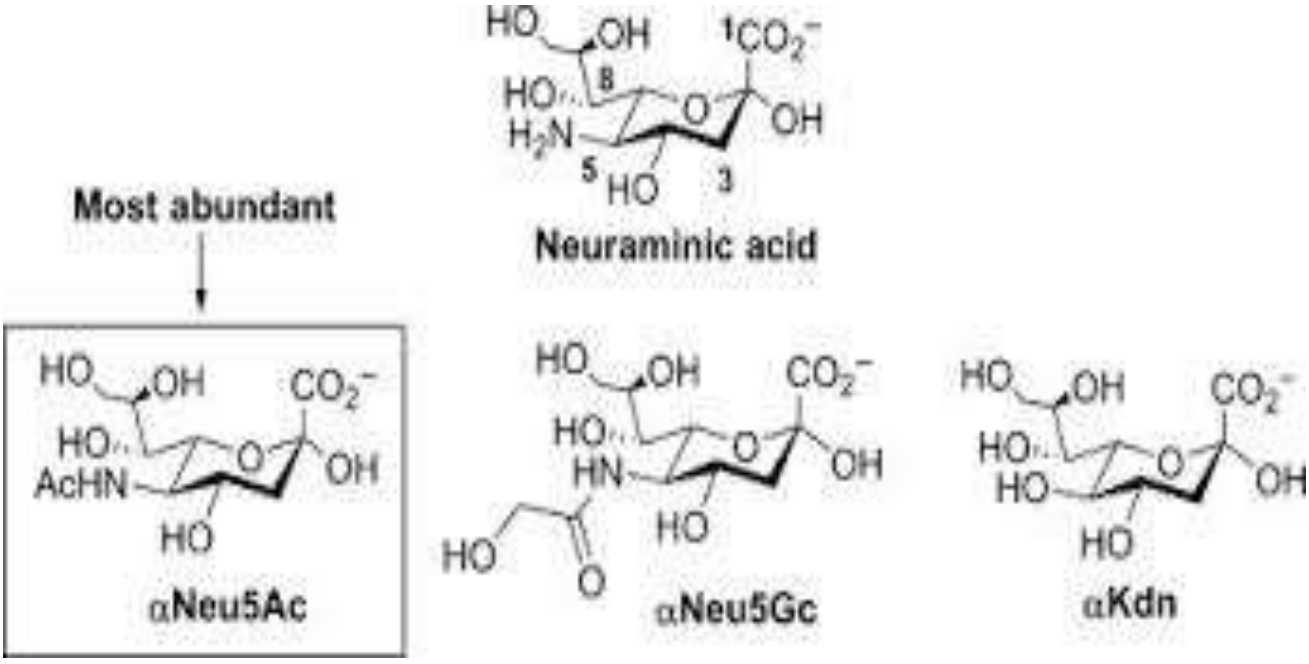

Figure 2.7: Structure of the three most important sialic acids (Stick and Williams, 2010).

2.14.2 Functions of sialic acids

The biological functions of sialic acid include binding and transport of positively charged molecules; attraction and repulsion phenomena between cells and molecules and protective shield functions for the terminal part of molecules or cells (Schauer and Kamerling, 2011).

Another important feature of sialic acid that seems to be in direct contrast to their recognition function is the masking of cells and molecules. A dense layer of sialic acid molecules covers erythrocytes, and during their lifetime in the peripheral circulation, sialic acids were removed stepwise from the surface of the cells as they age by the activity of serum sialidase hydrolysis of the surface sialic acids. Finally, the desialylated and unmasked erythrocytes were then bound to macrophages and phagocytosed (Esievo *etal*., 1982; Bratosin *etal*., 1995; Qi *et al*., 2009). There is ample evidence that sialic acids are important bioinformatic molecules, which play pivotal roles in biological, pathological, and immunological processes through modifying glycoproteins and glycolipids on the cell surface, including nervous system embryogenesis (Schnaar *et al*., 2014), cell-cell interactions (Teppa *et al*., 2016; Yaun *et al*., 2016), and signal transduction (Bate *et al*., 2016).

2.15 Sialylation

Sialylation is an enzymatic process that adds sialic acid to N- or O-linked sugar chains of glycoproteins or the terminal portions of the desialylated glycolipids. Sialylation is involved in a wide range of cellular activities including ganglioside biosynthesis (Okada *et al*., 2002), Lewis antigens formation (Kitagawa and Paulson, 1993; Okajima *et al*., 1999), tumor antigen 3 formation (Taylor-Papadimitriou *et al*., 1999) and T and B cells activation (Courtney *et al*., 2009; Hess *et al*., 2013). Sialylation of mammalian cell surface and secreted glycoproteins and glycolipids occurred through the action of a diverse family of sialyltransferases that acted on unique subsets of acceptor glycans and thus created four

distinct types of sialic acid linkages (Neu5Acα2,3Gal, Neu5Acα2,6Gal, Neu5Acα2,6GalNAc- and Neu5Acα2,8Neu5Ac-). The enzymes used a common CMP-Neu5Ac donor and a conserved donor binding site and catalytic residues assembled from sialylmotif sequences to carry out sugar transfer (Qi *et al*., 2009). Sialylated oligosaccharides possessed anti-adhesive effects against certain pathogens and that sialyllactoses might increase the adhesion of beneficial bacterial strains to the human intestine (Lane *et al*., 2012; Kavanaugh *et al*., 2013).

The enzymes that are responsible for sialylation are called sialyltransferases. Each sialyltransferase is specific for a particular sugar substrate (Ghosh, 2020). Sialylation, though essential for the production of recombinant therapeutic proteins (RTPs), and very important, availability of sialyltransferases is limited due to the low levels of a stable, soluble, and active protein produced in bacterial expression systems, which hampered biochemical and structural studies on these enzymes and thus restricted biotechnological applications (Ortiz-Soto and Seibel, 2016).

2.15.1 Sialyltransferases

Sialyltransferases (STs) transfer sialic acid (Sia) to glycan chains on glycoproteins and glycolipids. These sialylated structures are terminal structures on external cell surfaces, therefore, they are key players in cell-cell, cell-pathogen and cell-matrix interactions, and became important factors in protein stability and clearance (Bragonzi *et al*., 2000; Choi *et al*., 2015; Houeix and Cairns, 2019), neuronal development (Galuska *et al*., 2006), and immune regulation (Hennet *et al*., 1998; Priatel *et al*., 2000). Some STs can also modify

lactose to produce the sialyllactoses, 3′ sialyllactose, and 6′ sialyllactose, which are present in the new-born milk of many mammals. Human milk reportedly contained 12–14 g/L milk oligosaccharides and was particularly rich in sialyllactoses (~20%)(McJarrow and Van Amelsfort-Schoonbeek, 2004;Fierfort and Samain, 2008). Evidence suggested that sialylated oligosaccharides possessed anti-adhesive effects against certain pathogens and that sialyllactoses might increase the adhesion of beneficial bacterial strains to the human intestine (Lane *et al*., 2012; Kavanaugh *et al*., 2013). Bacterial STs are present only in select groups of bacteria (CAZy families GT30, GT38, GT42, GT73, and GT80) and many of these strains are highly pathogenic (Yamamoto, 2010; Meng *et al*., 2013; Watson *et al*., 2015).Most commercial STs are mammalian in origin, thus the enzymes have adapted to function in an environment of constant temperature, pH, and salinity. As a result, the enzymes are not necessarily optimal for *in vitro* reactions. On the other hand, non-mammal species function in much more variable environments and their enzymes would, therefore, be more variable in their kinetic parameters, substrate specificities, and stabilities (Siddiqui and Cavicchioli, 2006; Bauer and Osborn, 2015).

2.15.2 Sialyltransferase families

Sialyltransferases have been classified into five distinct families in the CAZy database (http://www.cazy.org/). However, all known eukaryotic ST sequences as well as viral sequences are grouped into a single CAZy family, GT29. To date, 20 different STs acting on glycoproteins and/or glycolipids had been characterized (Harduin-Lepers *et al*., 2001; Harduin-Lepers 2010), and classically split into four groups, depending on the type of linkage formed and the nature of the sugar acceptor as ST6Gal, ST6GalNAc, ST3Gal and ST8Sia and named accordingly (Tsuji *et al*., 1996; Datta 2009). The ST6Gal group

comprises only two protein members (ST6Gal-I and ST6Gal-II) that catalyzed the transfer of Neu5Ac residues to the hydroxyl group in C6 of a terminal galactose residue of type 2 disaccharide (Galβ1-4GlcNAc), and potentially to the N-acetylgalactosamine (GalNAc) residue of LacdiNAc motif (GalNAcβ1-4GlcNAc)(Rohfritsch *et al*., 2006). The ST6GalNAc group comprises six different members (ST6GalNAc-I to VI) that catalyzed a similar reaction but they used as acceptor a GalNAc residue found on O-glycoproteins (ST6GalNAc-I, II and IV) or glycolipids (ST6GalNAc-III, V and VI). The six protein members of the ST3Gal group (ST3Gal-I to VI) catalyzed the formation of an α2,3-linkage between Neu5Ac and terminal galactose residues found on glycoproteins and glycolipids. The six enzymes of the ST8Sia group (ST8Sia-I to VI) are known to mediate the transfer of Neu5Ac to the hydroxyl group in C8 of another terminal Neu5Ac residue also found on glycoproteins and glycolipids. The enzymatic properties of mammalian STs had been extensively studied in terms of substrate specificities toward synthetic acceptors as well as their glycoprotein and glycolipid preferences, which revealed the exquisite specificity of some of them (Datta 2009; Harduin-Lepers, 2010).

The crystal structuresof rat ST6Gal I (Fig. 2.8)(Meng *et al*., 2013), human ST8Sia III (Volkers *et al*., 2015), human ST6GalNAc II (Moremen *et al*., 2018), and several bacterial STs had been determined (Audry *et al*., 2011). In evolutionary terms, ST6Gal enzymes existed in all levels of the animal kingdom back to the echinoderms and hemichordates (Petit *et al*., 2018). In higher animals, both ST6GAL1 and ST6GAL2 paralogs existed, however, ST6Gal II had a much more restricted tissue distribution than ST6Gal I which was ubiquitously expressed. The earliest ST3Gal enzymes were found in echinoderms (Lehmann *et al*., 2008) and sponges (Petit *et al*., 2015). There were at least eight ST3Gal

paralogs (Teppa *et al*., 2016) with different kinetics and substrate specificities. The rat, human, and mouse enzymes were probably the best kinetically characterized by the STs (Lovering *et al*., 2007; Yuan *et al*., 2007; Igura *et al*., 2008; Maita *et al*., 2010).Most fully characterized STs are of mammalian origin and these have been used for *in vitro* and *in vivo* modification of glycans. Additional versatility could be achieved by the use of animal STs from other species that live in much more variable environments (Houeix and Cairns, 2019).The ST6GalI and ST3Gal1 are the two major sialyltransferases needed for fine control of the expression of sialyl glycoconjugates, resulting in a variety of developmental stage and tissue-specific glycosylation patterns. In mammals, the β-galactoside α2,6-sialyltransferases ST6Gal1 mediate the addition of α2,6-linked sialic acid to Galβ1–4GlcNAc disaccharides while ST3GAL1 is a sialyltransferase that catalyzes the transfer of sialic acid from cytidine monophosphate-sialic acid to galactose-containing substrates (Wu *et al*., 2018).

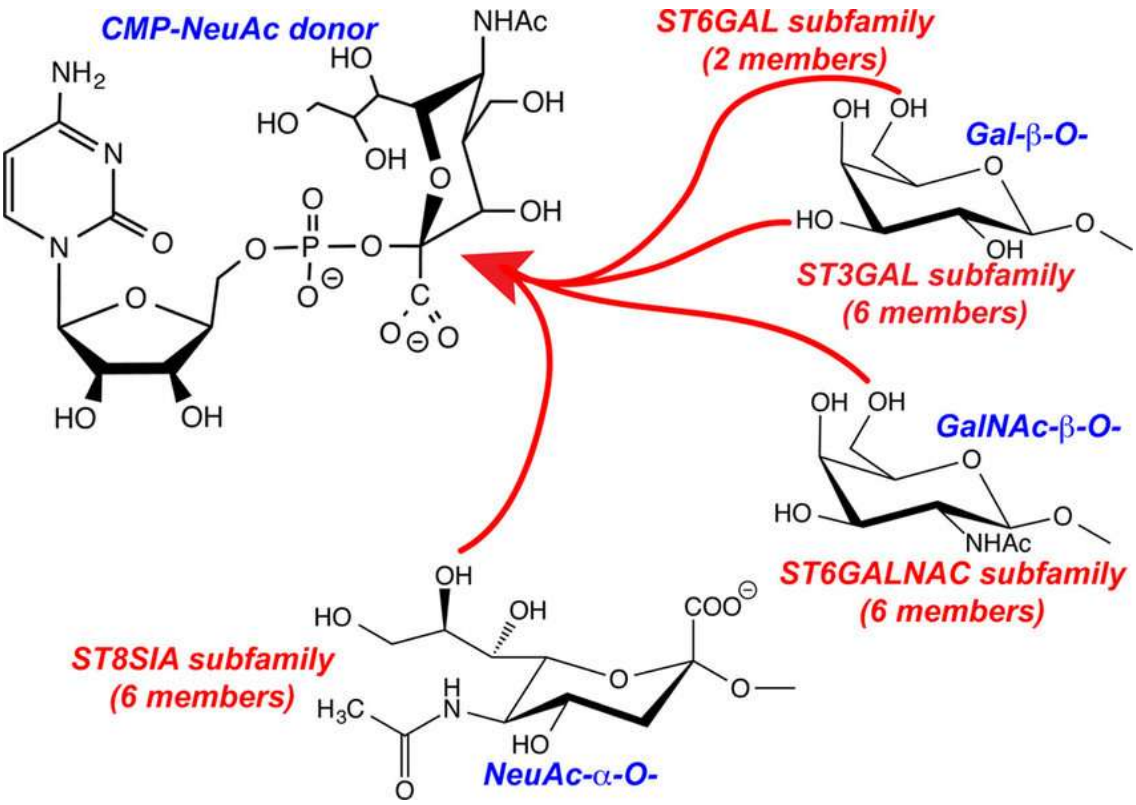

Figure 2.8: Structures of sialyltransferase subfamilies (Meng *et al*., 2013).

2.16 Quantitative Polymerase Chain Reaction (qPCR)

The polymerase chain reaction (PCR) is one of the most powerful technologies in molecular biology. Since it was first described (Mullis and Faloona, 1987), the PCR technique had undergone significant improvements in methodology that revolutionized molecular biology from conventional thoughts. It was a basic technique that could amplify a small amount of template DNA or reverse-transcribed RNA, also called complementary DNA (cDNA), into large quantities in a few hours. However, conventional end-point PCR had a drawback as it lacked reliable quantification and did not easily yield reproducible results. This limitation was resolved twenty years ago, with the invention of real-time PCR (Higuchi *et al*., 1992). Few years later, a novel "real-time" quantitative PCR (qPCR) method with greater specificity was also invented (Heid *et al*., 1996) and is now a widely used method for analyzing gene expression. It was developed by combining PCR with fluorescent techniques and it depends on collecting data throughout the PCR amplification, which was achieved by monitoring the increase in fluorescence intensity of a specific fluorescence dye, which correlated with the increase in PCR product concentration. The major progress of qPCR was that quantification did not have to be done in the plateau phase of amplification, which was a disadvantage of previous quantification methods (Gibson *et al*., 1996; Heid *et al*., 1996).

Polymerase chain reactionis normallydivided into four major phases: linear ground phase, early exponential phase, log-linear phase and plateau phase (Tichopad *et al*., 2003). During the linear ground phase, only background fluorescence could be detected. The early exponential phase started when the amount of fluorescence was significantly higher than the background. During the log-linear phase, when PCR had reached its optimal

amplification period, the amount of fluorescence rose exponentially. In an ideal reaction, the PCR products doubled after every cycle. Finally, when the reaction components became limited, the plateau phase was reached and the fluorescence could not increase anymore (Wong and Medrano, 2005).

The method uses two basic quantification methods which are increasingly used and suitable for different applications: Absolute quantification and relative quantification. The former is used to determine the absolute/exact quantity of a genomic DNA or RNA template within an unknown sample by using a standard curve that is prepared to form a dilution series of control template of known concentration. On the other hand, relative quantification is used to measure the relative concentration of template (or target gene) in unknown samples normalized to a stably expressed reference gene and compared relatively to a calibrator sample (for example time zero, or untreated sample). Real-time quantitative PCR (RT-qPCR) was used as a powerful tool for gene expression studies (Reiter and Pfaffl,2011; Mei*et al.*, 2012).

For accurate RT-qPCR analysis, it is necessary to correct non-specific experimental variations such as differences in starting material and quality between samples. These variations were controlled by normalizing gene expression data using one or more appropriate reference genes (Løvdal and Lillo, 2009; Vandesompele *et al.*, 2002). The selection of suitable references for the normalization of gene expression was a prerequisite for reliable results in quantitative RT- PCR (Vandesompele *et al.*, 2002). The ideal reference should be unaffected by experimental treatment and should be expressed at a constant level among different cells (Borowska, 2016). qPCR analysis does not involve complex bioinformatics analysis of the output and is considered straight forward relative to

RNA sequencing, but qPCR delivers results within hours after the cautious selection of primers and optimization of reaction. Relative quantification describes a real-time PCR experiment in which the expression of a gene of interest in one sample (i.e., treated) is compared to the expression of the same gene in another sample (i.e., untreated). The results are expressed as fold change (increase or decrease) in the expression of the treated compared to the untreated. A normalizer gene (such as β-actin) was used as a control for experimental variability in this type of quantification (Pfaffl, 2007).Many approaches are available for the relative quantification of gene expression. Most of them depend on the principle to define a threshold at which the PCR product fluorescence rises over the background fluorescence. The number of cycles needed until this threshold is reached, depending on the amount of template in a sample, is usually called Ct - the higher the template amount, the lower is the Ct value. Widely used approaches depending on this principle were the $2^{-\Delta\Delta Ct}$ or comparative Ct method (Livak and Schmittgen, 2001), the Pfaffl model (Pfaffl, 2001), or qBase software (Hellemans *et al*., 2007). While the comparative Ct method assumed the same amplification efficiency for all amplicons, other methods used serial dilutions of the samples to determine the amplification efficiencies from the increase in the Ct value with decreasing cDNA input, another approach determined the amplification efficiency or starting template amount from the increase in fluorescence during the PCR reaction (Liu and Saint, 2002; Ruijter *et al*., 2009).

Real-time PCR uses several different fluorescence detection technologies to detect PCR products. One of them is SYBR Green which is used as a dye for the quantification of double-stranded DNA (dsDNA) PCR products. This fluorescent dye must be added in the reaction mixture which contains template cDNA (or genomic DNA), gene-specific primers

(forward and reverse), and buffer. After annealing of the primers, a few dye molecules bind to the double-stranded DNA, resulting in a significant increase of molecules to emit light upon excitation. With each cycle, more and more dye molecules bind with newly synthesized DNA. If the reaction is monitored continuously, an increase of fluorescence can be viewed by using a computer. During the initial cycles of real-time PCR, the signal level can be denoted by the baseline of the reaction. Generally, there is little change in fluorescence signal within cycles of 3 to 15. The background or the noise of the reaction can be linked with the low-level signal of the baseline and to eliminate the background in the early cycles, it is necessary to take into consideration enough cycles when determining the baseline in the real-time PCR. However, the cycles in which the amplification signal begins to rise above the background should be avoided. The baseline value should be set in a way that the threshold cycle (Ct) could be determined accurately (Bustin *et al*., 2009).

The threshold of the real-time PCR reaction is the level of the signal that reflects a statistically significant increase over the calculated baseline signal. It is set to distinguish relevant amplification signal from the background. Usually, real-time PCR instrument software automatically sets the threshold above 10 times the standard deviation of the fluorescence value of the baseline. However, the positioning of the threshold can be set at any point in the exponential phase of PCR amplification. The threshold cycle(Ct) is the cycle number at which the fluorescence signal of the reaction crosses the threshold. The Ct is used to calculate the initial cDNA copy number because the Ct value is inversely related to the amount of starting template. The Rn value is the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye. The delta Rn value, or normalized reporter value, is the Rn value of an experimental

reaction minus the Rn value of the baseline signal generated by the instrument. After finishing a sufficient number of cycles (30 to 50), melting curve analysis could be performed to verify primer specificity when applying SYBR Green chemistry. Generally, a melting curve shows a single amplified product for a gene, but it is also possible to have a more amplified product from primer-dimer and genomic DNA, resulting in multiple peaks. Therefore, melting curve analysis could confirm the specificity of a quantitative reverse transcription PCR reaction. The melting curve can be found when double-stranded DNA (dsDNA) starts to melt by heating. As the temperature was raised, dsDNA became a single-stranded DNA and the dye (SYBR Green-based) dissociated from the DNA (Schefe*et al*., 2006; He *et al*., 2018).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 The Study Area

The experimental research was conducted in the Department of Veterinary Pathology, Faculty of Veterinary Medicine (FVM), Ahmadu Bello University (ABU),Samaru, Zaria, Kaduna state, Nigeria. Zaria lies in the Guinea Savannah belt, located at 2200 feet above sea level within latitudes 11°7', 11°12″ N and longitude 7°41″ E with a mean annual temperature of 27°C. Zaria experiences distinct wet and dry seasons. The wet season (May-October) is characterized by conventional rainfall that is followed by intense thunderstorms and lightening with a mean annual rainfall of about 1000 mm. The dry season (November - April) is characterized by a period of low temperatures (December-February) - this is the harmattan season; and the hot dry season (March - April) where temperatures are as high as 32ºC. Its relative humidity is high during the rainy season and low during the dry season (Sawa and Buhari, 2011; Abdulhamid *et al.*, 2016).

3.2 Ethical Approval

Ethical approval was obtained to conduct this study from Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC/2021/005).

3.3 Experimental Animals

A total of Eleven 11 mixed-breed of pigs, aged between 6 and 12 months, were used for the study. The animals were purchased from a reputable pig farm in Samaru, Zaria, Kaduna State, Nigeria, and their ages were obtained from the farm records, which were then verified using their dentition as described by Matschke(1967).Upon arrival, individual pigs were ear-tagged for proper identification and screened for endoparasites, ectoparasites and haemo-parasites using standard parasitological techniques. The animals were kept in a fly-proof experimental animal house and were dewormed with ivermectin (Ivomec®, Merial USA) at a dose rate of 0.4mg/kg body weight subcutaneously. They were also treated with Amprolium (Amprolium 50% Oral, Kepro®, Holland), a broad spectrum anticoccidial at a dose rate of 40 mg/kg body weight orally. They were regularly sprayed against ectoparasites using diazinon (Diazintol®, Animal Care, Nigeria) at a concentration of 2ml/liter of water. The pigs were fed on a compounded diet of 18% crude protein comprising of maize (45.0%), groundnut cake (25.0%), wheat (29.4%), premix (0.5%), lysine (0.1%), thymine (0.1%), table salt (0.15%) and water provided *ad-libitum*. The pigs were allowed to acclimatize for two months during which they were subjected to routine biweekly blood sampling for hematologicalevaluation and parasite screening, and were certified to be free of trypanosomes using the buffy coat centrifugation technique (Woo, 1970).Eleven of these pigs were used for the experiment while the remaining one served as donor animal.

3.4 Trypanosome Stock

The parasite *T. brucei brucei*(Federe strain) was obtained from the Department of Veterinary Parasitology and Entomology, University of Nigeria, Nsukka (UNN), Enugu State, Nigeria. The parasite was characterized morphologically and biologically based on resistance to human serum (Soulsby, 1982).The stabilate of this parasite was inoculated into two albino rats intraperitoneally. The inoculated rats were immediately transported to the Department of Veterinary Parasitology and Entomology, FVM, ABU, Zaria. These rats were kept in a cage and fed adequately. Blood samples were collected from each rat daily for the determination of parasitemia using the method of Woo (1970).

3.5 Infection and Detection of Parasitaemia in Donor Pig

After parasitaemia was detected in both rats on day 3 post-inoculation and peak parasitemia in the rats on day 6 post-inoculation, the rats were anaesthetized using chloroform and their jugular veins were then severed to collect sufficient blood into labelled heparin containing vacutainers; the blood was then used to inoculate the donor pig via the anterior vena cava. A blood sample was collected from the donor pig daily in a vacutainer containing Ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The blood was used to detect and determine the pre-patent period and parasitemia level that will be sufficient to infect the

experimental pigs using the modified method of Paris *et al*. (1982) as modified by Mutayoba *et al*. (1994).

3.6 Animal Grouping and Infection of Experimental pigs with *T. b. brucei*

The experimental pigs were allotted two groups; the first group of 5*T. b. brucei*-infected pigs and the second group of 5 non-infected (control) pigs. These groups were closely matched based on the mean hematocrit index (Infected group, 34.6±3.2%; control 34.3±4.5%). Parasitaemia was detected in the donor pig on day 5 post-inoculation and it reached its peak at 8 days post-inoculation. At the peak parasitemia (4×10^6 trypanosomes per ml of blood) in the donor pig, the blood of the donor pig was diluted with normal saline at a ratio of 1:8.Each pig from the infected group was then inoculated with 4 ml of the donor pig blood containing 2×10^6*T. brucei brucei* organisms through the anterior vena cava. Estimation of the number of trypanosomes was done using the method of Paris *et al.* (1982) as modified by Mutayoba *et al*. (1994).

