Studies on fowl spirochetosis in Khartoum state

By

Iman Mohammed El Nasri Hamza

B.V.Sc University of Khartoum
1985
M.Sc University of Khartoum
1997

A Thesis
Submitted to the University of Khartoum
in fulfillment of the requirements
for the degree of
Doctor of Philosophy (Ph.D.)

Department of Microbiology
Faculty of Veterinary Medicine
University of Khartoum

August
2008

TABLE OF CONTENTS

Chapter two: Materials and methods

Chapter three:Results

List of Tables

List of figures

DEDICATION

To the souls of my Dear Parents

To my husband Khalid my children Shahd ,Mohammed

And Muna

I dedicate this work

PREFACE

This work was carried out at the Department of avian pathology and diagnosis Central Veterinary Research Laboratory (CVRL) and Department of Microbiology, Faculty of Veterinary Science University of Khartoum from 2003 to 2007 under the supervision of Professor Mohammed Taha Shigidi and Co-supervision of professor Amal Mustafa Mohammed.

The experimental work is original and the dissertation has not been submitted partially or fully to any other university.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude and appreciation to my supervisor Professor Mohammed Taha Shigidi for the tremendous help and guidance throughout this work. My thanks are due to my co-supervisor professor Amal Mustafa for her help.

My thanks are extended to Professor M Salih Algabalabi previous Director of central Veterinary research laboratory and Professor Salah Mukhtar previous manager of assistant units (CVRL) for their unlimited help and for allowing this work to be done in the laboratory.

Appreciation are also extended to the technical staff of the avian pathology and diagnosis department for technical help and to any body at central veterinary research lab who directly or indirectly participated in helping me to finish this work.

With gratitude I acknowledge the financial support of the Sudan government for this study.

I would like to express my heartiest thanks to my family for the continuous help and encouragement during the course of the study.

My sincere thanks are due to Dr Mohammed Tag Eldin for his useful help on statistical work and Dr Hind Abd El Aziz for her real friendship, Continuous encouragement and moral support throughout the course of the study.

Summary

Spirochetosis was reported in different poultry farms in Khartoum state. Clinical disease was recorded among different ages and breeds. Based on morphology of the organism, presence of *argas persicus* clinical signs and post mortem lesions in inoculated chicken, the organism was confirmed as *Borrelia anserina.*

The disease was studied in three different age groups of chicken (Chick, grower and adult) using four field isolates .Short incubation period (2-3) days, severe clinical sings, extensive short duration of spirochete 3-4 day and enlargement and mottling of spleen were shown in older birds. Mild clinical signs, extensive prolong spirochetemia 10-15 days, slight enlargement of the spleen were the main features of the disease in chicks.

Histopathological examination of organs of infected birds showed difference between the isolates .Two isolates showed severe changes and damage of the spleen.

Disease in local breeds and pigeon was also studied.

A trail was carried out to isolate the causative agent in bacteriological media but the organism failed to grew albeit that the organism was isolated in embryonated eggs via different routes successfully.

Serological studies showed that Agglutination and immobilization tests were both comparable in detecting *Borrelia anserina* antibodies. Antigenic and pathogenic study of the 4 field isolate revealed that two isolates (namely:Butri and Soba1) were more pathogenic.

Cross protection test between the field isolates showed that the immune response induced by infection with two of the isolate will protect against challenge by the four isolate studied.

The effect of storage condition and duration on viability and infectivity of *Borrelia anserina* was also investigated.

In other part of the study, molecular characterization of *Borrelia anserina* was investigated. After extraction of DNA and amplification no results were obtained.

ملخص الاطروحة

بينت الدراسة وجود مرض زهرى الطيور في عدد من مزارع الدواجن بولاية الخرطوم. ظهرت الاصابة السريرية في اعمار و اجناس و سلالات مختلفة،و تم التاكد من الباكتيريا المسببة للمرض (البوريلية الوزية) من شكل الباكتيريا مع وجد القراد اللين والذي يعتبر العائل الاساسي للمسبب في جميع المزارع المصابة، بالاضافة الي الاعراض السريرية والتشريحية في الدجاج المصاب .