3.7 Rectal Temperature and Body Weight Determination

The rectal temperature of each pig was obtained daily using a digital clinical thermometer (MC-246, Omron) while their body weights were determined every week using a electronic weighing scale (Terraillon).

3.8 Blood Sample Collection

Starting from day 3 pre-infectionup to the time of termination of the experiment on day 21, 6ml of the blood was obtained from each of the infected and non-infected pigs through venipuncture of the anterior vena cava using a 19-gauge hypodermic needle and 10ml syringe. The site of the venipuncture was first aseptically prepared by disinfecting with 70% ethanol. Exactly1ml of blood was dispensed into vacutainers containing ethylene diamine tetra-acetic acid (EDTA) and the blood was used for the detection and determination of parasitemialevel in infected pigs and PCVin both infected and control pigs.

Two milliliters of the blood sample were dispensed into a screw-capped test tube containing 0.3 ml of reconstituted acid citrate dextrose (ACD) anticoagulant and used for the preparation of erythrocyte ghosts (membranes) which were used for the determination of erythrocyte surface sialic acid concentration.

The remaining 3ml was transferred into labelled plain screw-capped test tubes without anticoagulant for sera preparation. The blood samples for sera preparation were allowed to clot by leaving it undisturbed for 30 minutes at room temperature. The clots were trimmed off the tubes and centrifuged at 3000xg for 10 minutes. The sera were then aspirated into labelled serum vials and stored at -20^{o}C until required for the determination of free serum sialic acid concentration and serum sialyltransferase activity.

3.9 Laboratory Analyses

3.9.1 Determination of daily parasitaemia level

The level of daily parasitaemia in the infected pigs was determined by preparing a wet mount which was examined under x 40 objective lens of the light microscope.

3.9.2 Haematological determinations/analyses

Haematological values which included packed cell volume (PCV), haemoglobin concentration (HB), red blood cell count (RBC), total white blood cell (WBC), differential WBC and platelets counts were determined using methods described by Coles (1974). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration were determined using standard formulae (Esievo, 2017)

3.9.3 Determination of serum neuraminidase (NA) activity

Assay of serum NA was carried out using the TBA method (Aminoff, 1961). The substrate for the assay was Fetuin (Sigma® USA) at a concentration of 25 mg/ml (always prepared fresh), whilst the enzyme source was the serum sample. The volume of the substrate (Fetuin®) was kept constant at 0.01 ml for all samples. To every 0.01 ml of Fetuin, 0.05 ml of serum was added. Thereafter, 0.49 ml of acetate buffer (50 mM, at an optimum pH of 5.0) was added to give a total volume of 0.55 ml. The optimum pH of the buffer was pre-determined. The mixture was incubated at 37^{o}C for 3 h. Substrate control (0.01 ml Fetuin + 0.54 ml of acetate buffer pH 5.0), enzyme control (0.05 ml serum + 0.50 ml acetate buffer pH 5.0) and blank (0.55 ml acetate buffer pH 5.0) were prepared. The sialic acid liberated

from Fetuin was assayed using Aminoff's TBA method (Aminoff, 1961), which represented the NA activity.

3.9.4 Determination of erythrocyte surface sialic acid (ESSA) concentration

3.9.4.1 Preparation of haemoglobin free erythrocyte membranes (ghosts)

Hemoglobin-free erythrocyte membranes (ghosts) were prepared according to the method of Dodge *et al*. (1963). The blood samples collected into a test tube containing ACD as an anticoagulant were centrifuged at 8000g for 10 minutes and the plasma/buffy coat was aspirated using Pasteur pipette and discarded. The sedimented erythrocytes were washed several times with 3ml of isotonic phosphate buffer, (310 ideal milliosmoles (imOsm), pH 7.4) until a clear supernatant was obtained and the erythrocytes settled at the bottom. The washed erythrocytes were immediately haemolyzed by adding 3ml of hypotonic phosphate buffer (20 imOsm, pH 7.4) and agitated for 5 minutes. The resulting hemolysates were centrifuged at 2000 g for 20 minutes. The supernatant was decanted and the recovered erythrocyte ghosts were washed several times with 3ml of the hypotonic buffer until they become clear and free of haemoglobin. The erythrocyte ghosts were stored in labelled vials at -20^{o} C until required.

3.9.4.2 Assay of sialic acid from erythrocyte ghost cells

Quantitative determination of the sialic acid content of the erythrocyte ghost cells was determined using Periodate-thiobarbituric acid (TBA) assay as previously described

(Aminoff, 1961; Engstler *et al*., 1995). The procedure was accomplished in two phases; the first involves the hydrolysis or cleavage of sialic acid of the erythrocyte ghost cells. This was done by dispensing 50µl of the washed erythrocyte ghosts into a test tube after which 0.2 ml of 0.1N sulphuric acid (H_2SO_4) was added and incubated at 80^oC for 1 hour to liberate the sialic acids attached to the ghost cells (Warren, 1994). An aliquot (0.25 ml) of periodate acid was added to the liberated SA and the mixture was incubated at 37^oC for 20 min in a water bath. Thereafter, 0.1 ml of sodium arsenite was added followed by the addition of 1 ml TBA. The mixture was then placed in a boiling water bath for 15 min and later cooled in an iced water bath. Then, 2 ml of acid butanol was added, and the chromophore was extracted by vigorous vortexing (IKA® Works Inc. NC, US), and then centrifuged (Centurion® Scientific Ltd, West Sussex, UK) at 1000g for 5 minutes, after which each clear organic phase was transferred to a cuvette and optical density (OD) read using a UV-Spectrophotometer (Cole-Parmer®, US) at 549 nm (in triplicates) against a distilled water blank. The concentration of sialic acid in the samples was extrapolated from a standard curve prepared from *N*-acetylneuraminic acid (Sigma®, Germany).

3.9.5 Determination of free serum sialic Acid concentration

The FSSA was also determined using the TBA method as described by Aminoff (1961) and Engstler *et al*. (1995). Briefly, exactly 0.05 ml of serum was dispensed into a labelled screw-capped test-tube. This was followed by the addition of 0.25ml of periodate acid and the mixture was then incubated in a water bath at 37 ^0C for 20 minutes. Exactly 0.1ml of sodium arsenite was added to neutralize excess periodate followed by the addition of 1 ml of TBA. The mixture was later heated in boiling water bath for 15 minutes followed by cooling in an ice bath. The chromophore was extracted by vigorous vortexing following the

addition of 2 ml acid-butanol solution and later centrifugation at 1000 g for 5 minutes. The clear organic phase was then be transferred to a cuvette and the optical density (OD) read on a colorimeter (JENWAY 6051, ESSEX, UK) at 549nM against a blank (distilled water). The FSSA concentrations were extrapolated from a standard curve prepared from *N*-acetylneuraminic acid (Sigma®, Germany).

3.9.6 Determination of sialyltransferaseactivity

Sialyltransferase activity was determined as described by Kren and Thiem (1997) and the principle of the assay is based on the separation of the product sialylglycosides during sialyl transfer as well as the hydrolysis and colorimetric determination of released Sia by 2-thiobarbituric (TBA) assay.

Each assay mixture contained 25 µl buffer G, 25 µl BSA (5 mg/ml), 25 µl Asialofetuin (acceptor substrate) (1.05 mg/ml), 25 µl CMP-Sialic acid (donor substrate) (1.05 mg/ml buffer H) and 25 µl of enzyme solution. Controls lacked the acceptor substrate and 25 µl buffer G was used to replace it. The reaction was started by enzyme addition and then incubated for 30 minutes at 37 ^{0}C, after which, the reaction was stopped by dilution with 1 ml buffer Iand the mixture was immediately passed through Pasteur pipette filled with Dowex 1x8, 200 – 400 / (Cl^-) (3 cm length) and washed with 750 µl buffer I. Both unreacted CMP-Sialic acid and possible traces of free Sia are trapped by Dowex column. The Dowex was adequately equilibrated with buffer I before passing the reaction mixture. The combined eluates, 170 µl 1M H_2SO_4 was added and the mixture was heated for 1 hour at 80 ^{0}C to hydrolyze the sialyl-conjugates. The Sia released was determined by a modified TBA assay method (Warren, 1963). To the cooled samples (25 ^{0}C), 350 µl 0.2M $NaIO_4$ in

9M H_3PO_4 was added and shaken properly. After 20 minutes, 1.5 ml 10 % w/v $NaAsO_2$, in 0.05M H_2SO_4 containing 0.5M Na_2SO_4 was added and the tubes were shaken until a yellow colour disappears. 3 ml of TBA (0.6 % in distilled water) was added and the tubes were placed in boiling water for 15 minutes, then cooled in ice-cold water for 5 minutes and 0.5 ml saturated Na_2SO_4, after which the pink colour fades and a white precipitate appeared. After 5 minutes, 4 ml cyclohexanone was added and the tubes were vortexed. The red organic layer was aspirated and briefly centrifuged and its absorbance was measured against blank at 549 nm. A calibration curve was prepared using Neu5Ac: 0 – 60 µl of 1 µM Neu5Ac neutralized by $KHCO_3$ (0 – 60 nmol Neu5Ac) was pipetted into the tubes, 1.75 ml buffer I and 170 µl of 1M H_2SO_4 were added and the tubes were heated for 1 hour at 80 ^{0}C. After cooling, Sia was assayed by the above TBA assay method. Blanks contained no Neu5Ac.1 unit (U) of the enzyme incorporated 1 nmol/min of Neu5Ac in the product at 37 ^{0}C.

3.10 Tissue Sample Collection and Preservation

At termination of the experiment, all the pigs in the infected and control groups were humanely sacrificed. Post-mortem examination of the carcasses was carried out and tissue samples were collected from the thyroid gland, liver, kidneys, and small intestine of each pig. Each sample was divided into two parts: one part (5mm thick) was collected into an Eppendorf microcentrifuge tube containing 1 ml of RNA *later*®(Ambion, UK) and then stored at -20 °C until when required for RNA extraction and the other part (5mm thick) was

fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin wax and used for histopathological examination.

3.11 Molecular Characterization of Porcine ST3Gal1 and ST6Gal1

3.11.1 Ribonucleic acid extraction

Total sialyltransferase mRNA of liver, kidney, thyroid gland and small intestine from *T. b. brucei* infected and non-infected pigs was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Stabilized tissues that were previously stored in RNA *later* (Ambion, UK) solution at -20 °Cwere removed from the reagent using sterile forceps. Approximately 30 mg of the tissues were used for RNA extraction. FastPrep TM Lysing Matrix tubes containing ~30 mg of tissues was filled with 600µL of RLT buffer. To disrupt and homogenize tissues the FastPrep® FP120 Cell Disrupter was used. Each sample was homogenized for 45 sec at the speed of 6.5 m/sec. The lysate was centrifuged for 3 min at 16000 *g* to remove cell debris. Cleared lysate was mixed by pipetting with 1 volume of 70% (v/v) ethanol. The mix was placed in the RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 sec at 8000 *g*. The flow-through was discarded and 350µL of RW1 buffer was used twiceto wash RNA bound to the silica membrane by centrifuging for 15 sec at 8000 *g*. RPE buffer, diluted in absolute ethanol, was used twice (15 sec and 2 min) by centrifuging at 8000 *g*. After washing steps, the RNeasy column was placed in a new collection tube and centrifuged at 16,000 *g* for 1 min to remove any RPE buffer residues. Columns were moved to 1.5 collection tubes, 30µl of RNase-free water was placed onto the membranes, and centrifuged for 1 min at 8000 *g*. The eluate was re-applied

to the silica membrane and centrifugation repeated to increase the RNA yield. The RNA yield was quantified and purity checked using Nano-drop ND 1000 spectrophotometer.

3.11.2 Quantification and assessment of RNA/DNA purity by UV absorbance

The concentration of RNA/DNA was determined using the Nanodrop® ND-1000 spectrophotometer (Nanodrop Technologies®, Inc). A 1 µl aliquot of nuclease-free water was selected as the zero-absorbance reference. Another 1 µl aliquot of sample was loaded onto the measurement pedestal and the absorbance was measured over a continuous spectrum producing a graph. The A_{260}/A_{280} and A_{260}/A_{230} ratios were calculated; an A_{260}/A_{280} ratio in the values of 1.8 and 2.0 are generally accepted as pure for DNA and RNA respectively. The A_{260}/A_{230} ratio is used as a secondary measure of nucleic acid purity; values outside the 2.0-2.2 were considered to be due to organic chemical contamination such as phenol and thiocyanates. After this step, it was discovered that the extract from thyroid and small intestine samples did not have significant mRNA yield and the RNA integrity was not pure and hence not qualified for further downstream analyses.

3.11.3 DNase treatment

A DNA-free kit (Life Technologies®, Inc) was used to remove residual genomic DNA that might be contained in RNA samples. For every 40µL RNA suspension, 4µL (10% volume) 10XDNase I Buffer (100mM Tris-HCl pH 7.5, 25mM $MgCl_2$, 5mM $CaCl_2$) and 1µL recombinant DNase I (2U/µL) were added and incubated at 37°C for 25 minutes. Then 4µL (10% volume) resuspended DNase inactivation reagent was added and incubated at room temperature for 2 minutes, centrifuged at 10000g at room temperature for 1.5 minutes and

the upper aqueous layer was transferred to a fresh tube. RNA concentration and purity were measured in Nano-drop ND 1000 spectrophotometer.

3.11.4 First-strand reverse transcription using SOLIScript

The various mRNA samples were used to generate complementary DNA (cDNA) panels using the SOLIScript RT cDNA synthesis kit (Solis BioDyne) following the manufacturer's instructions. First-strand cDNA synthesis required mixing 1µg template RNA, 1µL of oligo(dT) primer (100µM) and sterile distilled water was added to make it up to 16µL. The mixture was then incubated at 65°C for 5 min and then placed on ice for 1 min. After a short spin, 2µL of 10x reaction buffer with dithiothreitol (DTT) reducing agent, 0.5µL of dNTP mix (20nM), 0.5µL of RiboGrip RNase inhibitor (40 U/µL) and 1µL of SOLIScript RT were added. The final mixture was incubated at 50°C for 30 min. The reverse transcription reaction was inactivated by heating to 85°C for 5 min. Complementary DNA was then divided into two (2), one part was used for downstream analysis, and the other part was stored at -80°C until required.

3.11.5 Optimization of primers for target and reference genes

Purified primers for target genes were generated (Invitrogen, Life Technologies®) and optimized to an equal annealing temperature of 60°C. Sequences of the set of primers (Table 3.1) were retrieved from literature (Park *et al*., 2011, Wang *et al*., 2015).

Table 3.1: Primer sequences used for RT-PCR

Genes	Primers	Sequences (5' to 3')
ST3Gal1	Forward	GCATCCTCTCCGTGATCTTC
	Reverse	CAAGATGGTTGTCACGTTGG
ST6Gal1	Forward	TGTGTGACCAGGTGGATGTT
	Reverse	TCCCAAGCAGGTAGATGTCC
β-actin (endogenous housekeeping)	Forward	CACGCCATCCTGCGTCTGGA
	Reverse	AGCACCGTGTTGGCGTAGAG

Key: ST3Gal1 = ST3 beta-galactoside alpha-2,3-sialyltransferase 1; ST6Gal1 = ST6 Beta-Galactoside Alpha-2,6-Sialyltransferase 1; β = Beta; G = Guanine; C = cytosine; T = Thymine and A= Adenine (Park *et al*., 2011, Wang *et al*., 2015).

3.11.6 Fluorescent dye-based (SYBRGreen) quantitativereverse transcriptase polymerase chain reaction

The SYBRGreen dye was a green fluorescent nucleic acid dye used in several applications including qPCR and its excitation and emission spectra were very close to those of fluorescein (FAM). This dye was readily compatible with instruments equipped with visible light excitation with wavelengths in that region. The dye was non-fluorescent by itself but became highly fluorescent upon binding to cDNA. It was non-mutagenic and non-toxic as it was impermeable to cell membranes. It was also less inhibitory towards qPCR and less likely to cause nonspecific amplification. The dye bound to double-stranded DNA (dsDNA) with a resultant increase in the amount of dsDNA as the fluorescence intensity increased (Rajeevan *et al*., 2001a; Rajeevan *et al*., 2001b). The Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) was used to amplify and detect products. The reaction mix prepared for each of the samples contained several components as shown in Table 3.2. The following PCR protocol was used: denaturation program (95°C for 10 minutes), amplification program repeated 40 cycles (denaturation: 95°C for 15 sec, annealing: 60°C for 20 sec, 72°C for 60 sec) and a cooling program to 40°C. Melting curves were generated after each run to confirm a single PCR product (from 60°C to 95°C, increasing 1°C/3 sec) (Jia *et al*., 2019; Dharavath *et al*., 2020).

Table 3.2: Protocol for qPCR reaction mix using cDNA and SYBRGreen (Solis BioDyne)

Components	**Volume**	**Concentration**
Template cDNA	4μL	
SYBRGreen Master Mix	4μL	5x
Forward Primer	0.3μL	10μM
Reverse Primer	0.3μL	10μM
H_2O	11.4μL	
Total volume	**20μL**	

Key: cDNA = complementary deoxynucleic acid; H_2O = water; μl = microlitre; μM = micromolar.

3.12 Analysis of Quantitative Reverse Transcriptase Polymerase Chain ReactionData

3.12.1 Polymerase chain reactionamplification efficiency

Polymerase chain reaction (PCR) efficiency is the ratio of the number of target gene molecules at the end of a PCR cycle divided by the number of target molecules at the start of the same PCR cycle. In the geometric phase, efficiency is a constant cycle-to-cycle.Efficiency can be represented as a ratio or a percentage. At maximum, a target sequence can double each cycle, because DNA only has two strands. A 2 or 100% efficiency means that the cDNA is doubled in every PCR cycle. In RT-PCR assay, the efficiency of both target and reference genes should be as close to 100% as possible because substantive differences could result in over or underestimation of expression ratio. To use $2^{-\Delta\Delta Ct}$ method for relative gene expression that is based on the normalization of the target gene to a reference gene, the efficiency should be close to 100% and the differences between them should not be more than 5% (Bustin *et al*., 2009; Rao *et al*., 2013). In general, an efficiency of 90% to 110% could be considered good enough for RT-PCR. The slope of the standard curve is used to calculate the efficiency using the expression:

$$E\ (\%) = 10^{-1/slope} \times 100$$ (Rao *et al*., 2013).

In this study, the efficiency of the PCR amplification of reference gene (ACTB) and the target genes (ST3Gal1 and ST6Gal1) was verified by preparing a pooled cDNA sample of an equal amount of infected and non-infected sample and a five-point standard curves of a 10-fold dilution series (1:1 – 1:10000) was plotted against the threshold cycles obtained in the RT-qPCR. The slope was then determined and it was used to determine the efficiency

(%). Each dilution was amplified in triplicate and the average Ct was taken and used for the plot.

3.12.2 Relative gene expression using $2^{-\Delta\Delta Ct}$ method

Data obtained from the RT-qPCR experiment was analyzed using the $2^{-\Delta\Delta Ct}$ method, which is also known as the Livak method (Livak and Schmittgen, 2001). This relative gene expression method was based on the assumption that both the target and reference genes were amplified with efficiencies near 100 % and are within 5 % of each other. It was therefore vital to fulfilling the conditions before using the method. For quantification of gene expression of ST3Gal1 and ST6Gal1, the cycle of threshold (Ct) for each gene transcript was determined. Ct is the point at which the fluorescence rises appropriately above the background fluorescence. The baseline signal and threshold signal of fluorescence were determined automatically by the qPCR machine. Table 3.3 shows the required Ct values that were required for relative gene expression involving normalization of target genes by the reference gene ($2^{-\Delta\Delta Ct}$ method).

Table 3.3: Required information for relative quantification of the target genes (ST3Gal1 and ST6Gal1) using a reference gene (ACTB) as normalizer.

	Infected	Control
Target gene	$Ct_{(target, infected)}$	$Ct_{(target, control)}$
Reference gene	$Ct_{(reference, infected)}$	$Ct_{(reference, control)}$

Key: Ct = cycle threshold; (target, infected) = target gene and infected sample; (target, control) = target gene and control sample; (reference, infected) = reference gene and infected sample; (reference, control) = reference gene and control sample.

The $2^{-\Delta\Delta Ct}$ method involves three (3) steps calculation. In the initial step, the Ct value of the target gene is normalized to the Ct value of the reference gene for both the *T. b. brucei* infected and control sample.

$\Delta Ct_{(infected)} = Ct_{(target, infected)} - Ct_{(reference, infected)}$

$\Delta Ct_{(control)} = Ct_{(target, control)} - Ct_{(reference, control)}$

In the second step, the ΔCt of the *T. b. brucei* infected tissue samples were normalized to the ΔCt of the control.

$\Delta\Delta Ct = \Delta Ct_{(infected)} - \Delta Ct_{(control)}$

In the final step, the normal expression ratio is calculated as the difference in the expression between the gene in the two (2) samples

$$\text{Relative quantification (RQ)} = 2^{-\Delta\Delta Ct}$$

All Calculation were conducted in Microsoft Excel, 2019. One-sample Student's t-test was performed in GraphPad Prism 8.0 to compare the difference in the expression of ST3Gal1 and ST6Gal1 isolated from liver and kidney tissue samples of *T. b. brucei* infected and control pigs.

3.13 Data Analysis

Descriptive statistics were performed for all the data obtained and were expressed as the mean ± SD using GraphPad Prism Version 8.0 for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com). The Student's t-test was used to compare

means between the two groups. The values of $p<0.05$ were considered significant. The analyzed data werepresented in charts.

CHAPTER FOUR

4.0 RESULTS

4.1 Mean Log_{10}Parasitaemia of*T. b. brucei*-Infected Pigs

The mean log_{10}parasitemiaof*T. b. brucei* pigs (Figure 4.1) show T. *b. brucei* organism was first detected in the blood of pigs on day 3 post-infection and by day 5 post-infection, all the pigs in the infected group were parasitaemic. Hence, the mean pre-patent period for this experimental infection was 4.29 ± 0.35 days. From day 3 post-infection Log Equivalent value (LEV) of 0.4 ± 0.2, a fluctuating parasitemia was observed with Peak LEVs of 3.0 ± 0.4, 5.2 ± 1.2 and 3.6 ± 0.9 on days 7, 13 and 20, respectively which corresponded to the peak period of each parasitaemic wave (Fig. 4.1). The second wave of parasitemia was the highest followed by the third with observable minimal mean parasitemia level in between the peaks.

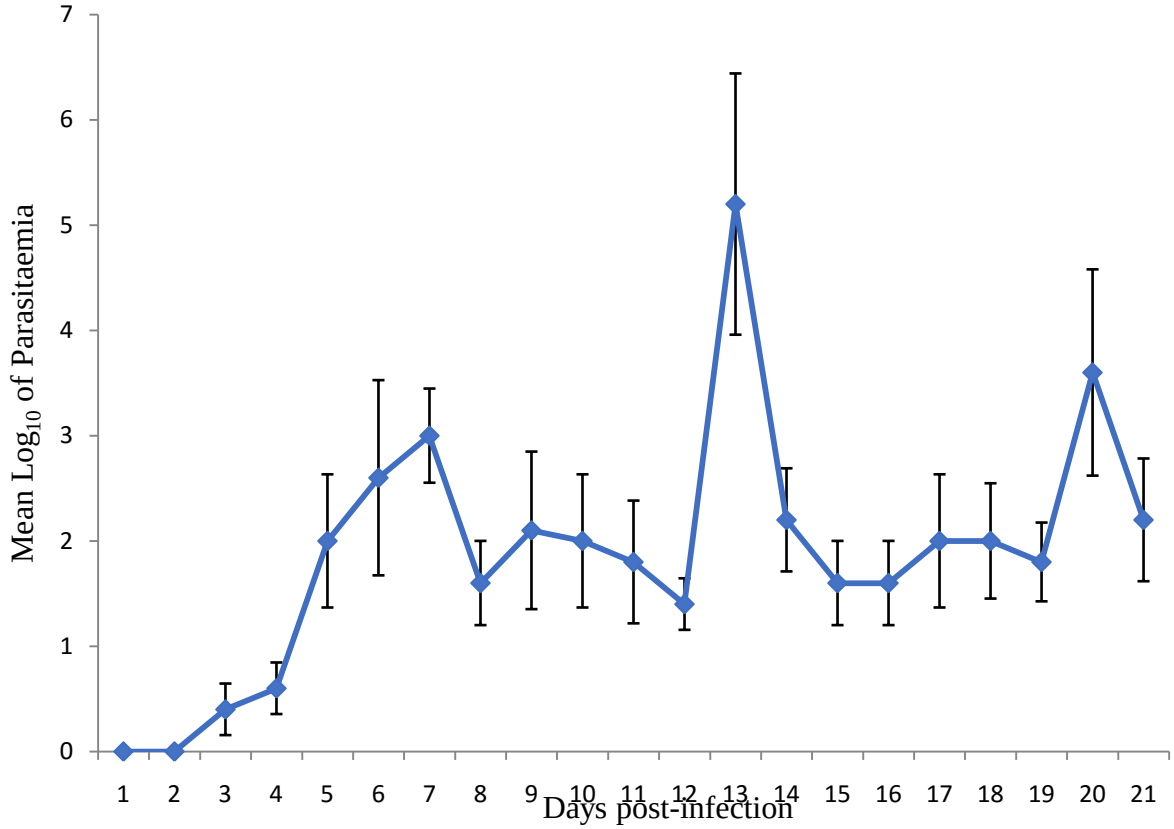

Figure 4.1: Mean Log_{10} of *T. b. brucei* parasitaemia of infected pigs.

4.2 Clinical Observationsof*T. b. brucei*Infected Pigs

4.2.1 Variation ofrectal temperature

The mean rectal temperatures in *T. b. brucei* infected and control pigs are presented in Figure 4.2. At the start of the experiment (day 0), the rectal temperatures of all the experimental pigs (infected and control) ranged from 37.1 to 37.8 ^{0}C with mean values of 37.96 ± 0.45 ^{0}C and 37.90 ± 0.37 ^{0}C for the infected and control groups, respectively. The rectal temperature of the infected group gradually rose to 39.62 ± 0.20 ^{0}C on day 3 post-infection which coincided with first detection of parasitemia. This was then followed by flunctuating increases with highest peak values of 40.46 ± 0.15 ^{0}C on day 7 and 40.62 ± 0.32 ^{0}Con day 13. Remarkably, these were the days of 1st and 2nd peaksof parasitemia, respectively. A gradual drop in rectal temperature was then observed with occasional spikes until termination of the experiment at day 21 post-infection. The mean rectal temperature of the control group varied but remained within the reference range up to termination of the experiment.

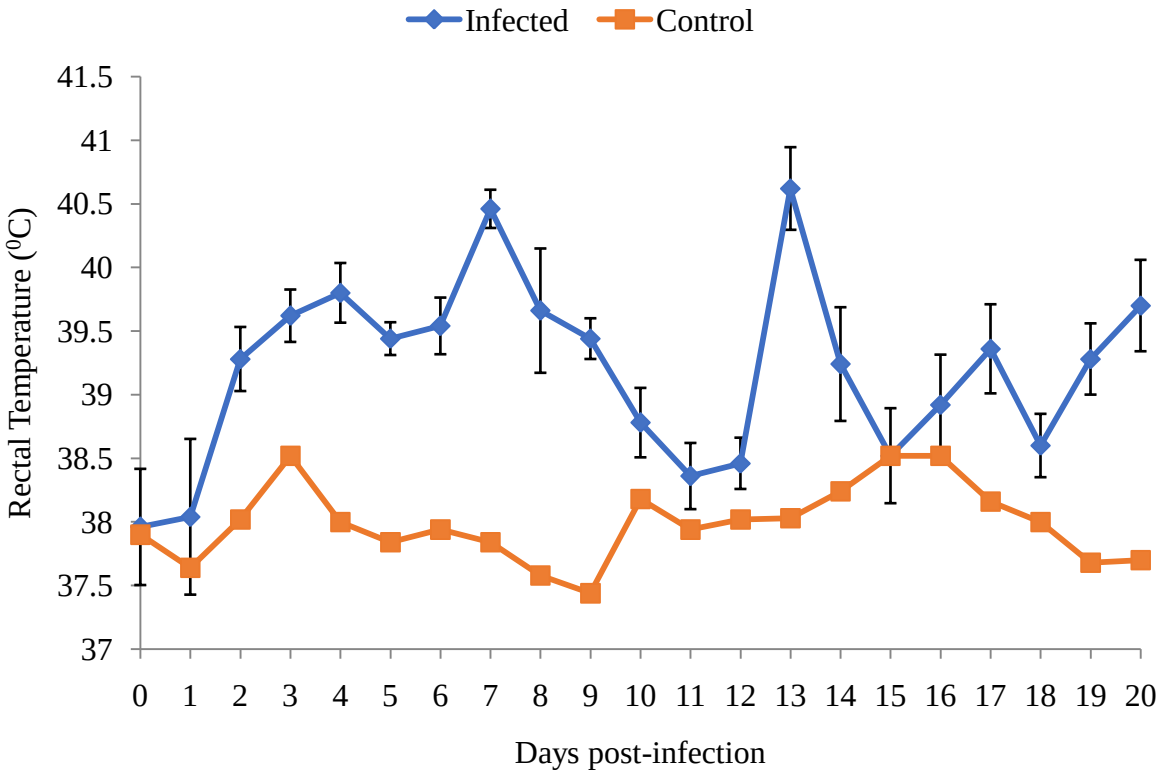

Figure 4.2: Variation in the mean rectal temperatures of*T. b. brucei*-infected and control pigs.

4.2.2 Variation in body weight

Themean body weight of all the experimental pigs were between 16.8 and 42.5 kg at the start of the experiment on day 0 with mean values of 22.88 ± 2.04 and 30.60 ± 3.00 kg for the infected and control group, respectively (Figure 4.3). Post-infection, the bodyweight of the *T. b. brucei*-infected pigs rose to 25.05 ± 1.62 kg on day 7 and then dropped to 22.60 ± 1.54 kg on day 14 and finally increased to 23.60 ± 2.16 kg by the end of the experimental period at day 21 ($P>0.05$). Meanwhile, the mean body weight of the control pigs increased progressively from 30.60 ± 3.00 kg at day 0 to 34.58 ± 2. 94 kg by day 21.

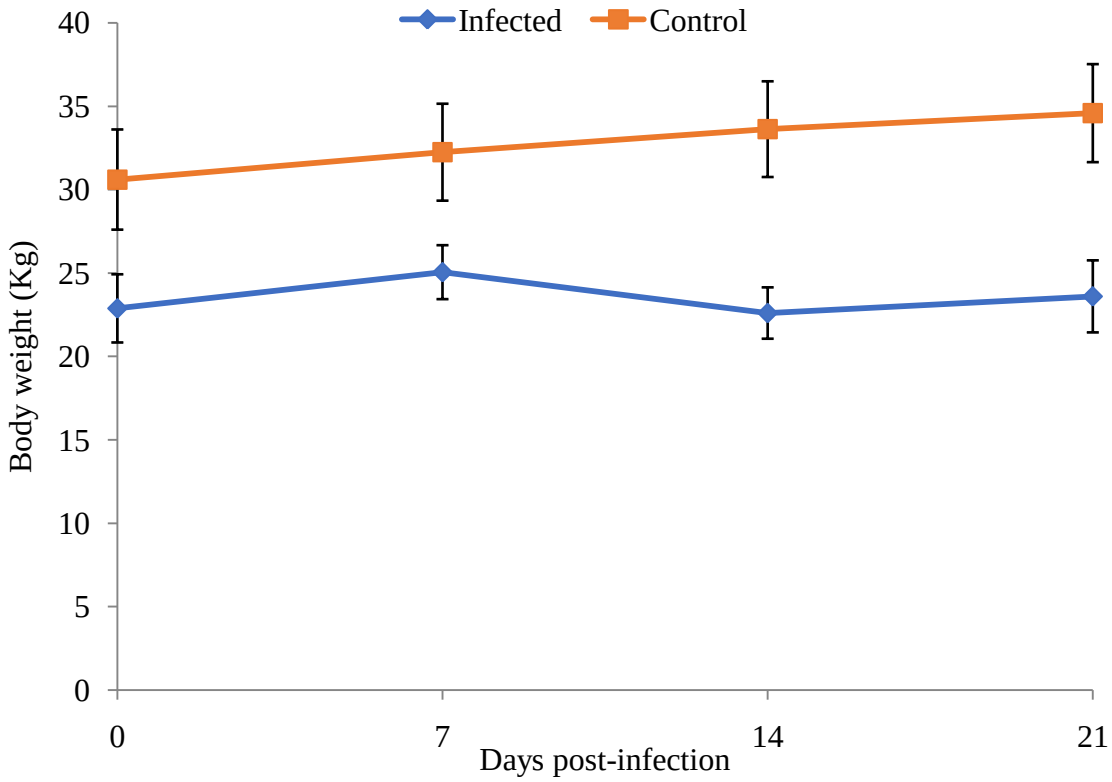

Figure 4.3: Mean body weights of *T. b. brucei*-infected and control pigs.

4.3 Haematological Changes of*Trypanosoma brucei brucei*-Infected and Control Pigs

4.3.1 Variation in packed cell volume

The mean PCV values of experimental pigs (infected and control), ranged from 34.0 to 47.0 % at the start of the experiment on day 0 with mean values of 41.40 ± 1.33 and 39.00 ± 2.55 % for infected and control groups, respectively (Figure 4.4). Following infection of the pigs with trypanosomes, there was a gradual and progressive drop in the mean PCV values in the infected group until a minimum value of 19.40 ± 1.66 % was recorded on day 13 post-infection. Following the attainment of this minimal mean PCV, a gradual increase to the highest value of 22.60 ± 1.81 % was observed on day 21 post-infection when compared to the control. A significant ($P<0.05$) decrease in PCV value in the infected group became evident beginning from day 4 post-infection. The mean PCV in the control group was maintained within a steady limit and the values did not vary significantly ($P>0.05$) throughout the experimental period. The lowest mean PCV level (19.40 ± 1.66 %) recorded in the infected group differed significantly ($P<0.05$) from the corresponding value (37.04 ± 2.36 %) in the control group.

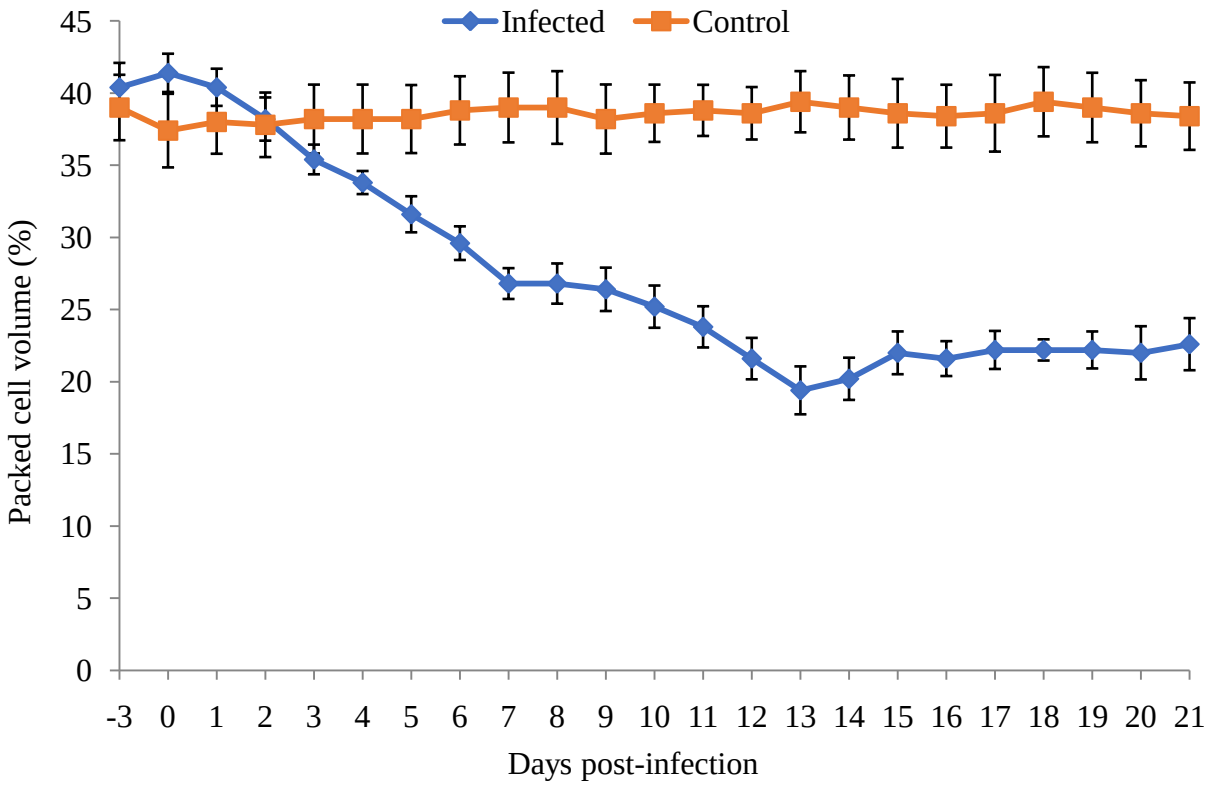

Figure 4.4: Packed cell volume (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.2 Variation in haemoglobinconcentration

At the start of the experiment on day 0, themean HBconcentrations were 13.24 ± 0.84 and 13.44 ± 1.28 mg/dl for infected and control groups, respectively (Figure 4.5). Following infection of the pigs with trypanosomes, there was a gradual and progressive drop in the mean HBof the infected group to reach a minimum value of 6.60 ± 0.76 g/dl at the termination of the experiment on day 21. Significant ($P<0.05$) decrease in haemoglobin concentration became evident beginning between days 4 to 21 post-infection. The mean haemoglobin concentration of the control group did not vary significantly ($P>0.05$) and was maintained within a steady limit and the values throughout the experimental period. The lowest mean haemoglobin concentration (6.60 ± 0.76 g/dl) recorded in the infected group differed significantly ($P<0.05$) from the corresponding value (11.78 ± 1.46 g/dl) in the control group.

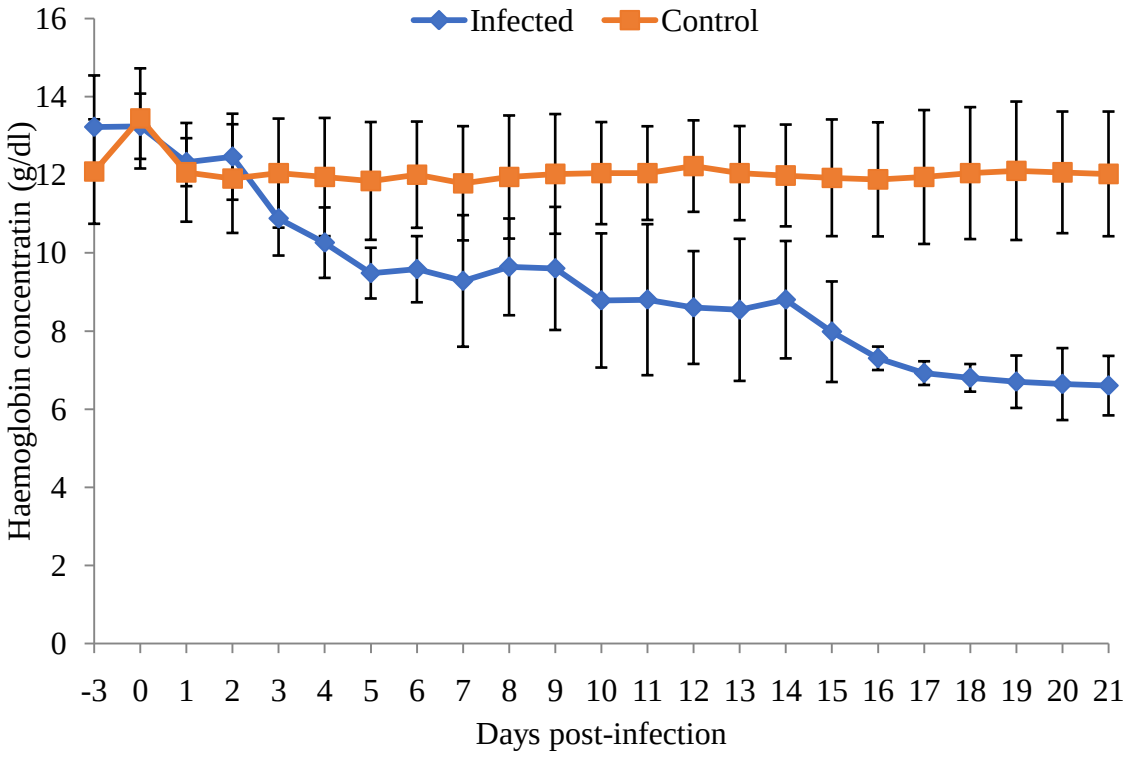

Figure 4.5: Haemoglobin concentrations (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.3 Variation in red blood cellcount

The mean red blood cell counts of all experimental pigs ranged between 4.7 and 6.3 x 10^{12}/L at the start of the experiment on day 0 with mean values of 5.60 ± 0.52 and 5.22 ± 0.36 x 10^{12}/L for infected and control groups, respectively (Figure 4.6). Following infection of the pigs with trypanosomes, the mean red blood cell counts of the infected group remained within the normal range until day 4 when it started to decline progressively until a lowest value of 3.50 ± 0.72 x 10^{12}/L was recorded at the termination of the experiment on day 21. Significant ($P<0.05$) decrease in mean red blood cell counts became evident beginning from day 4 to day 21 post-infection. Unlikein the control group where the mean values did not vary significantly ($P>0.05$) throughout the experimental period. The lowest mean value (3.50 ± 0.72 x 10^{12}/L) recorded in the control group differed significantly ($P<0.05$) from the corresponding value (5.14 ± 0.53 x 10^{12}/L) in the infected group at the termination of the experiment.

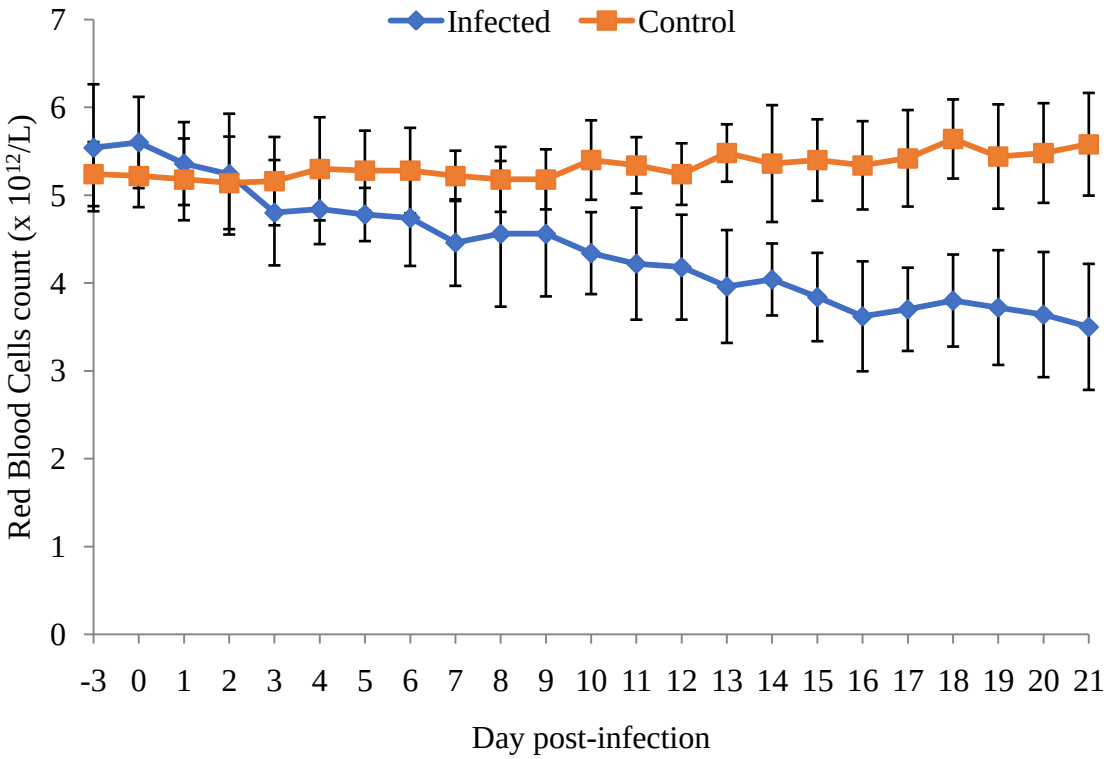

Figure 4.6: Red blood cell count (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.4 Variation in mean corpuscular volume

The mean corpuscular volume (MCV) of*T. b. brucei* infected and control pigs are presented in Figure 4.7. The MCV values of all experimental pigs varied between 50.22 and 65.90 fL at the start of the experiment on day 0 with mean values of 60.11 ± 3.57 and 59.12 ± 6.61 fL for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the mean values for the infected group remained within the normal range until day 6 when the value increased to 71.43 ± 5.08 fL with a further increase to 81.74 ± 10.fL by day 10;thereafter, fluctuating increases and decreases followed until a mean value of 69.64 ± 12.38 fL was obtained at the termination of the experiment on day 21. Significant ($P<0.05$) increase in mean corpuscular volume was recorded on days 10, 17, and 18 post-infection. The mean MCV of the control group was maintained within a steady limit and did not vary significantly ($P>0.05$) throughout the experimental period.

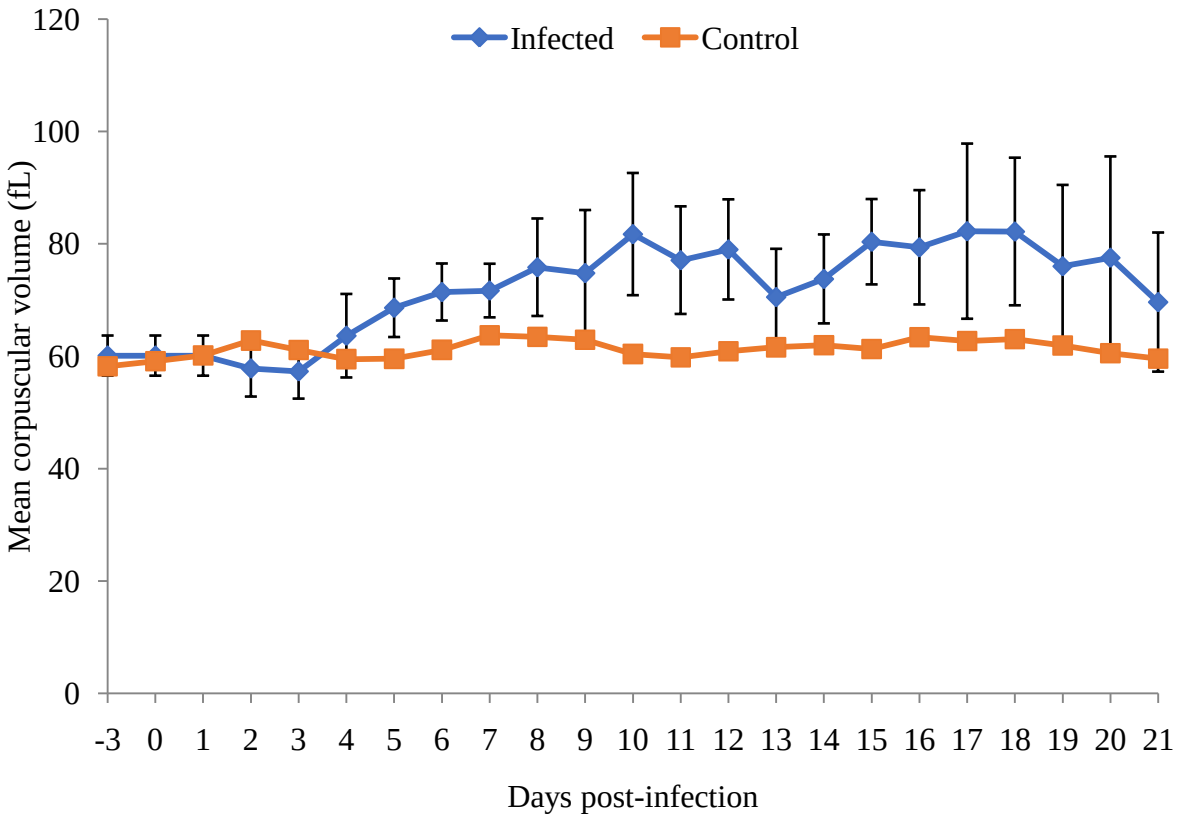

Figure 4.7:Mean corpuscular volume (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.5 Variation in mean corpuscular haemoglobin concentration

The mean corpuscular hemoglobin concentrations (MCHC) of*T. b. brucei* infected and control pigs are presented in Figure 4.8. The values for all experimental pigs varied between 29.9 and 35.8 g/dL at the start of the experiment on day 0 with mean values of 32.03 ± 0.83 and 32.00 ± 2.48 g/dL for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the mean MCHC for the infected group showed fluctuating decreases from day 5 to attainthe lowest value 27.23 ± 1.58 g/dL on day 7 post-infection. Even though the variations were not statistically significant (P>0.05). This was followed by a fluctuating increase and recovery to pre-infection value on day 12 which was maintained until termination of the experiment on day 21. The mean MCHC value of the control group was maintained within a steady limit and did not vary significantly (P>0.05) throughout the experimental period.

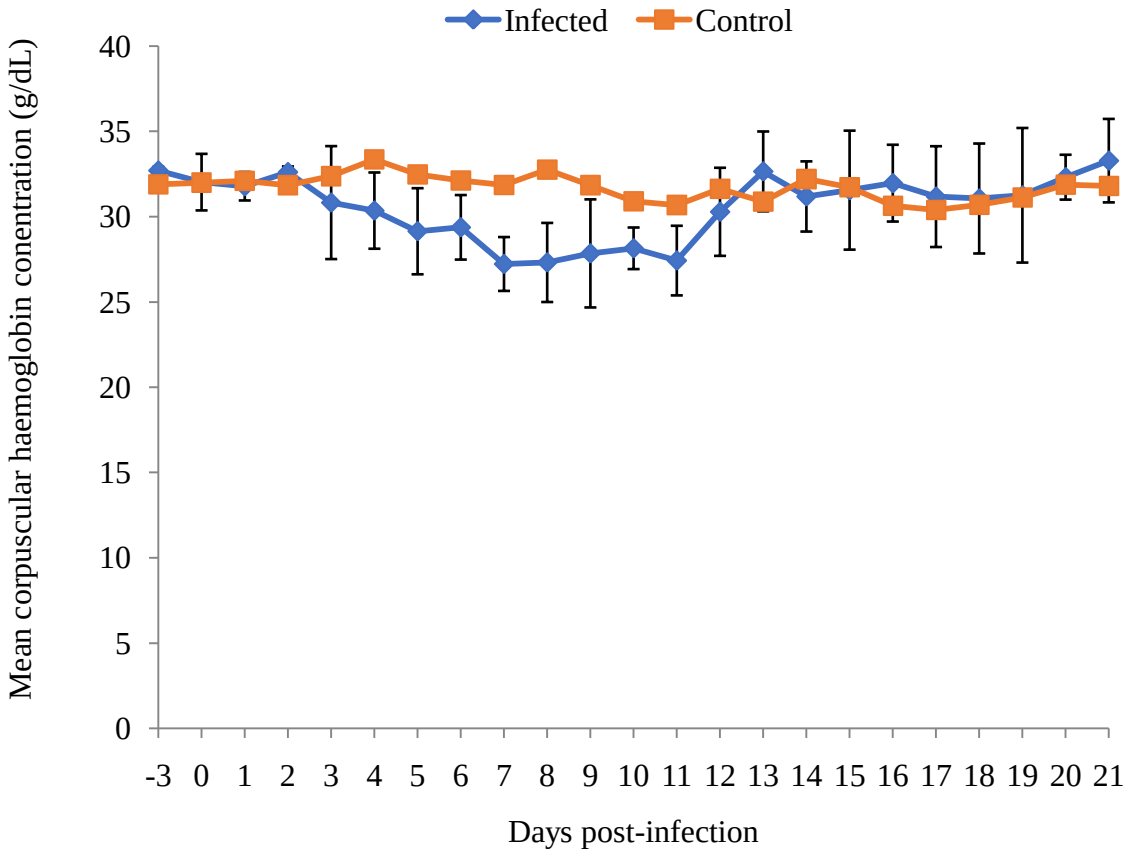

Figure 4.8: Mean corpuscular haemoglobin concentration (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.6 Variation in mean corpuscular haemoglobin

The mean corpuscular hemoglobin (MCH) of*T. b. brucei* infected and control pigs are presented in Figure 4.9. The values for all experimental pigs varied between 18.2and 23.6pg at the start of the experiment on day 0 with mean values of 19.23 ± 1.02 and 18.91 ± 0.98 pg for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the mean MCH values for the infected group were maintained within narrow limits up to day 21 when the value attained a non-significant ($p > 0.05$) increase to 23.01 ± 1.21pg and pattern of increases were sustained until termination of the experiment on day 21. The MCH values of the control group were maintained within a steady limit and did not vary significantly ($P>0.05$) throughout the experimental period.

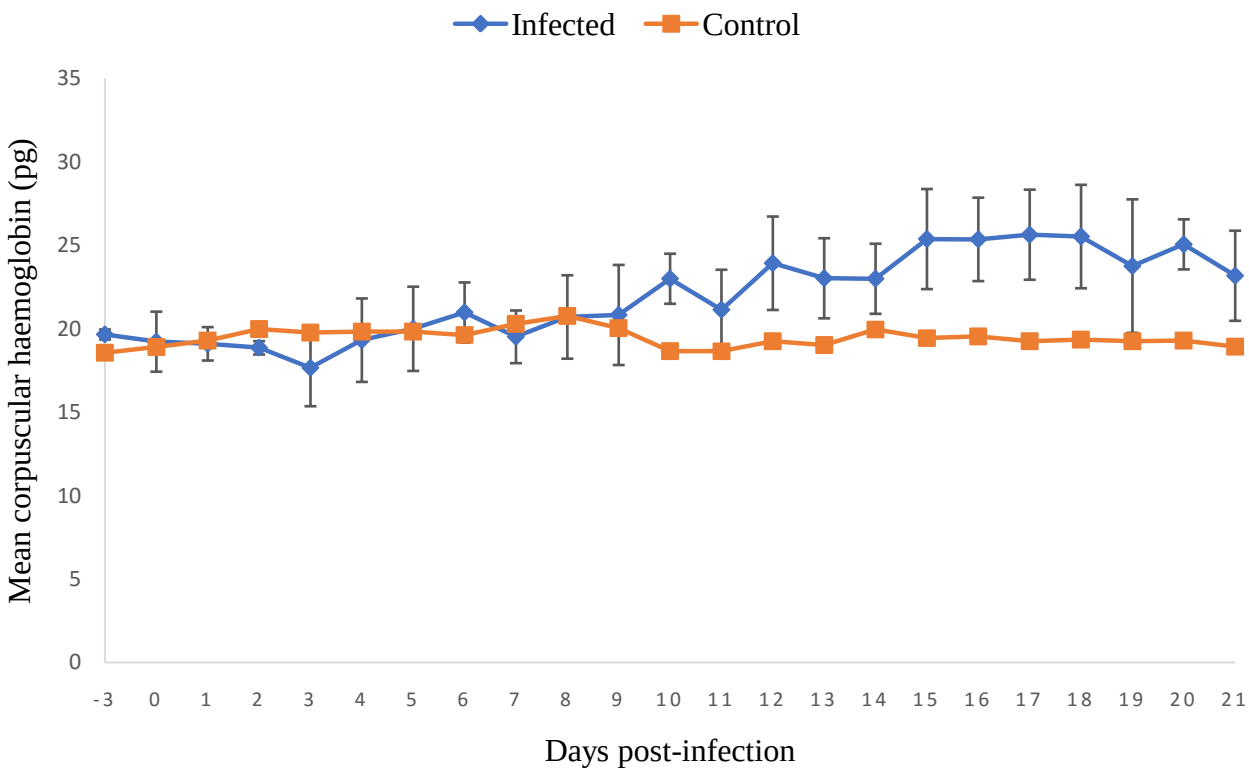

Figure 4.9: Mean corpuscular haemoglobin (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.6 Variation in platelet counts

The values of mean platelet countof*T. b. brucei*-infected and control pigs are presented in Figure 4.10. The values for all experimental pigs varied between 203.0 and 320.0 x 10^9/L at the start of the experiment on day 0 with mean values of 263.6 ± 46.34 and 259.0 ± 30.6 x 10^9/L for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the mean platelets countfor the infected group remained within the normal range until day 11 when the value started to declineto reach a lowest valueof 126.2 ± 31.8 x 10^9/L at the termination of the experiment on day 21. Significant ($P<0.05$) decrease in platelet count were recorded from day 8 to day 21 post-infection. The mean platelet counts of the control group were maintained within a steady limit and did not vary significantly ($P>0.05$) throughout the experimental period.

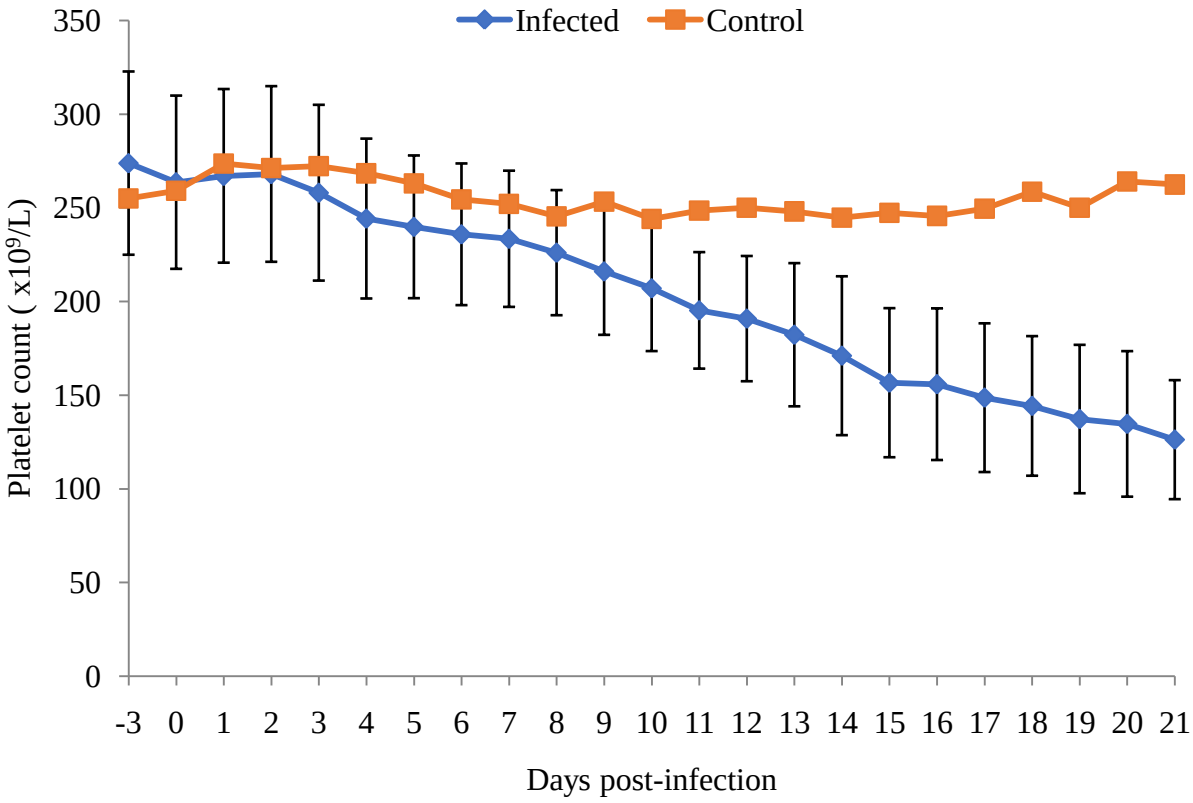

Figure 4.10: Platelet counts (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.7 Variation in total white blood cell counts

The mean white blood cell (WBC) counts of*T. b. brucei* infected and control pigs are presented in Figure 4.11. The values of all experimental pigs varied between 10.9 and 15.7 x 10^9/L at the start of the experiment on day 0 with mean values of 12.92 ± 1.48 and 13.28 ± 0.87 x 10^9/L for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the mean values of the infected group increased significantly ($P<0.05$) from 12.00 ± 3.12 x 10^9/L on day 5 to 17.10 ± 4.24 x 10^9/L on day 6 with a peak value of 27.48 ± 4.77 x 10^9/L obtained at the termination of the experiment on day 21. The mean WBC count of the control group was maintained within a steady limit and did not vary significantly ($P>0.05$) throughout the experimental period.

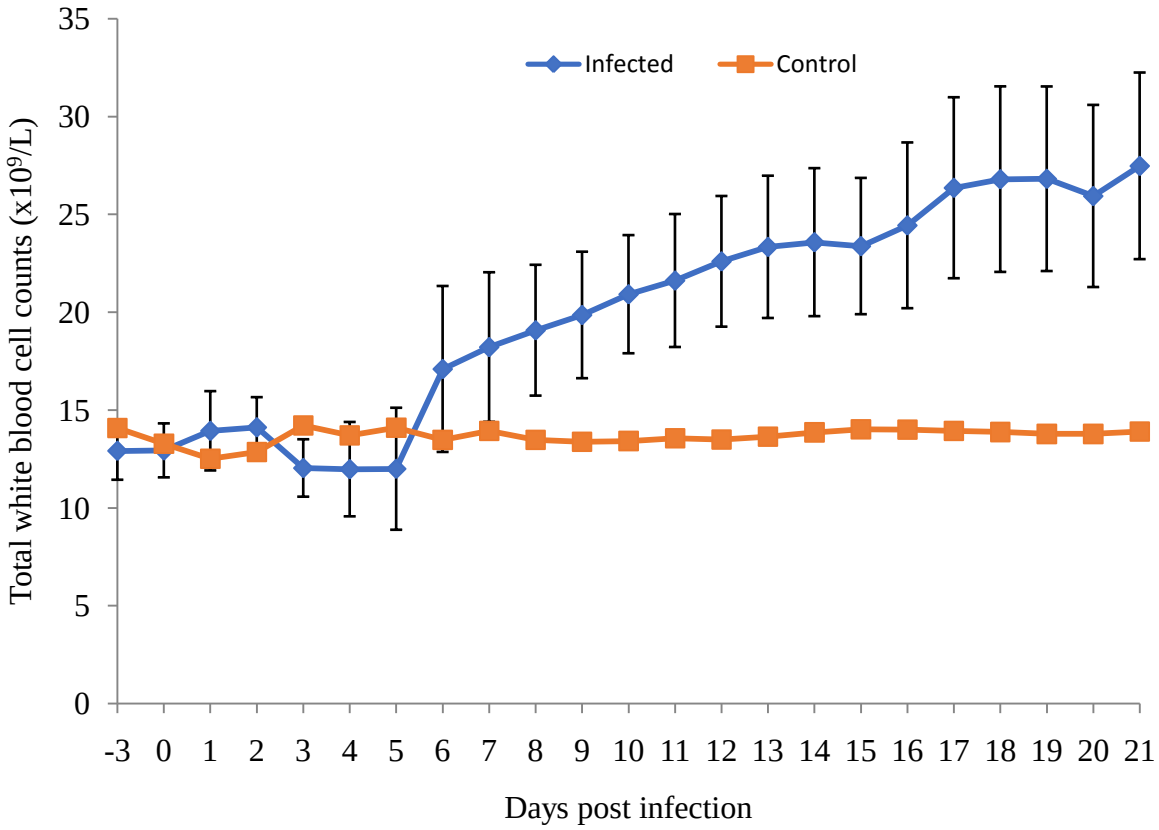

Figure 4.11: White blood cell counts (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.8 Variation in differential leukocyte counts

4.3.8.1 Variation in neutrophil count

The mean neutrophil countsof*T. b. brucei*-infected and control pigs are presented in Figure 4.12. The values in all experimental pigs varied between 4.2 and 6.5 x 10^9/L at the start of the experiment on day 0 while the mean values of 5.22 ± 0.71 and 6.04 ± 0.41 x 10^9/L for infected and control groups, respectively. The infection of the pigs with trypanosomes, the neutrophil count showed a slight increase on day 2 followed by a drop on day 3, and then a statistically significant ($P<0.05$) increase to reach 8.98 ± 0.62 x 10^9/L on day 9 after which the value continued to increase throughout the experimental period with a highest peak of 15.46 ± 2.51 x 10^9/L attained on day 21 post-infection. The mean neutrophil counts of the control group was maintained within a normal reference limit and only showed slight non-significant ($P>0.05$) fluctuations throughout the experimental period.

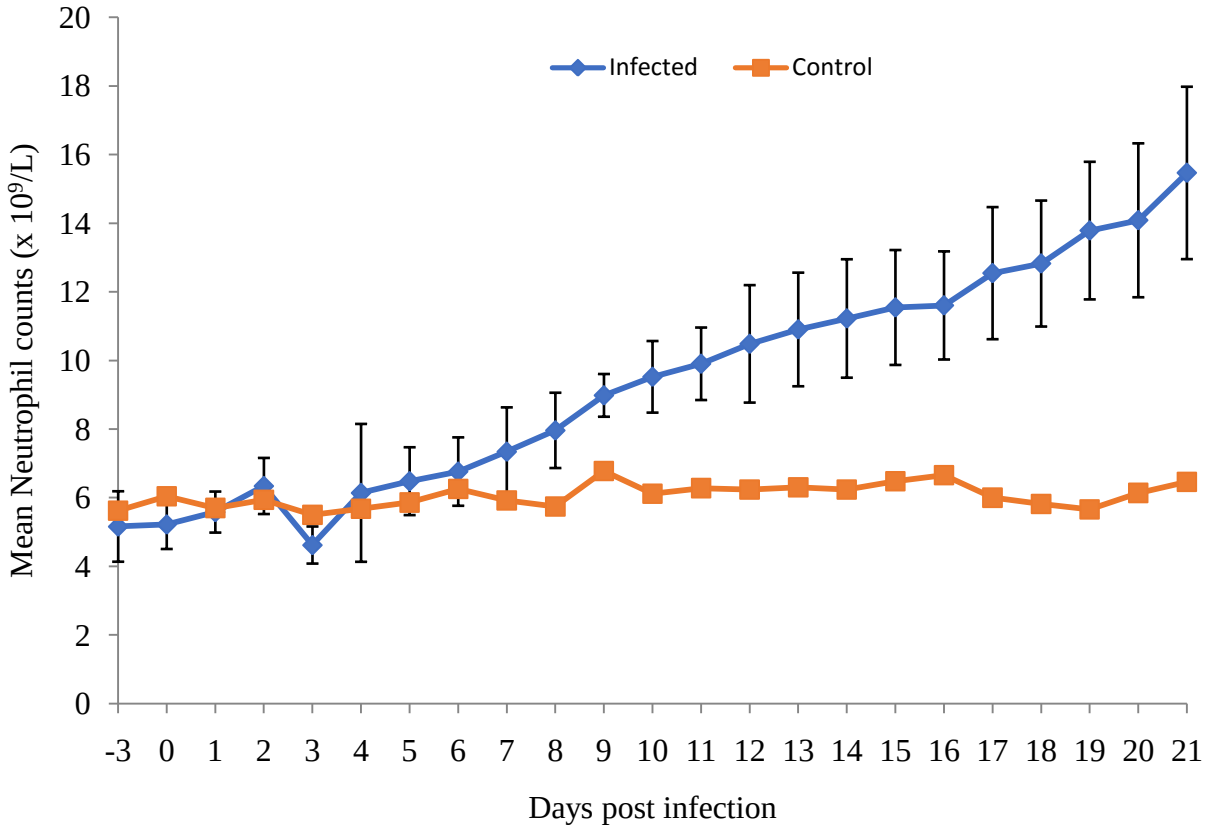

Figure 4.12: Neutrophil counts (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.8.2 Variation in lymphocyte counts

The mean lymphocyte counts of*T. b. brucei* infected and control pigs are presented in Figure 4.13. The lymphocyte counts in all experimental pigs varied between 5.4 and 9.5 x 10^9/L at the start of the experiment on day 0 with mean values of 7.30 ± 1.56 and 6.50 ± 1.11 x 10^9/L for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the lymphocyte counts showed progressive non-significant ($P>0.05$) increase from day 5 to day 11 post-infection, followed by a statistically significant ($P<0.05$) increase to a value of 13.72 ± 2.65 x 10^9/L recorded on day 9 post-infection and the value continued to increase until termination of the experiment with a highest value of 21.92 ± 2.89 x 10^9/L at day 21 post-infection. The mean lymphocyte counts of control group only showed some fluctuationsthat were not significant ($P>0.05$), throughout the experimental period.

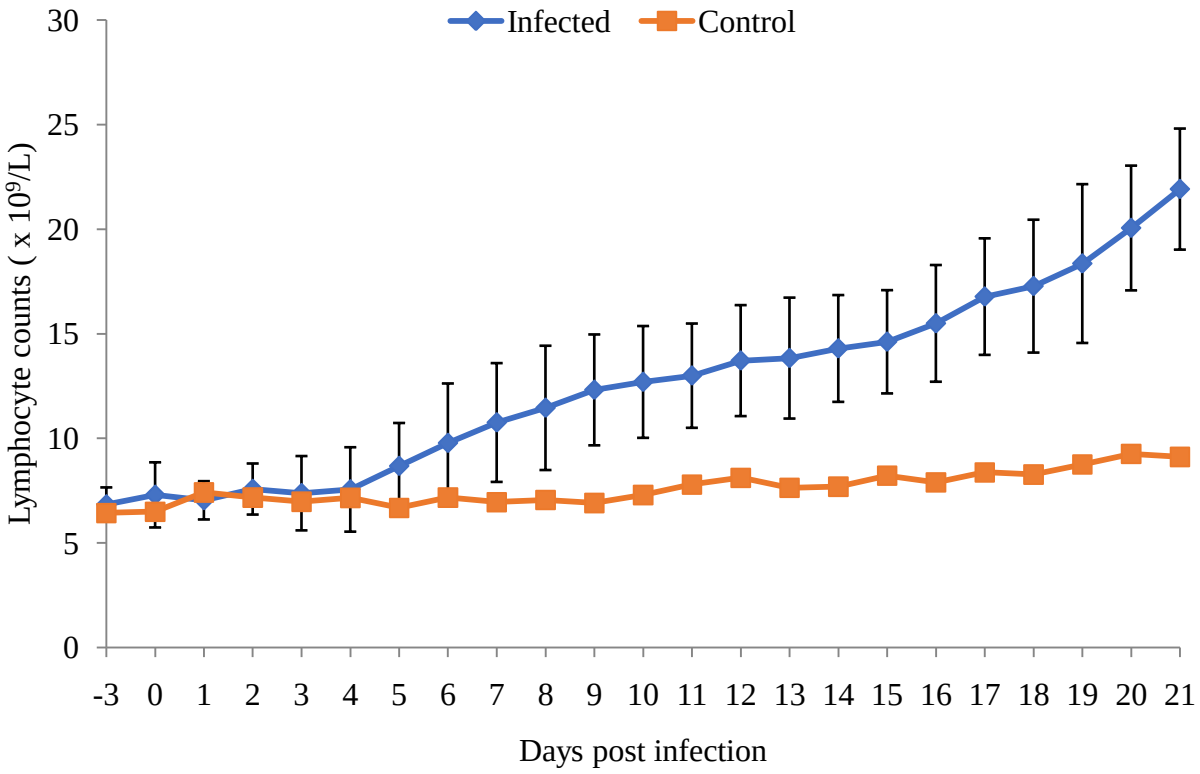

Figure 4.13: Lymphocytes counts (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.3.8.3 Variation in eosinophil counts

The mean eosinophil countsof*T. b. brucei*-infected and control pigs are presented in Appendix III. The eosinophil count in all experimental pigs varied between 0.0 and 1.0 x 10^9/L at the start of the experiment on day 0 with mean values of 0.32 ± 0.40 and 0.18 ± 0.13 x 10^9/L for infected and control groups, respectively. After infection, the mean eosinophil count of the infected group showed non-significant ($P>0.05$) fluctuations with a highest value of 1.02 ± 0.73 x 10^9/L recorded on day 20 post-infection. Meanwhile, the mean value for the control group only showed fluctuations that were not significant.

4.3.8.4 Variation in monocyte counts

The mean monocyte counts of*T. b. brucei*-infected and control pigs are presented in Appendix III. The monocyte counts in all experimental pigs varied between 0.0 and 0.3 x 10^9/L at the start of the experiment on day 0 with mean values of 0.12 ± 0.13 and 0.12 ± 0.13 x 10^9/L for infected and control groups, respectively. During infection, the mean monocyte counts of the infected and control groups fluctuated within the reference limits throughout the experimental period.

4.4 Neuraminidase Activity in *T. b. brucei*-Infected Pigs

The neuraminidase activity of*T. b. brucei* infected and control pigs are presented in Figure 4.14. The activity in all experimental pigs varied between 0.9 and 2.1 µmol/min at the start of the experiment on day 0 with mean values of 1.46 ± 0.38 and 1.48 ± 0.48 µmol/min for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the mean neuraminidase activity started to increase from day 5 and attained a statistically significant ($P<0.05$) value of 3.96 ± 2.38 µmol/min by day 7 post-infection. Thereafter, the mean activity showed a slight decrease overthe next 2 days and followed by an increase on day 10 until the highest peak of 5.22 ± 1.49 µmol/min was obtained on day 11 ($P<0.05$). This was followed by a fluctuation in values that were non-significant ($P>0.05$) until termination of the experiment. The mean neuraminidase activity of the control group fluctuated within a narrow limit throughout the experimental period.

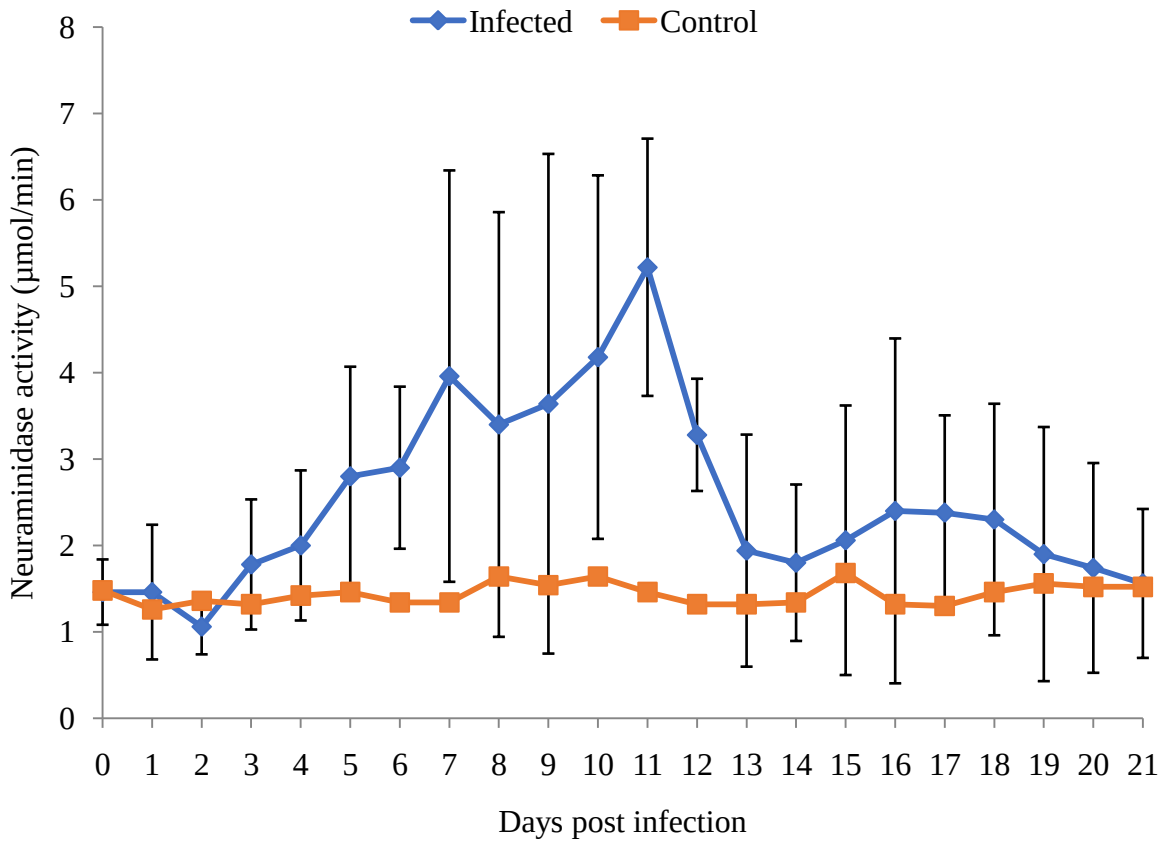

Figure 4.14: Neuraminidase activities (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.5 Sialic Acid Concentration of*T. b. brucei*-Infected Pigs

4.5.1 Variation in erythrocyte surface sialic acid concentration

The erythrocyte surface sialic acid (ESSA)concentration in *T. b. brucei* infected and control pigs are presented in Figure 4.15. The ESSA concentration in all experimental pigs varied between 1.2 and 3.8 mg/dl at the start of the experiment on day 0 with mean values of 2.58 ± 0.90 and 2.38 ± 1.05 mg/dl for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the mean ESSA concentrationbegan to drop below normal value from day 6 post-infection, three days after parasites were first detected in the blood of infected pigs. Thereafter, the mean ESSA concentration increased over two days followed by a progressive significant ($P<0.05$) drop to 0.72 ± 0.37 mg/dl on day 13 post-infection, which was approximately a week after the initial drop in concentration. The ESSA concentration then showed an apparent progressive rise to reach a value of 2.34 ± 1.97 mg/dl at the termination of the experiment on day 21 post-infection. The mean ESSA concentration of the control group fluctuated within a narrow limit throughout the experimental period.

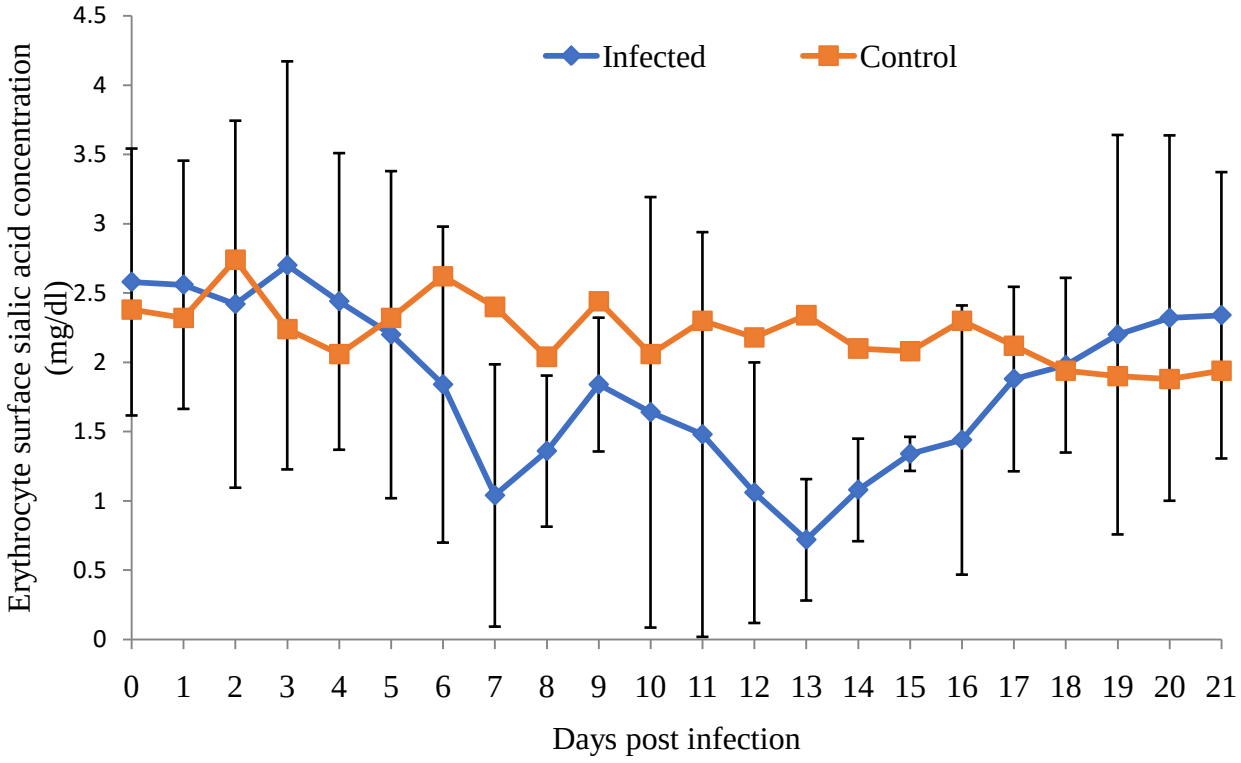

Figure 4.15: Erythrocyte surface sialic acid concentrations (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.4.2 Variation in free serum sialic acid concentration

The values of mean free serum sialic acid (FSSA)concentration of*T. b. brucei* infected and control pigs are presented in Figure 4.16. The FSSA concentration in all experimental pigs varied between 1.3 and 2.4 mg/dl at the start of the experiment on day 0 with mean values of 1.90 ± 0.42 and 1.86 ± 0.34 mg/dl for infected and control groups, respectively. Following infection of the pigs with trypanosomes, the mean FSSA concentrations began to increase from day 4 and attained a statistically significant ($P<0.05$) value of 2.98 ± 0.72 mg/dl by day 7 post-infection. Thereafter, the mean FSSA concentration showed a progressive increase until the highest peak of 4.08 ± 2.26 mg/dl was reached on day 11 post-infection. This was followed by an apparent but fluctuating decrease in values all through the experimental period until a close to the pre-infection value of 1.88 ± 0.51 mg/dl was obtained on day 21 post-infection. The mean FSSA concentration of the control group fluctuated within a narrow limit throughout the experimental period.

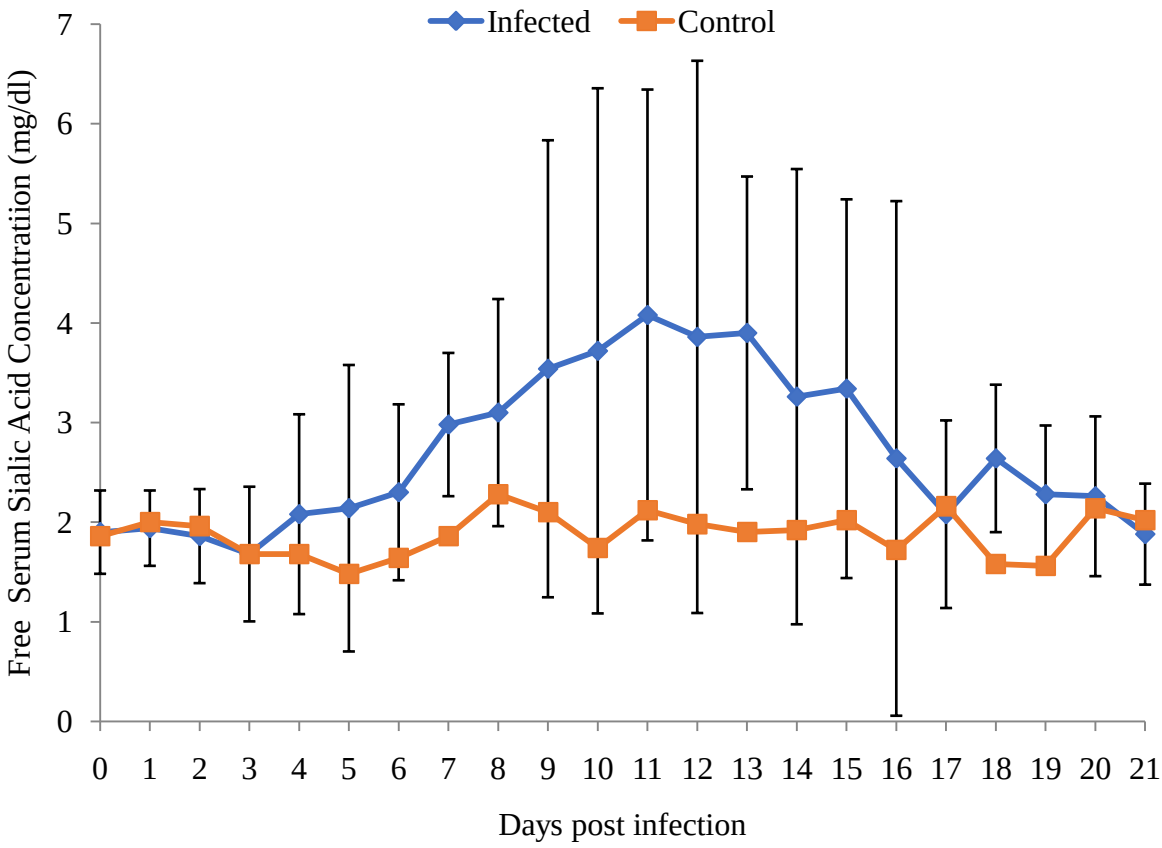

Fig 4.16: Free serum sialic acid concentrations (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.6 Sialyltransferase Activity of*T. b. brucei*-Infected Pigs

4.6.1 Variation in serum sialyltransferase activities

The sialyltransferase activity of*T. b. brucei* infected and control pigs are presented in Figure 4.17. The sialyltransferase activity in all experimental pigs varied between 0.83 and 1.97 x 10^{-2} mmol/min at the start of the experiment on day 0 with mean values for infected and control groups were 1.50 ± 0.43 and 1.29 ± 0.19 x 10^{-2} mmol/min respectively. Following infection of the pigs with trypanosomes, the mean sialyltransferase activity began to increase from day 3 and attained a first peak value of 3.00 ± 1.94 x 10^{-2} mmol/min on day 6. Thereafter, the mean decreased before increasingto reach a second peak value of 3.59 ± 1.09 x 10^{-2} mmol/min recorded on day 12 almost a week after the first peak. The highest peak value observed was 4.30 ± 2.46 x 10^{-2} mmol/min ($P<0.05$) on day 16 post-infection. A progressive non-significant ($P>0.05$) decrease in the activity up to termination of the experimental period;the lowest value of 1.09 ± 0.25 x 10^{-2} mmol/min was obtained on day 21. The mean sialyltransferase activity of the control group fluctuated within a narrow limit throughout the experimental period.

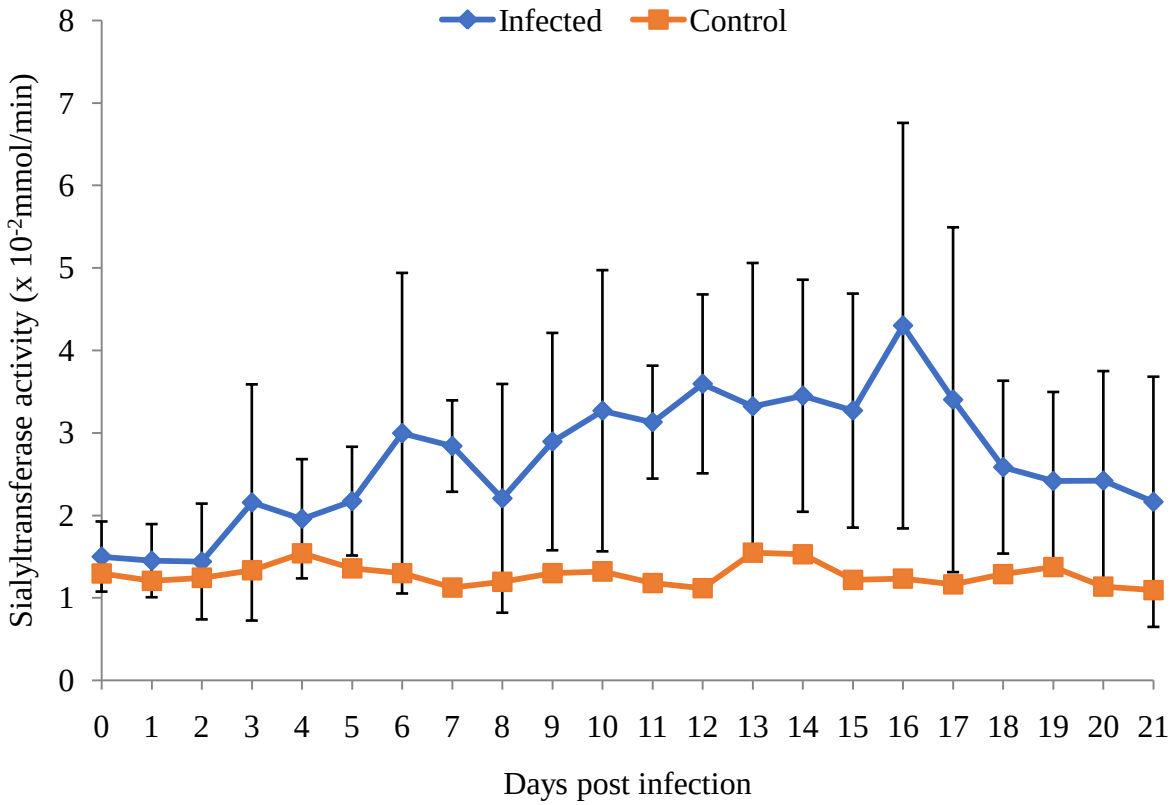

Figure 4.17: Serum sialyltransferase activities (Mean ± SD) of*Trypanosoma brucei brucei*-infected and control pigs.

4.6.2 Variation in sialyltransferase activity of the thyroid gland

At the termination of the experiment on 21 post-infection, the mean sialyltransferase activityin the thyroid gland of *T. brucei brucei*-infected and control groups were 1.84 ± 0.31 and 0.92 ± 0.27 x 10^{-2} mmol/min, respectively; the activities ranged from 1.51 to 2.14 and 0.59 to 1.39 x 10^{-2} mmol/min in the infected and control groups, respectively. There was a statistically significant ($P<0.05$) difference between the two mean activities.

4.7 Relationships Between Serum Sialyltransferase Activity and Parasitaemia, Packed Cell Volume, Neuraminidase Activity, Erythrocyte Surface, and Free Serum Sialic Acid Concentrations of*T. b. brucei*-Infected Pigs.

4.7.1 Relationship between sialyltransferase and parasitaemia

The relationship between serum sialyltransferase activity and parasitemia in *T. b. brucei* infected pigs are presented in Figure 4.18. The relationship was significant ($P<0.05$) and direct with a correlation coefficient (r) of 0.5.

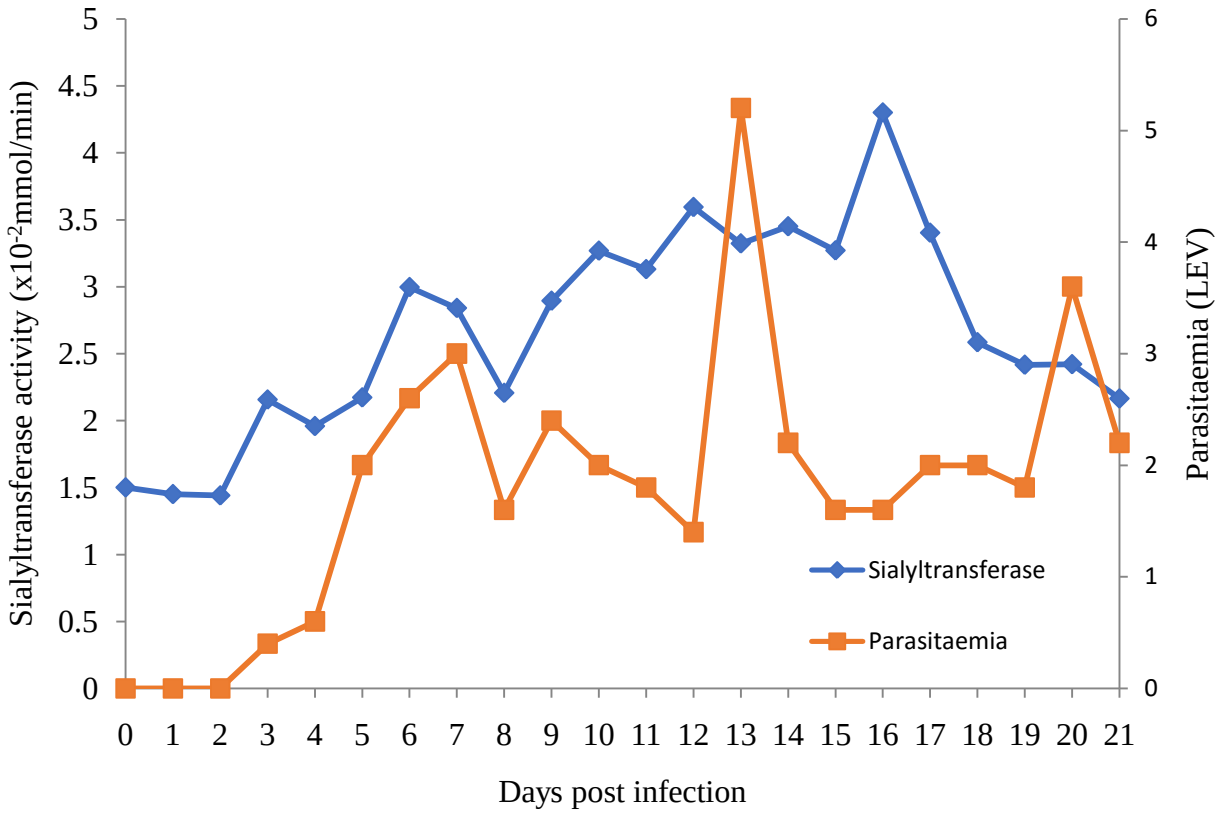

Figure 4.18: Relationship between serum sialyltransferase activities and parasitaemia of*Trypanosoma brucei brucei*-infected pigs.

4.7.2 Relationship between serum sialyltransferase and packed cell volume

The relationship between serum sialyltransferase activity and packed cell volume in *T. b. brucei* infected pigs are presented in Figure 4.19. The relationship was significant ($P<0.01$) but inverse with 'r' value of - 0.8.

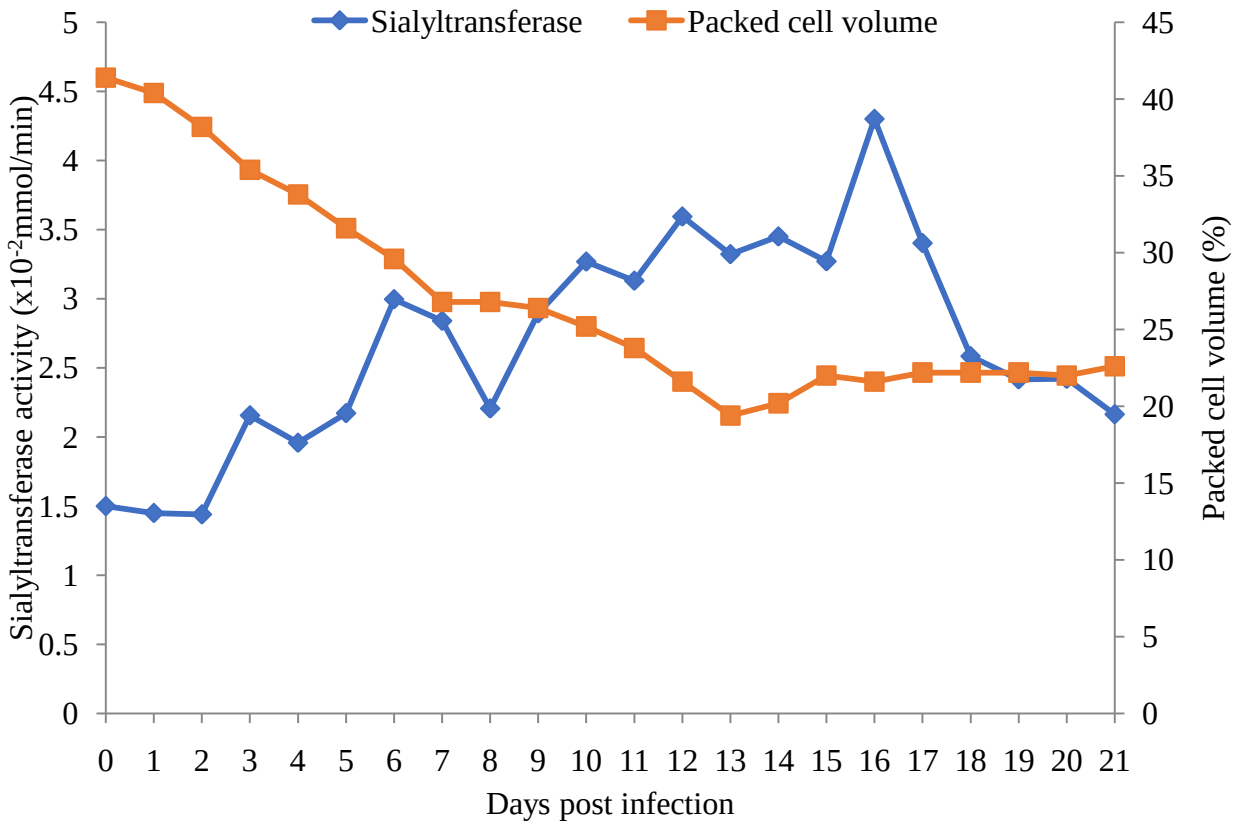

Figure 4.19: Relationship between serum sialyltransferase activities and packed cell volume of *Trypanosoma brucei brucei*-infected pigs.

4.7.3 Relationship between serum sialyltransferase and neuraminidase activities

The relationship between the serum sialyltransferase and neuraminidase activities in *T. b. brucei* infected pigs are presented in Figure 4.20. The relationship was significant ($P<0.05$) and direct ($r = 0.4$).

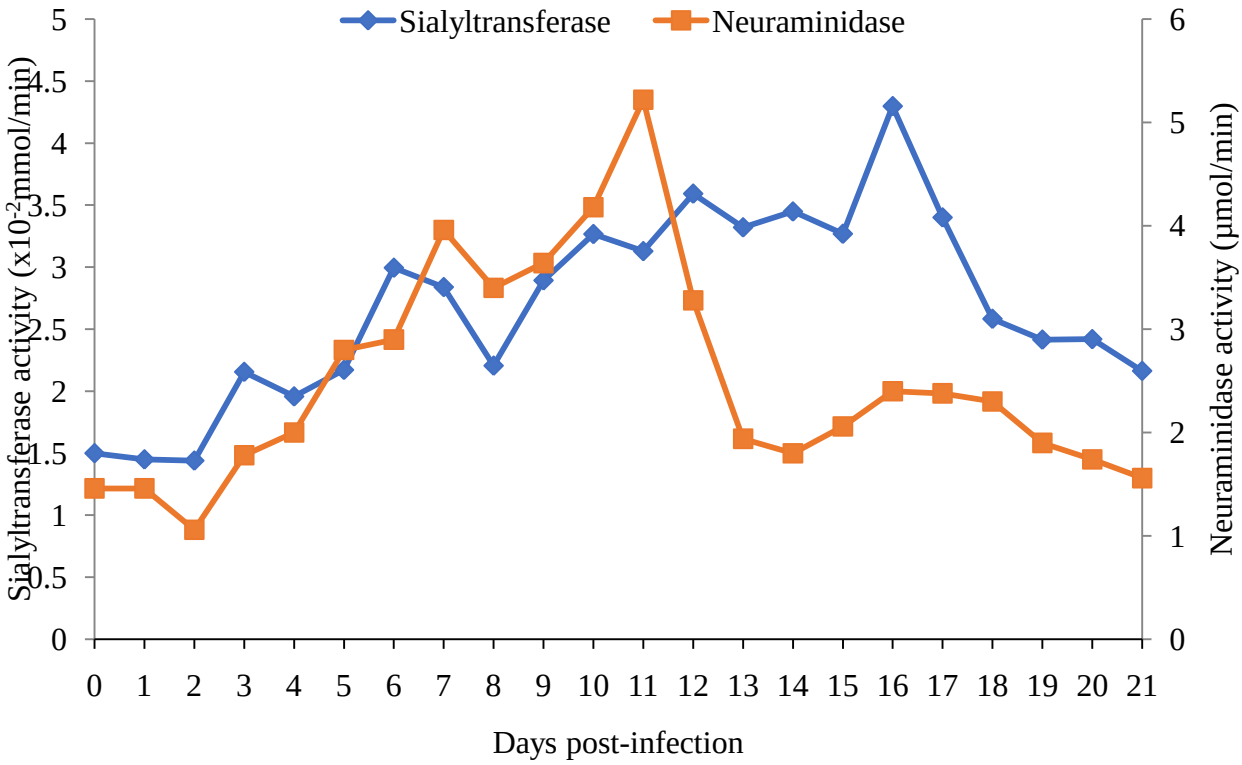

Figure 4.20: Relationship between serum sialyltransferase and neuraminidase activities of*Trypanosoma brucei brucei*-infected pigs.

4.7.4 Relationship between serum sialyltransferase and erythrocyte surface and free serum sialic acid concentration

The relationship between the serum sialyltransferase activity and erythrocyte surface and free serum sialic acid concentrations in *T. b. brucei* infected pigs are presented in Figure 4.21. The relationship between the serum sialyltransferase activities and erythrocyte surface sialic acid was significant ($P<0.01$) and inverse ($r = -0.8$) while the relationship between the serum sialyltransferase activity and free serum sialic acid concentration was significant ($P<0.01$) and direct ($r = 0.7$).

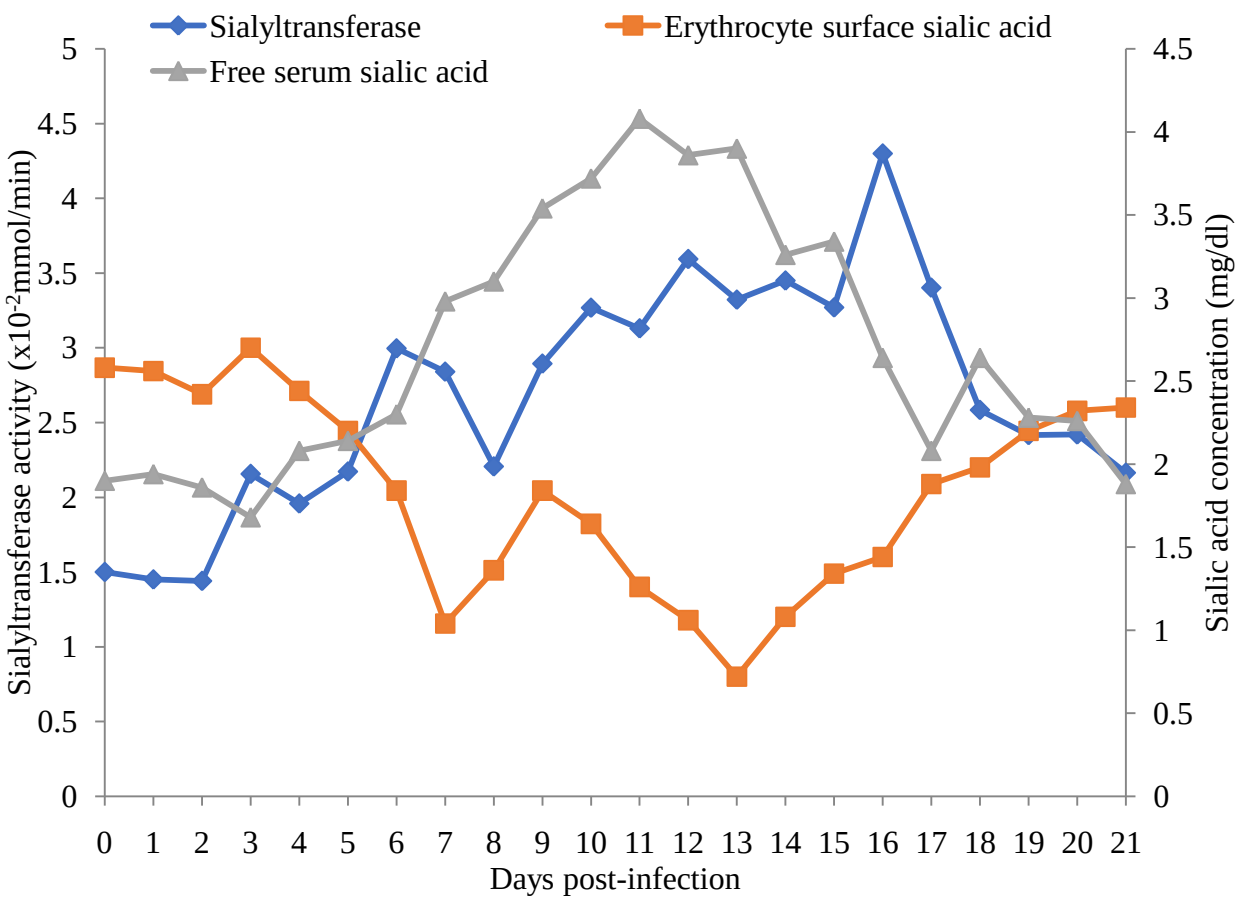

Figure 4.21: Relationship between serum sialyltransferase activities and sialic acid concentrations of *Trypanosoma brucei brucei*-infected pigs.

4.8 Histopathological Findingsof *T. b. brucei*-Infected Pigs

Histopathology of the kidneys revealed hypercellularity of the glomeruli with dilation of renal tubules and mononuclear cell infiltration around the glomeruli and within the tubules (Plate I). Areas of necrosis and atrophy of some glomeruli were also observed. In the liver,there were centrilobular congestion, degeneration of hepatocytes, haemosiderosis and infiltration with lymphocytes and plasma cells (Plate II). The thyroid glands had hyperplasia of follicular and cells with perivascular mononuclear cell infiltration (Plate III).

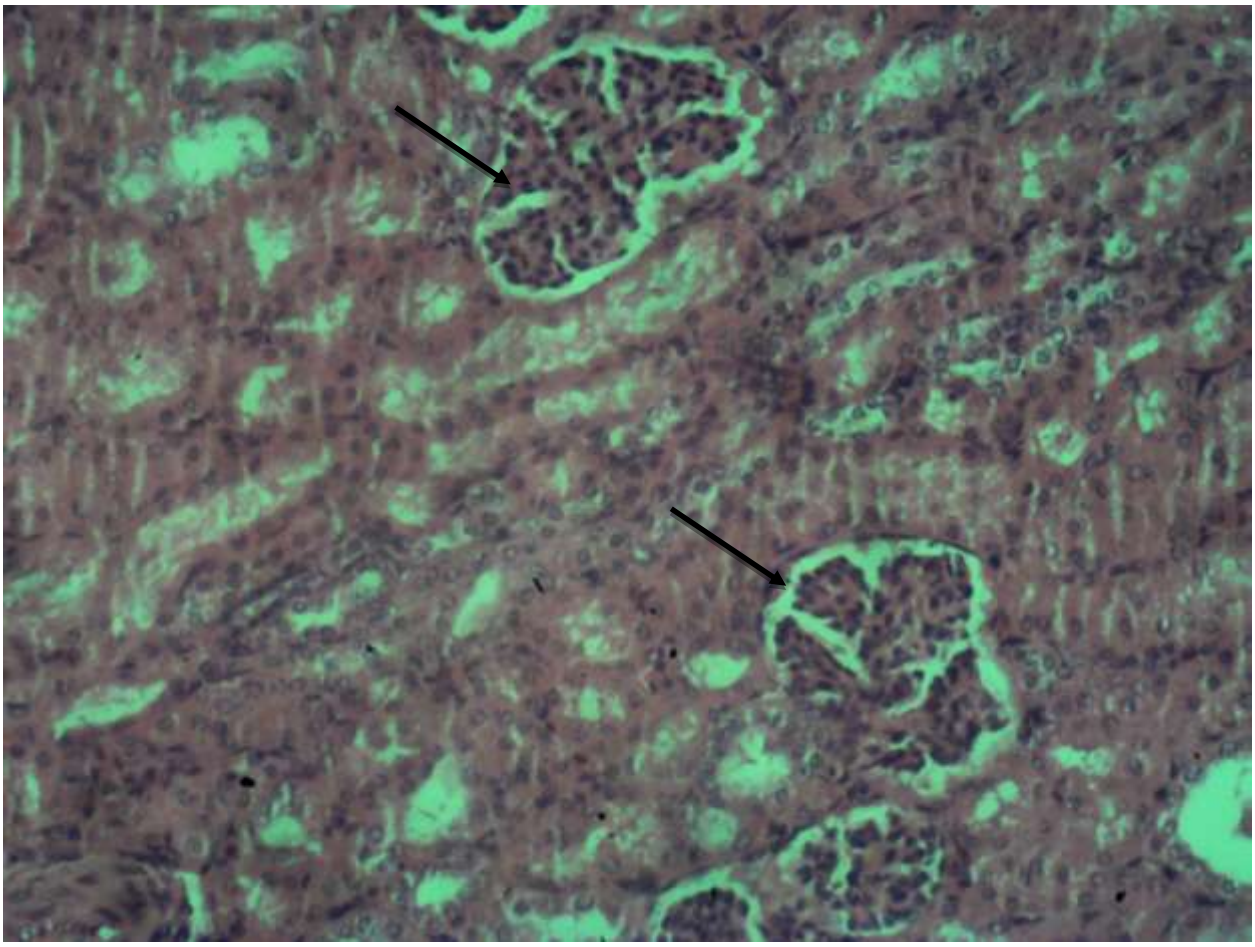

Plate I: Photomicrograph of a section of the kidney of a *T. b. brucei* infected pig showing hypercellularity of the glomeruli (arrowed). H & E X400.

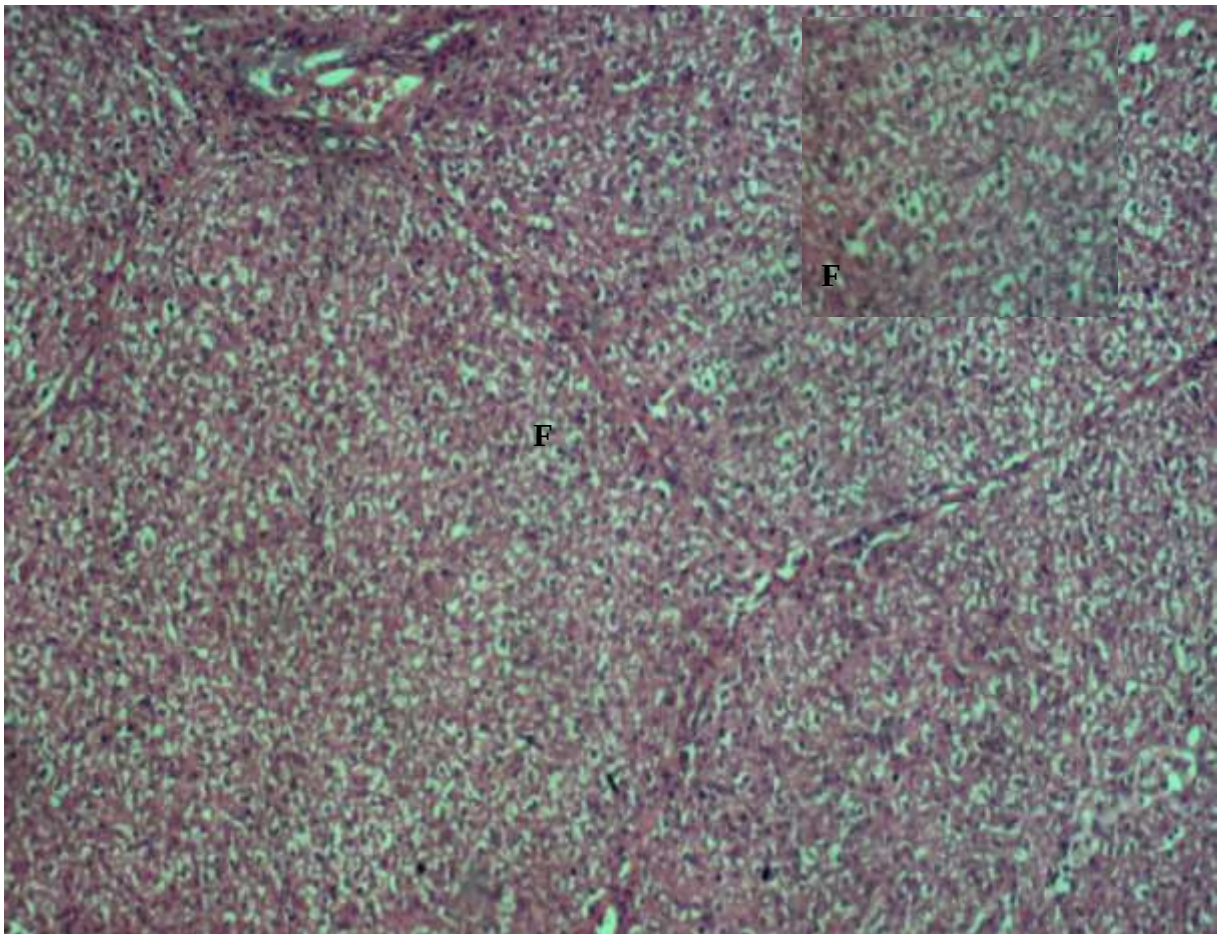

Plate II: Photomicrograph of a section of the liver of *T. b. brucei* infected pig showing fatty infiltration of hepatocytes (insert F). H & E X40.

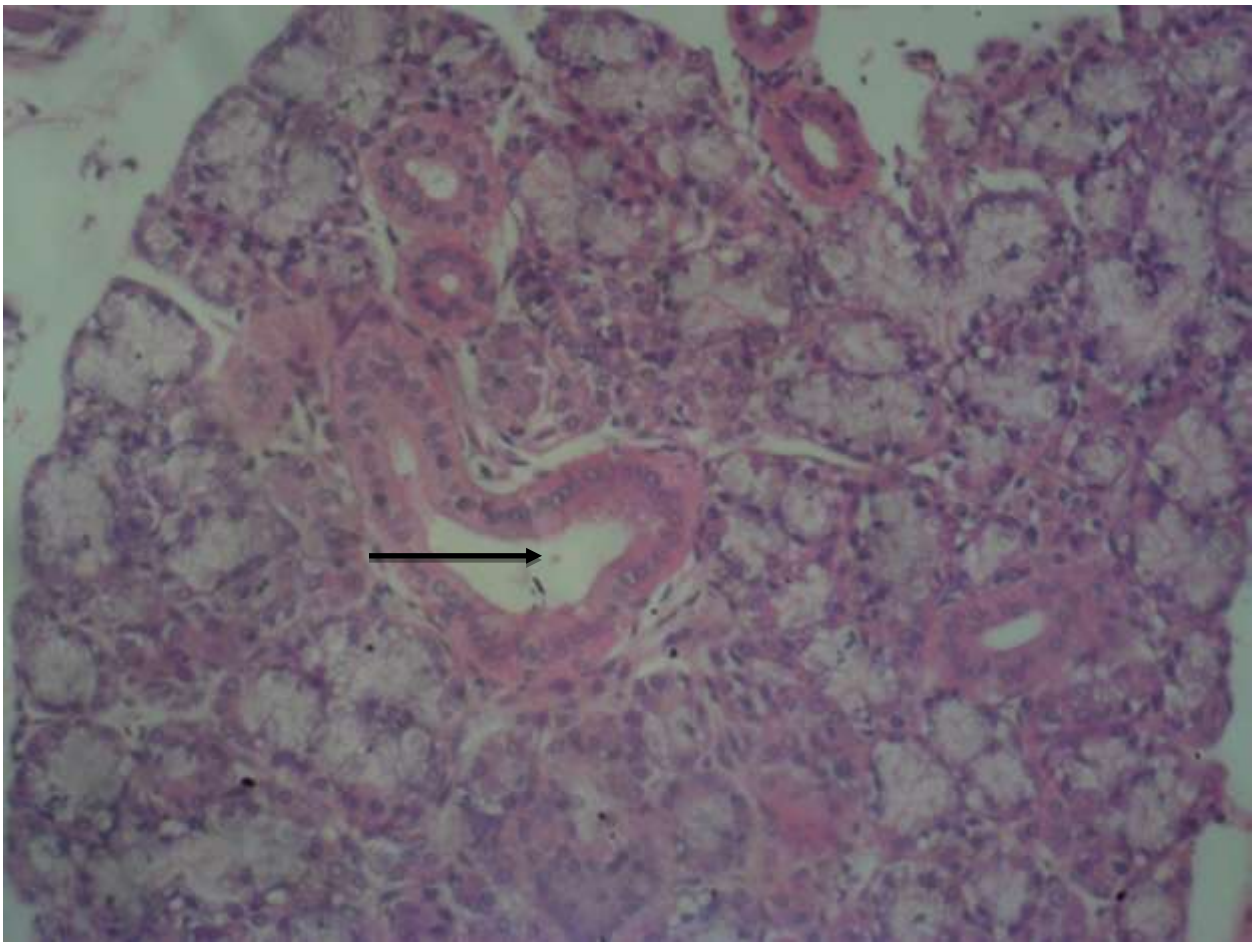

Plate III: Photomicrograph of a section of the thyroid gland of *T. b. brucei* infected pig showing colloidal secretionoffollicular cells (arrowed). H & E X400.

4.9 Molecular Expression of ST3Gal1 and ST6Gal1

4.9.1 Purity of RNA and cDNA

The purity of RNA samples and cDNA templates were analyzed by NanoDrop. For RNA samples, 260/280 values were greater than 1.80 and their 260/230 values ranged from 0.40 to 0.92. The complementary DNA templates generated by reverse transcription from RNA samples had 260/280 values greater than 1.85 and 260/230 values greater 1.0. The ratio of absorbance at 260nm and 280nm was used for the assessment of the purity of the nucleic acids. A ratio of 260/280 >1.8 wasconsidered an indication of adequate purity of the RNA product. A reduced ratio may be caused by contamination with proteins or phenol that absorb strongly near 280nm. The ratio of 260/230 was another measure of nucleic acid purity used. Expected values of 260/230 range from 2.0 to 2.2, which is normally higher than the respective 260/280 values. A lower 260/230 ratio may indicate the presence of Ethylenediaminetetraacetic acid (EDTA), carbohydrates, or phenol, which absorb near 230nm. However, lower 260/230 does not interfere with the PCR reaction. In this present study, only the RNA and cDNA samples of the liver and kidney passed the RNA integrity test (had desirable 260/280 ratios but lower 260/230). The low 260/230 might be due to the contamination from this phenol reagent used to extract RNA from the cells or other contaminants (such as carbohydrates) with absorbance at 230nm. Furthermore, 280/260 values of RNA above 2.0 demonstrated no genomic DNA contamination. Thus, the RNA sample was thought to be of sufficient quality for reverse transcription. As all cDNA 260/280 above 1.85 theoretically excluded protein contamination, they were used as purified templates for qRT-PCR. Protein extraction from thyroid gland and small intestine

does not yield sufficient protein and had 260/280 and 260/230 ratio that were within DNA range and hence not suitable to downstream analysis.

4.9.2 Efficiency of quantitative reverse-transcription PCR (qRT-PCR)

The efficiencyof qRT-PCR was calculated from standard curves of serial dilution PCR of cDNA (Figures 4.22 – 4.24). All the investigated transcripts showed good efficiencies: ACTB, E=95.8%; ST3Gal1, E=96.5% and ST6Gal1, E=96.9% (Table 4.1). As shown in Figure 4.25, both the reference (ACTB) and reference (ST3Gal1 and ST6Gal1) fulfilled the condition required for the use of the $2^{-\Delta\Delta Ct}$method that was utilized for the relative gene quantification of ST3Gal and ST6Gal1 in this study.

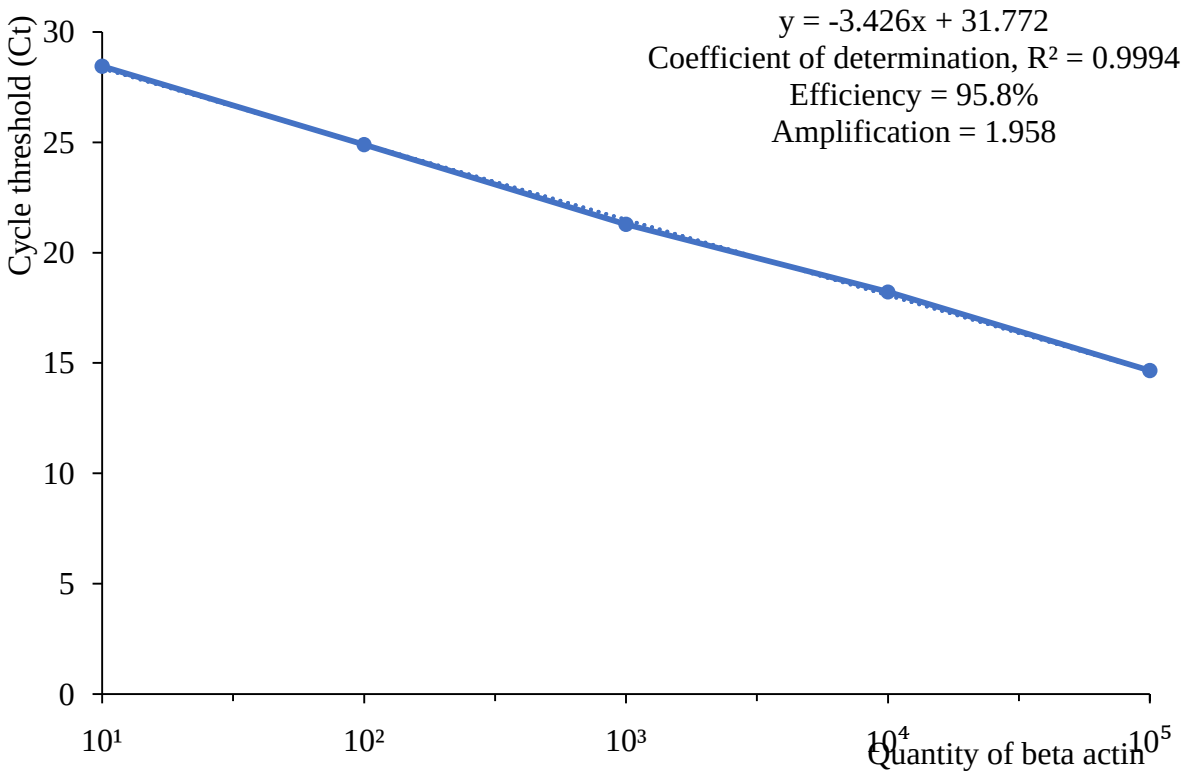

Figure 4.22: Standard curve of Ct plotted against the quantity ACTB (reference gene) for the five points covering a 10-fold dilution series. The equation of the regression line, the coefficient of determination (R^2), the amplification and the efficiency (%) are shown above the graph.

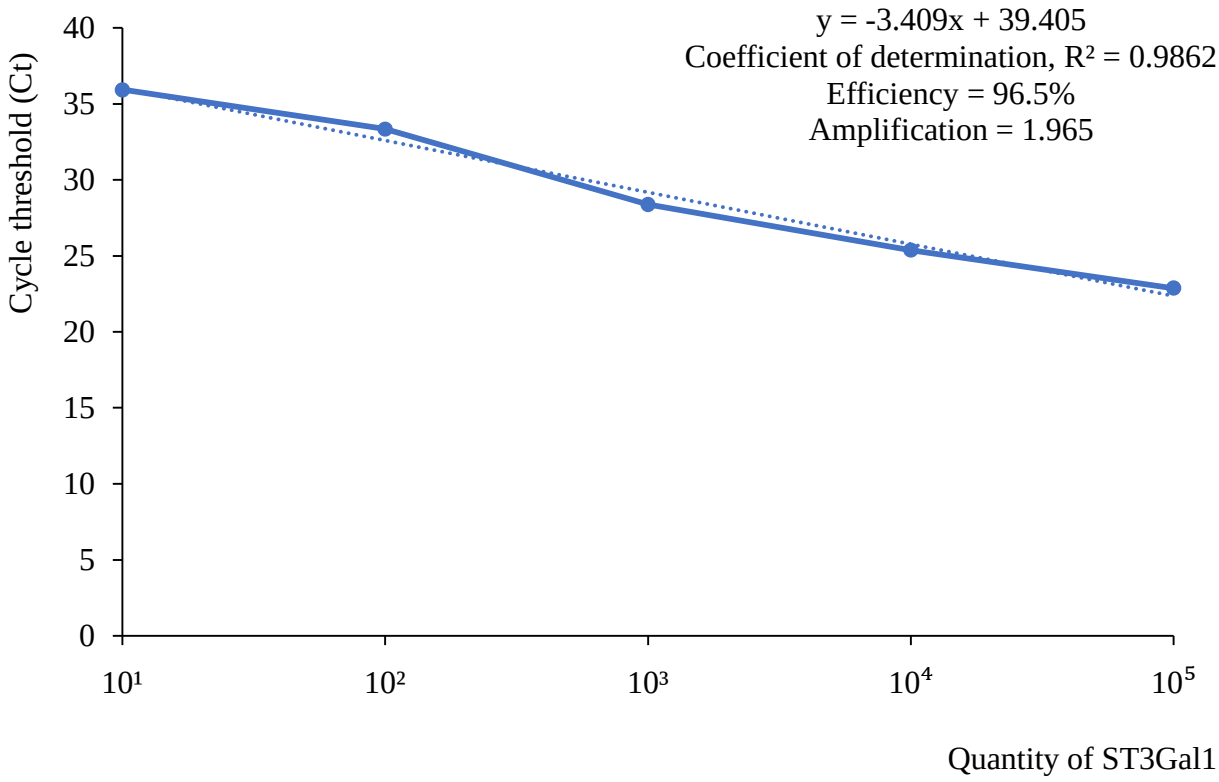

Figure 4.23: Standard curve of Ct plotted against the quantity ST3Gal1 (target gene) for the five points covering a 10-fold dilution series. The equation of the regression line, the coefficient of determination (R^2), the amplification and the efficiency (%) are shown above the graph.

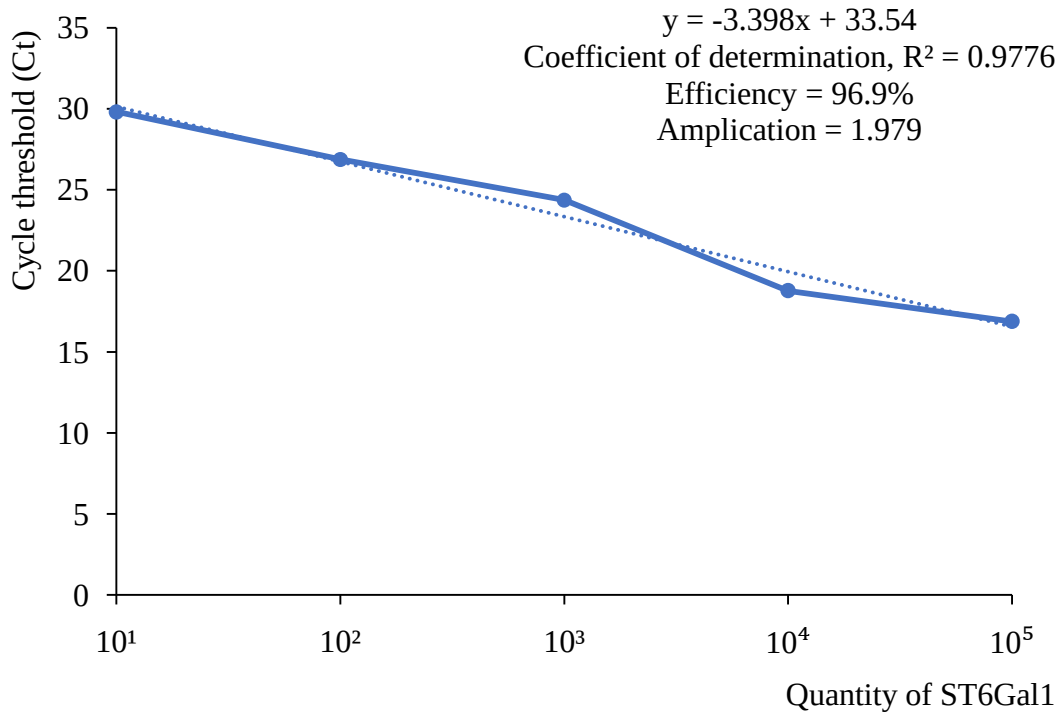

Figure 4.24: Standard curve of Ct plotted against the quantity ST6Gal1 (target gene) for the five points covering a 10-fold dilution series. The equation of the regression line, the coefficient of determination (R^2), and the amplification efficiency are shown above the graph.

Table 4.1: Slope, coefficient of determination (R^2), and the efficiency (%) of ACTB, ST3Gal1, and ST6Gal1.

Gene	Slope	R^2	Efficiency (%)
ACTB	-3.426	0.999	95.8
ST3Gal1	-3.409	0.986	96.5
ST6Gal1	-3.389	0.978	96.9

Key: R^2 = coefficient of determination. From the Table 4.1, it is evidence that the efficiency (%) of the house-keeping gene and those of the gene of interest were close to 100% and their differences were within 5% of each other and therefore fulfilled the condition for the use of $2^{-\Delta\Delta Ct}$ method in this study.

4.9.3 Relative gene expression of hepatic and renal ST3Gal1 and ST6Gal1

The expression levels of mRNA of ST3Gal1 and ST6Gal1 were quantified in the liver and kidney of *Trypanosoma brucei brucei*-infected and non-infected (control) pigs and are shown in Figures 4.25 and 4.26. At the time of evaluation, the relative gene expressions between the infected and control pigs were significantly different. The mRNA expressions of *T. b. brucei*-infected and non-infected (control-derived) kidney and liver genes showed that hepatic ST6Gal1, Renal ST6Gal1, Hepatic ST3Gal1 and Renal ST3Gal1 gene expression were significantly ($P < 0.0001$) upregulated (41.87-fold; 19.17-fold; 15.16-fold; and 5.00-fold respectively) compared to the control.

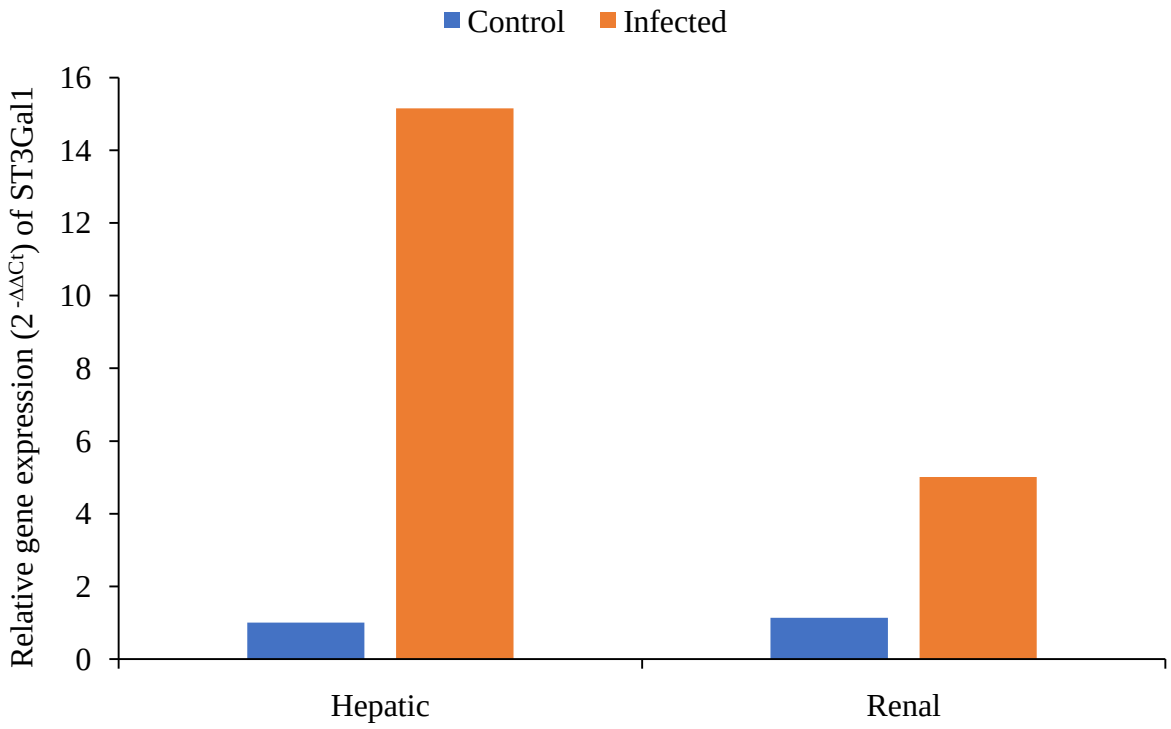

Figure 4.25: Relative gene expression of Hepatic and Renal ST3Gal1 of *T. b. brucei* infected and control pigs.

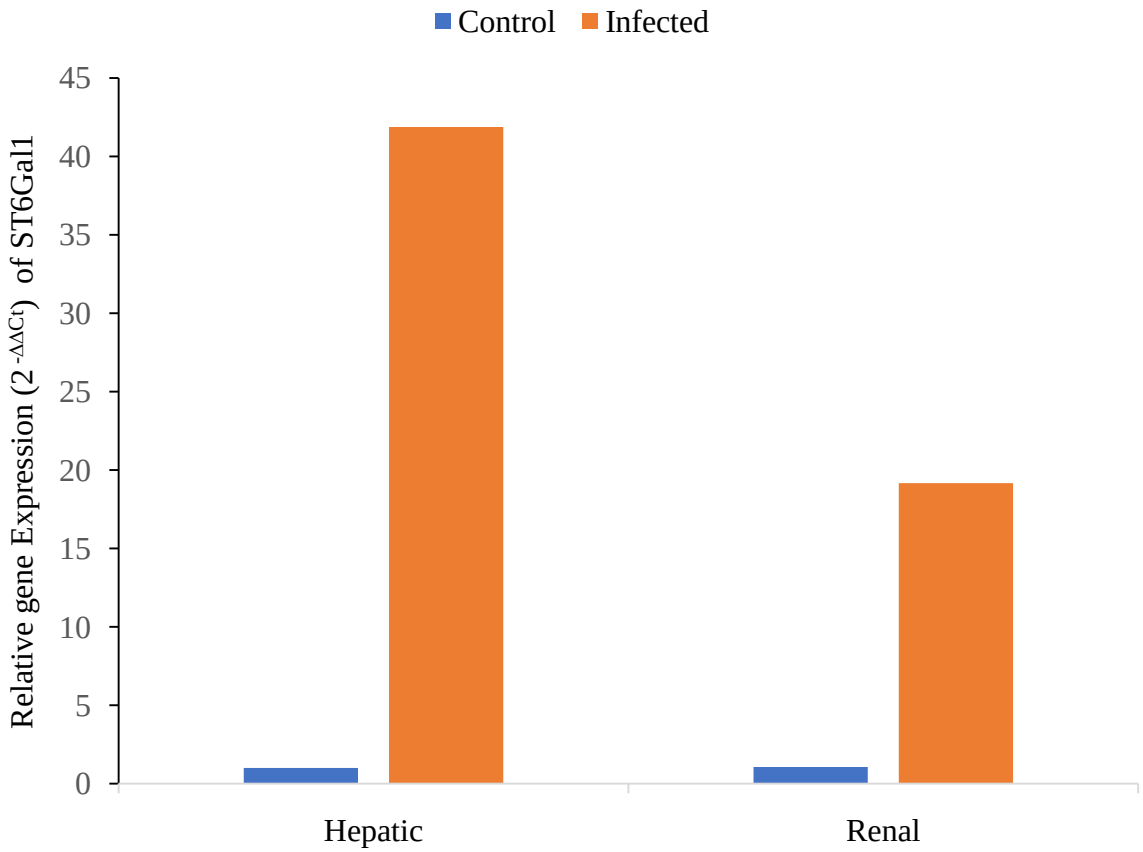

Figure 4.26: Relative gene expression of Hepatic and Renal ST6Gal1 of *T. b. brucei* infected and control pigs.

CHAPTER FIVE

5.0 DISCUSSION

In this present study, the Federe strain of *Trypanosoma brucei brucei* multiplied and was maintained in the experimental pigs. This was evidenced by the mean prepatent period of 4.29±0.35 days, indicating the infectivityand virulence potential of the trypanosomes in pigs. The mean prepatent period observed was similar to those reported by Ezeokonkwo *etal*., 2004; Abenga, 2011; Allam *etal*., 2011) but shorter than that reported by Akpa *etal*. (2008). The difference in the observed prepatent period could be attributed to differences in strains and infectivity of the *Trypanosoma brucei* species.

The clinical signs observed in *T. b. brucei* infected pigs were fever, anemia, occasional shivering, urticarial swellings, lethargy, loss of body weight and edema of the foot pads. All the clinical signs observed were consistent with those already reported of experimental trypanosome infection in pigs (Abenga, 2011; Allam *etal*., 2011), although other clinical signs such as keratitis, hemorrhages in the anterior eye chamber, motor disturbances, edema and petechial hemorrhages of serous membranes were reported by other workers (Reid *etal*., 2001; Mijares *etal*., 2010; Padmaja, 2012; Hussain *et al*., 2016). The appetite of the experimental pigs remained reasonably unaffected during the experimental period and was unrelated to weight loss observed. The loss of body weight and the subsequent emaciation reported in this study could have resulted from the febrile response observed in this study and the complex immunological and physiological changes reported to be caused by the parasite resulting in increased catabolic activity (Seed and Hall, 1985).

The undulating waves of parasitemia in the present study, that occurred throughout the experimental period are consistent with previous findings (Allam *etal*., 2011; Abenga *etal*., 2017) and were related to the phenomenon of antigenic variation. It was reported that *T. brucei* exhibited antigenic variation during infection in several vertebrate host species (Morrison *etal*., 2005; Stockdale *etal*., 2008; Mugnier *etal*., 2015). In the present study, the parasitaemic wave was in synchrony with the undulating increases in rectal temperature observed and this correlates with findings in previous investigations on African trypanosomiasis (Aquino *et* al., 1999; Taylor and Authie, 2004).

In this study, consistent reduction in packed cell volume (PCV) observed from day four (4) post-infection up-to the termination of the experiment followed similar patterns of variation in hemoglobin concentration and erythrocyte counts which give credence to the earlier reports of anemia in*T. b. brucei*-infected pigs (Abenga, 2011; Allam *etal*., 2011). The anemia of *T. brucei*-infected pigs occurred in the early phase of the infection and it was short-lived due to self-recovery during the chronic phase of the infection, this recovery phenomenon was not observed in this study probably because of the difference in the durations of the experiments with the one in the present study being shorter. The low PCV values occurring concomitantly with the peak parasitemia in this study may be attributed to the hemolysis arising from the activities of the parasite (Anosa, 1988; Adamu *etal*., 2008).Anemia is considered as a major and consistent indicator of trypanosomiasis infection in African animal and African human trypanosomiases. The anemia results from the parasitic infection partly because a large number of erythrocytes from the circulationare removed due to mononuclear phagocytic response in spleen and haemal lymph nodes (Eyob

and Matios, 2013). Hyperthermia is a major contributory factor to red cell damage and the development of anemia in African trypanosomiasis (Mbaya *etal*., 2012).

Several factors such as immunological mechanisms, hemolytic factors, adherence of trypanosomes to red blood cells, RBC fragmentation, high body temperature, and hyperactivity of the mononuclear phagocyte system (Tizard, 1985; Anosa, 1988; Nok and Balogun, 2003) had been associated with events leading to anemia in trypanosomiasis. Consequently, the anemia caused vascular dysfunction resulting in anoxic condition and increased oxidation of erythrocytes (Gutierrez *etal*., 2005; Eyob and Matios, 2013). Severe anaemia resulted in death in trypanosomiasis but low mortality was observed with animals that were capable of managing the reduction in PCV and erythrocyte counts. Although the degree of reduction in the PCV and erythrocyte counts observed in this study, cannot be regarded as severe, the observation that the PCV gradually increased after the minimal value was attained on day 13, which was sustained until the termination of the experiment on day 21 could be a reliable indication of the ability of the infected pigs to control the infection and survive. It is also reasonable to attribute this increase and subsequent stabilization of PCV in the *T. bruceibrucei*-infected pigsto the activity of sialyltransferase, among other factors, which coincidently increased in infected pigs. The role of this enzyme is resialylation of the erythrocytes and thereby minimizing the rate of their binding to macrophages thereby prolonging their lifespans (Adamu*et al*., 2009).

Red cell indicesused to classify the anemia (Esievo, 2017) in this study showed that mean corpuscular volume (MCV) of the infected group increased dramatically from day 6 post-infection and remained so throughout the experimental period and was accompanied by a decrease in mean corpuscular hemoglobin concentration (MCHC) although the latter was

followed by a sharp increase and return to normal level on day 12. These suggest a regenerative anaemia typically associated with haemolytic anaemias (Esievo, 2017). These variations of the erythrocytic indices observed in the present study indicated a macrocytic hypochromic anemia which may be associated with increase in reticulocytes in peripheral circulation - an index of regenerative anemia. This observation agrees with previous finding with *T. evansi* infection in camels (Abd El-Baky and Salem, 2011; Eyob and Matios, 2013). Abenga *etal*. (2017) also reported macrocytic hypochromic anemia in the initial phase of *T. b. brucei* infection in pigs with a gradual return to normal values during the later stage of the infection. Our findings were in contrast with those of Yusuf *etal*. (2013) who reported an initial microcytic hypochromic followed by macrocytic hypochromic anemia in the course of *T. b. brucei* infection in rats. Differences in the type of experimental animalspecies, strain of infecting trypanosomes and duration of infection would likely explain the differences in these observations.

The thrombocytopenia reported in the *T. b. brucei*-infected pigs agrees and supports the observationsby other investigators (Esien and Ikede, 1978; Kagira *etal*., 2006; Da Silva *etal*., 2009; Ngotho *etal*., 2011; Abenga *etal*., 2017). This low platelet count observed in this study could be a result of hyper-destruction of platelets by toxic products reported to be produced by the infecting trypanosomes (Da Silva *etal*., 2010; Da Silva *etal*., 2011). Other factors responsible for the thrombocytopenia include: pooling of blood in the spleen, removal of platelets by the mononuclear phagocytic system and increased ‘consumption’ of platelets in disseminated intravascular coagulation reaction which was widely reported in trypanosomiasis (Tanowitz *et al*., 1996; De La Rue *etal*., 1997; Da Silva *etal*., 2010; Oliveira *etal*., 2011).In addition, the thrombocytopenia was associated with the possible

involvement of *Trypanosoma* neuraminidase or transialidase from the parasite that altered the platelet surface sialic acid content (Thompson and Jakubowski, 1988; Tribulatti *etal*., 2005). Also, other molecules derived from the immune response against the infection, like cytokines or other inflammatory products, might also collaborate this process (Tribulatti *etal*., 2005; Savino *etal*., 2007).

The early (marked) leukocytosis observed throughout the experiment in *T. b. brucei*-infected pigs was due to marked neutrophilia and lymphocytosis. This recent works partly agreed with the work of Abenga *et al*. (2017) and Erin *et al*. (2020) who reported leukocytosis due to marked lymphocytosis, neutrophilia, and mild monocytosis in experimental infection of pigs and rats, respectively. In addition, Yusuf *et al*. (2013) also reported leukocytosis characterized by lymphocytosis, mild neutrophilia, eosinopenia and monocytosis in *T. b. brucei* infected rats.Earlier, Njiru *et al*. (2000) reported marked leukocytosis in *T. evansi*-infected dromedary camels. The observation of leukocytosis is however in contrast with previous reports on African trypanosomiasis in which pancytopenia characterized by leukopenia, neutropenia, lymphopenia, and eosinopenia were observed (Omotainse and Anosa, 1995; Taylor and Authie, 2004; Allam *etal*., 2011). An experiment on trypanosomiasis in goats stated that leukocytosis was not a reliable indicator of infection (Dargantes *etal*., 2005). In infected camels, Choudhary and Iqbal (2000) noted a significant decrease in lymphocyte with a visible increase in leukocytes and neutrophils. In other experimental infections of Norwegian lemmings with *T. lemmi,* the leukocyte counts remained unchanged **(**Wiqer, 1978). Hosts, trypanosome species, and duration of the experiment may account for these variations in leukocyte responses (Taylor and Authie, 2004). Although leukopenia associated with general depression of

granulopoiesis, massive peripheral utilization, splenic sequestration and phagocytosis in other organs such as the bone marrow and the liver occurred more frequently in African trypanosomiasis (Taylor and Authie, 2004), the etiology of leukocytosis in *T. brucei* infection had been tied to sustained lymphocytosis probably resulting from trypanosome antigenic challenge leading to increased proliferation of immune-competent cells into antibody and or lymphokine producing cells (Emeribe and Anosa, 1991). The lymphocytosis reported in this present study could be regarded as a good prognostic sign (increased antibody production), although its effectiveness in limiting disease pathogenicity is still debatable. Meanwhile, the neutrophilia observed in this study could be associated with stress, inflammation and secondary bacterial infection. Another likely mechanism includes early activation and proliferation of neutrophils by indirect mechanisms such as dispersal of trypanosome antigens to the bone marrow and generation of tissue break down products due to extravascular activities by trypanosomes (Abenga *etal*., 2017).

In this study, the gradual increase in serum sialidase activitycoinciding in some cases with the decrease in erythrocyte surface sialic acid and the increase in free serum sialic acid concentrations, suggests that *T. b. brucei* produced sialidase which cleaved red cells sialic acid into plasma, further supporting the reports that trypanosomiasis decreased the sialic content of erythrocyte membranes and increased sialic acid content in plasma (Walia *etal*., 1996; Montagna *etal*., 2002). The appearance of the trypanosomes, with the highest sialidase activities being recorded during the peak of parasitemia gives credence to the production of sialidase by *T. b. bruceiin vivo*. Therefore, the decline in erythrocyte surface sialic acids with the increase in free serum sialic acid may be readily explained. It has been shown that *T. vivax* produced neuraminidase enzyme both in vitro and in vivo (Esievo *etal*.,

1982) in *T. vivax*-infected cattle that cleaved off erythrocyte surface sialic acid in cattle (Esievo *et al*., 1982). Therefore, the finding in this study that reduced erythrocyte surface sialic acid occurred concurrently with an increasing number of trypanosomes in circulation, accompanied by the development of anemia has been carefully tied to the production of sialidase by *T. b. brucei*, which then, in turn, cleaves off erythrocyte surface sialic acid and thus increasing the concentration of free serum sialic acid as observed in this study. The pathophysiological mechanisms involved in the *Trypanosoma* sialidase-induced destruction of red blood cells is linked to the removal of sialic acid from the exposed epitopes of the erythrocytes and hence predisposing the cells to physicochemical alterations which make them bind to β-D-galactose-specific lectin on surfaces of macrophages and, consequently, resulting in the uptake and clearance of the desialylated erythrocytes (Durocher *et al*., 1975; Nok *etal*., 2003; Schauer, 2009).

Drop in the erythrocyte sialic acid concentration following parasitemia in *T. b. brucei*-infected pigs re-affirm the findings of previous reports (Esievo *etal*., 1983; Olaniyi *etal*., 2001; Nok and Balogun, 2003; Shehu *etal*., 2007; Adamu, 2009; Abenga, 2011). Ode *etal*., 2017 also reported a higher percentage reduction in erythrocyte surface sialic acid in the trypanosome-positive Bunaji compared to the trypanosome-positive Muturu cattle and therefore suggests that the Bunaji cattle would be more vulnerable to erythrophagocytosis and subsequent development of anemia. The significant reduction in ESSA concentration in the *T. b. brucei* infected pig, as observed in the present study, was most probably the result of elaboration and subsequent release of sialidase; a sialic acid-hydrolyzing enzyme produced bythe infecting trypanosomes as similarly reported earlier (Esievo, 1982; Nok and Balogun, 2003; Umar *et al*., 2008). This claim is supported by the report that tissue

invasive *T. brucei*produces sialidase which plays a major role in the development of anemia in pigs (Montagna *et al*., 2002).Esievo *etal*. (1982) observed that the period of decrease in red cell surface sialic acid concentration coincided with the first drop in packed cell volume and increase in free serum sialic acid concentration and subsequent development of clinical anemia in the experimental animals. It was observed that *T. vivax* possesses sialidase which hydrolyzes erythrocyte surface sialic acid of infected animals and that the activity of this trypanosome enzyme is proportional to the concentration of trypanosomes in-vitro (Esievo, 1979; Esievo, 1983) and this phenomenon was therefore concluded to be responsible for aging of erythrocyte with resultant reduction in life span and their subsequent removal of senescent red blood cells by erythrophagocytosis (Esievo *etal*., 1982; Nok and Balogun, 2003). Experimentally-induced anemia in both human and animal trypanosomiasis revealed that the entire erythroid cells suffer the same fate of phagocytosis by macrophages in the reticuloendothelial system because sialic acids also form a protective shield for other immature erythrocytic cells (Anosa *etal*., 1992; Anosa *etal*., 1997; Rossi *etal*., 2017; Igbokwe, 2018).

The observation of an increase in mean free serum sialic acid concentrations of *T. b. brucei* infected pigs following the onset of parasitemia on day 7 post-infection agrees with the findings of previous studies (Esievo *etal*., 1982; Olaniyi *etal*., 2001; Nok and Balogun, 2003; Shehu *etal*., 2007; Adamu, 2009; Abenga, 2011). The early increase in free serum sialic acid concentration observed in this study could be attributable to the activities of the circulating trypanosomes which produce neuraminidase that removed sialic acid from the surface of erythrocytes and immediately rendered them more prone to phagocytosis, as earlier reported (Esievo *etal*., 1982). The rise in the free serum sialic acid concentration,

which accompanied a reduction in erythrocyte surface sialic acid concentration, suggests that erythrocytes were the major source of the free serum sialic acid in *T. b. brucei* infected pigs.

In this present study, the ESSA concentration in the *T. brucei brucei*-infected pig improved greatly at later stages in the course of the infection but it failed to return to levels comparable to those of the control pigs. This observation may be attributable to the combined effect of *Trypanosoma* sialidase and the autoinduction of the activity of sialate-pyruvate-lyase (aldolase, EC 4.1.3.3); an enzyme that is localized in the cytosol of mammalian cells and regulates the recycling of the sialic acid by hydrolyzing it to pyruvate and the corresponding acyl-mannosamine (Traving and Schauer, 1998).

The return to comparable normal levels of FSSA concentration, following an initial rise, in *T. brucei brucei*-infected pigs was as a result of the activity of sialyltransferase enzyme, which depletes in a process the FSSA to resialylate cells, glycoconjugates and glycolipids (Bosshart and Berger, 1992; Hennet *etal*., 1998; Kaufmann *etal*., 1999; Ellies *etal*., 2002; Borman, 2004; Wang, 2005).

The reported observation of increased serum sialyltransferase activities in *T. b. brucei*-infected pigs occurring concomitantly with the onset of parasitaemia, increases in rectal temperature, reduction in ESSA and decline in FSSAwere in agreement with previous findings (Adamu, 2009; Abenga, 2011; Baraya, 2015). The pattern of increase in the activities of sialyltransferase in this study shows that peak activities follow a period of increased parasitemia and that after the first peak, the enzyme activities became more easily increased in the plasma of infected pigs.

Sialyltransferases areenzymes that catalyze the transfer of sialic acids to desialylated terminal positions on cell surfaces, glycoproteins and glycolipids (Borman, 2004; Wang, 2005). Sialyltransferase was found responsible for resialylation of erythrocyte surface by picking and replacingFSSA onto the surface of desialylated erythrocyte in *T. brucei* infected hosts (Abenga, 2011). Indeed, Esievo *et al*. (1982) hypothesized that sialyltransferase present in the thyroid gland regenerated sialic acid on the surface of erythrocytes after their removal by the trypanosome sialidases thus preventing the rise in free serum sialic acid concentration and thereby resulting to self-cure from anemia. In the present study, the sustained surge in the sialyltransferase activities which signaled the period of progressive increase in ESSA concentration, a relative drop in FSSA concentration and the improvement in the value of the packed cell volume, despite persistent parasitemia suggested that the increased activities of the sialyltransferase in this phase of *T. b. brucei* infection in pigs was responsible for metabolizingFSSA(cleaved off erythrocytes by trypanosomes sialidase) onto the surface of the desialylated erythrocyte to complete their process being salvaged from eminent destruction. Thus, the increased sialyltransferase activities played an active role the recovery of anemia and from this observation the early recovery of anemia in *T. b. brucei* infected pigs depends predominately on increased activities of sialyltransferase. It has been speculated that increased sialyltransferase activities are more prominent in trypanotolerant animals since the ability to resist and recover from anemia in trypanosomiasis is the most important attribute of Trypanotolerance (Naessens *etal*., 2002). Abenga (2011) suggested that recovery of experimental pigs from trypanosomiasis induced anemia was partially due to increased sialyltransferase activities andincreased erythropoietin level; it was further suggested that the erythropoietic response from both erythropoietin and sialyltransferase

activitywere similar. Serum erythropoietin was found rich in sialic acid which determined their bioactivity serum lifespan (Narhi *etal*., 1991; Egrie and Browne, 2001) and hence subjected to desialylation by sialidase (Jeong *etal*., 2009); it was therefore postulated that the serum erythropoietin activities in trypanosomiasis were potentiated by the serum sialyltransferase activities.

The mechanism by which sialyltransferase activities were regulated in this study could be likely due to stress as evidence by the observed neutrophilia and eosinopenia; this should have been further confirmed by lymphopenia but in this case, lymphocytosis was observed which could have been due to antibody response. A study reported that stress led to over 40% elevated sialyltransferase activities in the spleen, liver, and cerebrum of experimental rats and therefore hypothesized that increased stress hormone level playeda role in the upregulation of sialyltransferase activities. The parasitemia and hyperthermia observed in this study may serve as stressors that could potentiate the activities of sialyltransferase in *T. b. brucei* infected pigs. ST6Gal-I; an isoform of sialyltransferase was upregulated and highly expressed during inflammation and the degree of its expression was used as a serological clinical marker for inflammation (Yasukawa *etal*., 2005). The increased sialyltransferase activities observed in this study could have been potentiated by the inflammation induced by the activities of the tissue invasive *T. b. brucei* in the experimental pigs.

The observed increased activities of sialyltransferase level in the thyroid gland of the *T. b. brucei*-infected pigs at the termination of the experiment which strongly supportedthe fact that the thyroid gland was a source of serum sialyltransferase in infected animals as previously reported (Abenga, 2011). This is supported by the active follicles demonstrated

by the histopathological sections of the thyroid gland of the *T. b. brucei* infected pigs which revealed lesions characterized by marked colloidal secretions and hyperplasia of the follicular epithelium as well as the parafollicular cells which are indicative of increased physiological activities of the thyroid gland.

The strong relationship between sialyltransferase activities and drop in the ESSA concentration, increase in FSSA concentration and increase in packed cell volume in the *T. b. brucei* infected pigs in the phase of persistent parasitemia is an indication that the secretion of sialyltransferase is triggered by the biochemical interplay of the aforementioned factors. This finding was in agreement with the previous finding on African trypanosomiasis (Adamu, 2009; Abenga, 2011, Baraya, 2015).

The absence of observable microscopic lesions in the brain of the infected pigs give credence to the fact that pathological changes observed in *T. b. brucei* infected pigs were those of early stage of the infection. The histopathological lesions in the liver such as distention of the hepatic sinusoids, fatty degeneration and advanced necrosis of hepatocytes and those of the kidney,such as, proliferative glomerulonephritis might have compromised effective erythropoiesis and effective recovery of anemia.

In the present study, it was demonstrated for the first time that the upregulation of Renal ST3Gal 1, Renal ST6Gal 1, Hepatic ST3Gal 1, and Hepatic ST6Gal 1 is important in alleviating *Trypanosoma brucei brucei* associated anemia in pigs. The upregulated ST3Gal 1 and ST6Gal 1 reported in this study may increase Siaα2,3Galβ1,3GalNAc-R, and Siaα2,6Galβ1,4GlcNAc-R, respectively, on glycolipid and glycoprotein within the liver and kidney of the infected as evidenced by the increasing number of sialic acids available for

re-sialylation as reported in this study. It could, therefore, be postulated that the increase in sialylation (re-sialylation) observed in the anemic pigs which correspond with the period of their recovery from anemia may be in part due to the production of "new" sialic acids due to the upregulation of both renal and hepatic ST3Gal1 and ST6Gal1 which would, in turn, increased the number of free serum sialic acids that are to be replaced on the surface of diseased desialylated erythrocytes or partly due to the inhibition of nitric oxide production (NO) by the activated macrophages which is important in the induction of anaemia in trypanosomiasis and hence recovery from anemia reported in this study. In this study, the increased serum activity sialyltransferase corresponds to the increased relative mRNA expression of both hepatic and renal ST6Gal 1 and ST3Gal1. Even though their serum activities were not measured individually,a study on ST6Gal expression observed that there was tremendous inconsistency in the correlation between the ST6Gal mRNA level and the measurable enzymatic activity in lactating mouse (Dalziel *et al*., 2001). The availability of sialyltransferase substrate (sialic acid) was involved in the regulation of mRNA expression of ST3Gal1 and ST6Gal1 in embryonic mouse cell (Bork *et al*., 2017). Recent evidence suggested that the molecular expression of sialyltransferase could be regulated by the secretion of NO and its derivatives by activated macrophages (Vilcaes *et al*., 2020).

Our data suggest that both ST6Gal1 and ST3Gal1 sialyltransferases originated mostly from the liver and to a lesser extent kidney, are constantly released into circulation, where we suspect they target marrow hematopoietic stem and progenitor cells (HSPCs). This suspicion is due to the research on human pluripotent stem cell, ST6GAL1 that reported its critical involvement in the regulation of cellular pluripotency, essential for efficient induction of pluripotency in somatic cells, which further provided evidence for the

functional significance of a sialyltransferase and macromolecule sialylation in regulating the pluripotent state in human cells (Wang *et al*., 2015). In another study, the downregulation of ST6GAL1 in undifferentiated HPSCs was shown to significantly alter the expression of many genes that orchestrated cellular differentiation and organ development (Nasirikenari *et al*., 2014). Hepatic ST3Gal 1 and ST6Gal 1 were upregulated in GalT knockout pig liver cells as compared to controls resulting in an increase in both α2,3ST and α2,6ST in heterozygote GalT knockout livers compared to control livers(Park *et al*., 2011). In knockout mice, ST6Gal I showed severe immunosuppression phenotypes, such as reduced serum IgM levels, impaired B cell proliferation in response to IgM and CD40 cross-linking and attenuated antibody production to T-independent and T-dependent antigens. This indicated that ST6Gal I and its products played important roles in the immune system (Hennet *et al*., 1998). Analysis of ST3Gal I knockout mice revealed that ST3Gal I was involved in the sialylation of core1 O-glycans in T lymphocytes, which played an important role in the survival of CD8 T cells in apoptosis (Priatel, 2000). ST6Gal I played a role in inflammation andin mammals, transient up-regulation occurred during acute phase reaction when the organism experienced trauma or infection (Jamieson *et al*., 1993; Dalziel *et al*., 1999; Thorne-Tjomsland *et al*., 2000; Teppa *et al*., 2016). Hepatic ST6GalI mRNA expression was downregulated in human alcoholics as compared to the control indicating decreased hepatic activity of the enzyme due to decreased synthetic rate (Gong *et al*., 2007). Therapeutic administration of recombinant, bioactive ST6Gal-1 (rST6G) mitigated acute inflammation in a murine model mimicking acute exacerbations experienced by patients with chronic obstructive pulmonary disease (COPD) (Nasirikenari *et al*., 2019). The overexpression of ST3GAL1 increased cell growth, migration, and

invasion *in vitro* whereas under-expression reduced cell growth, migration, and invasion (Wu *et al*., 2018).

In the present study,ST3Gal1 and ST6Gal1 were better expressed in the liver than the kidney suggesting that these enzymes are constantly been produced and released into circulation where might act on erythropoietinogen to initiate the development of bone marrow hematopoietic stem and progenitor cells.Both ST3Gal1 and ST6Gal1 investigated played a key role in the re-sialylation ("renewal") of erythrocytes as demonstrated by the findings of this study, they are also involved in tumor renewal (Cheng *et al*., 2016) in various tumor cells. Hence, the renewed interest the gene expression studies of STs in cancer therapy because of the role of the enzymes proliferation of tumor cells and thereby increasing cancer progression due to their upregulation in various types of tumors (Hsieh *et al*., 2017; Jones, 2018; Wu *et al*., 2018, Ortiz-Soto *et al*., 2019; Ou *et al*., 2020; Xu *et al*., 2020) and therefore, the inhibition of sialyltransferases is recently been exploited as an emerging potential strategy to prevent metastasis in several cancers (Cheng *et al*., 2020; Natoni *et al*., 2020). Conversely, to what is hitherto believed, the downregulation of ST3Gal1 and ST6Gal1 was reported in oral squamous cell carcinoma tissues (Mehta *et al*., 2020), and also in inflammatory induced cystic fibrosis (Barnes *et al*., 2019). ST3Gal1 mRNA was shown to be decreased (downregulated) in pre-malignant and malignant tissues compared to normal tissues (Roa-de La Cruz *et al*., 2018). Another study also reported that ST3Gal1 remained unchanged under stress in the liver of tumor-bearing mice (Caballero-Hernandez *et al*., 2016). The pivotal roles of sialic acid and sialyltransferase-mediated macromolecule sialylation in many physiological and pathological processes, including the development of embryos, cancer progression, the regulation of immune systems and host-

pathogen interactions, have been identified by numerous studies (Angata and Varki, 2002; Schwarzkopf *et al*., 2002; Chen and Varki, 2010; Wang *et al*., 2015; Qi *et al*., 2020). The activities of sialyltransferase indicated by their increased expression in various tissues led to the formation of the so-called sialome,specific for each tissue of all the biological systems of the vertebrate species (Cohen and Varki 2010; Varki, 2011). Because many biological processes, governed by carbohydrate-protein interactions involving sialic acids, the evolutionary approach to gain further insights into the biological relevance of sialyltransferases provided particular interest (Varki, 2007; Schauer, 2009). It is therefore visibly true that the upregulation of STs should generally not to be treated as a "bad omen" because the pathophysiological mechanism it uses in protecting cancer cell could also be exploited in the protection of patients against chemotherapy and radiotherapy-induced anemia which is major worry and source of complications in cancer therapy (Xu *et al*., 2020).

CHAPTER SIX

CONCLUSIONS AND RECCOMENDATIONS

6.1 Conclusions

It was concluded from this study that:

i. During the period of active infection, the activity of *T. b. brucei* neuraminidase was also increased confirming its role in cleaving sialic acid from the surface of red blood cells and hence the development of anaemia.
ii. *Trypanosoma b. brucei* induced parasitaemia in pigs caused a rapid and simultaneous decreases in erythrocyte surface sialic acid concentrations and packed cell volume.
iii. The parasitaemia observed was also related to rise in free serum sialic acid concentrations with the highest level observed during the peak parasitaemias.
iv. *T. b. brucei* infection was associated with increased activity of sialyltransferase which coincided with decreasing concentration of erythrocyte surface sialic acid, packed cell volume and increasing concentration of free serum sialic acid.
v. The lymphocytic leucocytosis reported in this study could enhance hosts' ability to tolerate and survive the early phase of *Trypanosoma brucei* infection in animals and man.
vi. The thrombocytopenia reported could be associated with the activity of neuraminidase which may alter the sialylation of the platelet surface directly by reducing their sialic acid contents and the life span of platelets.

vii. The expression of ST3Gal1 and ST6Gal1 isolated from the liver and kidney of *T. b. brucei*-infected pigs were upregulated.

viii. The increased serum activity of sialyltransferase corresponds to the increased relative mRNAexpression of both hepatic and renal ST3Gal1 and ST6Gal1.

ix. The observation of correlation in the upregulation of ST3Gal1 and ST6Gal1 with the increased activity of serum sialyltransferases are strong evidences that the enzyme is responsible for resialylation of erythrocytes which is therefore involved in the mechanism of natural recovery from anaemia in Trypanosome-infected animals.

x. Both ST3Gal1 and ST6Gal1 were better expressed in the liver than the kidney suggesting that these enzymes are constantly been produced and released into circulation.

6.2 Limitations

The main limitation of this study is:

i. Difficulty in sourcing for *Trypanosoma brucei brucei.*

ii. Lack of previous research on the tissue expression pattern of sialyltransferase in *Trypanosoma brucei brucei*-infected pigs.

6.3 Recommendations

It is recommended that,

i. The upregulation of hepatic and renal ST3Gal1 and ST6Gal1 observed in this study should be explored as potential targets for therapeutic intervention for African Animal trypanosomiasis.
ii. The result of this investigation should also be used as a guide to understand and design empirical solutions to the menace of African Human Trypanosomiasis since pig is also a monogastric and *T. b. brucei* is phylogenetically related to both *T. b. gambiense* and *T. b. rhodensiense* that affect man.
iii. A more detailed study should be conducted to understand the sequential expression of ST3Gall1 and ST6Gal1 in serum of Trypanosome-infected animal and to relate the fold change to the level of parasitaemia and observed anaemia.
iv. An in-depth study should be conducted in trypano-tolerant and trypano-resistant animals to compare the expression pattern of sialyltransferase and the role of host gene and immunopathology in the expression pattern of sialyltransferase in these groups of animals.
v. Further studies should be done on sialyltransferase using the tools of Western immunoblotting, immunohistochemistry and flow cytometry.

6.4Contribution to Science

The result of this study has contributed the following information to science:

i. To the best of our knowledge, this study is the first to analyse the expression of sialyltransferase gene in Trypanosome-infected animal.
ii. The upregulation of both hepatic and renal ST3Gal1 and ST6Gal1 was responsible for the recovery from anaemia in Trypanosome-infected pigs.
iii. Trypanosoma neuraminidase could be implicated in the development of thrombocytopenia in Trypanosome-infected pigs.

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APPENDICES

Appendix I: Preparation of Reagents for Sialic Acids, Neuraminidase and Sialyltransferase Assays

Preparation of the anticoagulant acid citrate dextrose (ACD)

Acid citrate dextrose (ACD) was prepared by dissolving 0.8 g of citric acid, 2.2 g of sodium citrate and 2.5 g of D-glucose in 100 mL of distilled water. ACD is usually freshly prepared before use.

Preparation of Sodium arsenite solution

Sodium arsenite solution was prepared by adding 95.7 mL of distilled water to 2 g of sodium arsenite, followed by addition of 4.3 mL of conc. hydrochloric acid (HCl) and the resultant solution will be agitated gently.

Preparation of Acid-butanol solution

A solution of acid-butanol was prepared by mixing concentrated hydrochloric acid (HCl) to n-butanol in the ratio of 1:9 followed by gentle agitation (precisely, 20 mL of conc. HCl was added to 380 mL of n-butanol to prepare a total of 400 mL of the solution.

Preparation of Sodium periodate solution

A solution of sodium periodate solution was prepared by first weighing out 1.14 g of sodium periodate to which 199.3 mL of distilled water was added followed by the addition of 0.7 mL of concentrated sulphuric acid (H_2SO_4).

Preparation of 1N (normal) Sodium hydroxide solution

Molecular weight of sodium hydroxide (NaOH) is 40 g (i.e. Na = 23 g, O = 16 g and H = 1). Therefore, to prepare 1N solution, 40 g NaOH pellets was dissolved in 1000 mL of distilled water or as needed (e.g. 4 g → 100 mL, 0.4 g → 10 mL etc.).

Preparation of Thiobarbituric acid (TBA) solution

Thiobarbituric acid solution was prepared by dissolving 7.21 g of thiobarbituric acid in 500 mL of distilled water. The *p*H of the solution was adjusted to 9.0 by sequential addition of drops of 1N sodium hydroxide solution.

Preparation of isotonic and hypotonic solutions

The buffers for the preparation of erythrocytes ghost were prepared from two solutions, Solution A and B. Solution A: was prepared by dissolving 2.68 g of sodium dihydrogen orthophosphate (NaH_2PO_4) in 125 mL distilled water. Solution B: was prepared by dissolving 7.31 g of disodium hydrogen orthophosphate ($NaHPO_4$) in 500 mL of distilled water. Isotonic buffer: Forty (40) millilitres (mL) of solution A was added to 440 mL of solution B, the solution was mixed thoroughly. Hypotonic buffer: One (1) millilitre of the prepared isotonic buffer was added to every 14.5 mL of distilled water. Since, much of the hypotonic solutions will be needed for washing erythrocyte ghosts, the needed amount was prepared.

Preparation of sodium phosphate citrate buffer (0.2 M, *p*H 6.8)

Two solution A and B was prepared: Solution A was prepared by dissolving 28.4 g of disodium hydrogen orthophosphate (Na_2HPO_4) in 1 litre of deionized water (giving a *p*H of 9.3). Solution B was prepared by dissolving 21.02 g citric acid in 1 litre of deionized water (giving a *p*H of 2.2). Equal volumes of solution A and B were mixed (and the *p*H noted). The *p*H of the mixed solution was adjusted either up or down to 6.8 using either a solution prepared from *p*H 4.0 buffer tablet or distilled water (*p*H 7.0).

Preparation of Fetuin

Preparation of the enzyme substrate Fetuin will be as follows. Twenty-five (25) milligram (mg) of freeze dried Fetuin will be dissolved in 1 mL of 0.2M sodium phosphate citrate buffer (*p*H 6.8)

Preparation of solutions of *N*-acetylneuraminic acid for standard curve

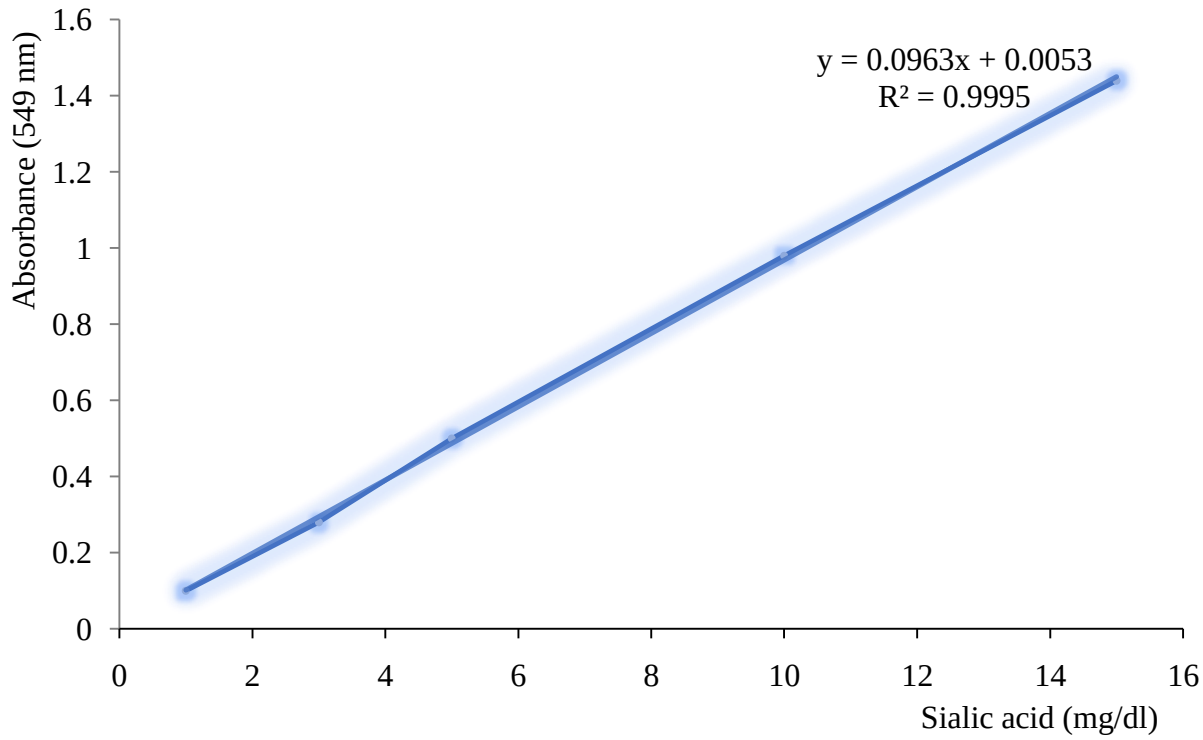
Solutions of *N*-acetylneuraminic acid (Sigma-Andrich®, Germany) containing 1.0, 3.0, 5.0, 10.0 and 15.0 mg/mL was prepared and their respective absorbance at 549 nm determine by the thiobarbituric acid method of Aminoff (1961) to establish a sialic acid standard curve.

Preparation of Sodium acetate buffer (50mM)

Exactly, 4.1 g of sodium acetate (molecular weight of 82.2 g) was weighed. Five hundred (500) millilitres (mL) of distilled water was added by first adding small volumes and mixing thoroughly to ensure dissolution. This was called solution A and the *p*H noted (i.e. 8.3). Then 3000 µl (3 mL) of acetic acid (98.8% purity) was dispensed in a 1 L volumetric

flask and 997 mL distilled water was added. This was solution B. The *p*H of solution B was noted (i.e. 3.2). For every 100 mL of solution B taken, solution A was sequentially added to adjust the *p*H to the desired test *p*H using a *p*H meter (Jenway® 3510, Essex UK) to obtain a 50 mM sodium acetate buffer at that desired *p*H. All *p*H readings were taken in duplicate.

Appendix II: Data and Standard Curve for Sialic Acid Determination



Appendix III: Variation in the mean values of eosinophil and monocyte counts in *T. b. brucei*-infected and control pigs.

	Eosinophils counts ($x10^9$/L)		Monocytes counts ($x10^9$/L)	
Days PI	**Infected Group**	**Control Group**	**Infected Group**	**Control Group**
-3	0.42 ± 0.37	0.18 ± 0.24	0.10 ± 0.12	0.12 ± 0.22
0	0.32 ± 0.40	0.18 ± 0.24	0.12 ± 0.13	0.12 ± 0.30
1	0.72 ± 0.65	0.28 ± 0.47	0.56 ± 0.46	0.32 ± 0.43
2	0.14 ± 0.21	0.32 ± 0.23	0.06 ± 0.90	0.14 ± 0.21
3	0.04 ± 0.05	0.32 ± 0.28	0.02 ± 0.04	0.04 ± 0.05
4	0.28 ± 0.41	0.38 ± 0.63	0.08 ± 0.11	0.08 ± 0.11
5	0.30 ± 0.33	0.44 ± 0.91	0.14 ± 0.21	0.30 ± 0.33
6	0.42 ± 0.41	0.46 ± 0.32	0.18 ± 0.19	0.22 ± 0.29
7	0.40 ± 0.54	0.48 ± 0.33	0.12 ± 0.08	0.20 ± 0.20
8	0.18 ± 0.25	0.50 ± 0.48	0.12 ± 0.13	0.18 ± 0.25
9	0.18 ± 0.19	0.50 ± 0.55	0.22 ± 0.20	0.18 ± 0.19
10	0.24 ± 0.21	0.50 ± 0.58	0.12 ± 0.13	0.24 ± 0.21
11	0.22 ± 0.19	0.50 ± 0.48	0.10 ± 0.12	0.22 ± 0.19
12	0.22 ± 0.22	0.50 ± 0.55	0.20 ± 0.19	0.22 ± 0.22
13	0.36 ± 0.38	0.50 ± 0.64	0.12 ± 0.34	0.16 ± 0.17
14	0.62 ± 0.57	0.50 ± 0.58	0.20 ± 0.18	0.42 ± 0.30
15	0.30 ± 0.35	0.52 ± 0.83	0.22 ± 0.19	0.12 ± 0.13
16	0.78 ± 0.46	0.54 ± 0.87	0.36 ± 0.38	0.26 ± 0.20
17	0.90 ± 0.70	0.56 ± 0.52	1.08 ± 0.77	0.24 ± 0.34
18	0.46 ± 0.36	0.56 ± 0.42	1.04 ± 1.34	0.16 ± 0.21
19	0.50 ± 0.75	0.62 ± 0.79	0.86 ± 1.00	0.30 ± 0.40
20	1.02 ± 0.73	0.62 ± 0.56	0.76 ± 0.42	0.42 ± 0.33
21	0.92 ± 0.93	0.88 ± 0.37	0.36 ± 0.39	0.32 ± 0.31