شمل البحث دراسة المرض في الدجاج في ثلاثة اعمارمختلفة باستخدام اربع عترات محلية تم عزلها من اصابات حقلية .فى الطيور الكبيرة (Growers and Adults) كانت فترة الحضانة 2-3يوم كما اظهرت اعراض حادة ويستمر وجود الباكتيريا في الدم لفترة قصيرة تتراوح بين2-3 يوم، وتضخم وتبقع شديد في الطحال.اما الطيور الصغيرة فقد اظهرت اعراض سريرية خفيفة و فترة حضانة تتراوح بين 2- 4يوم مع وجود اعداد كبيرة من الباكتيريا في الدم لفترة طويلة تتراوح بين 11-15يوم و قد لوحظ تضخم خفيف في الطحال .

فحص الانسجة المريضة للطيور المصابة بالعترات الحقلية اوضح اختلاف كبيرفي ضراوتها. اظهرت اثنين منهم تضخم شديد و تبقع قي الطحال مع عدة اعراض تشريحية اخرى .

لم تنجح العترات في احداث المرض في الدجاج البلدى والحمام والنمو في الاوساط البكتيرية ولكن تمت عزلها بنجاح في اجنة الدجاج بعدة طرق.

كما تمت مقارنة طرق تشخيص المرض بواسطة الاختبارات المصلية (agglutination and immobilization tests) وقد وجد ان الاختبارين جيدين من حيث تحديد الاجسام المضادة مع اختلاف طفيف بينهما .دلت دراسة الامراضية و الا ستضداية للعترات الاربعة على وجود مستضد مشترك بينهما ولكنها غير متماثلة . اظهرت اثنين منهم(بترى و سوبا1)ضراوة اكثر من (طيبة والكدرو) كما اثبتت الدراسة ان العترات المعزولة محليا متشابة من حيث الوقاية المتبادلة بينهما بالرغم من الاختلاف في ضراوتها .

تضمن البحث دراسة تاثير ظروف وفترة التخزين علي حيوية وضراوة البكتيريا. اوضحت التجارب ان الباكتيريا تعيش لمدة 23شهر متواصلة في الدم المصاب في درجة حرارة -196 درجة مئوية (النتروجين السائل). كما احدث الدم المحفوظ المرض مع وجود عدد كبير من الباكتيريا في دم الدجاج المحقون.

تم االستخلاص الحامض النووي للباكتيريا لدراسة الصفات الجزيئية ولكن لم يتم التوصل لنتائج .

Introduction

Spirochetosis is an acute or subacute, septicaemic tick-borne disease. It affects many bird species including geese, ducks , turkey , pigeon and chicken of all ages and breeds (Gross,1984).The disease occurs world wide; it was reported in Europe, Africa and Asia,in parts of Australia and Central and South America (Cooper and Bickford,1993).

The disease is prevalent mainly in North Africa, Middle East and Asia in tropical and Subtropical areas.

Spirochetosis has an important economical impact, as it causes high mortality among birds which may reach up to 100%, beside its effect on lowering egg production in layers and carcass down grading in broiler chicken. The disease is caused by aerobic Gram negative, highly motile bacteria *Borrelia anserina* (Barnes 1994) and is transmitted mainly by an arthropod vector, the soft tick *Argas persicus*. The bird can also get infected as a result of ingestion of ticks, their eggs, contaminated droppings and via cannibalism.

Clinical signs of the disease vary according to the virulence of the strain, but appear as fever, greenish diarrhea, dullness, paleness of comb and wattles and loss of weight. The characteristic post mortem lesion is the enlargement and / or mottling of the spleen.

The disease is diagnosed by demonstration of *Borrelia anserina* in peripheral blood or detection of antibodies after disappearance of the organism from the blood. Serological tests such as agglutination and immobilization are in use.

In Sudan, the disease has not received the appropriate attention. It has been reported as early as 1906 in chicken and latter in ducks in 1943.Very little work has been done , mainly on hematological and histopathological changes (Ginawi, 1980).

In the country the main problem is that the majority of poultry farms are open system ones in which birds are more likely to be exposed to *Borrelia anserina* due to the endemic nature of the vector *Argas persicus* in the country .

Objectives of the study:

1- To study the prevalence of spirochetosis in Khartoum state.

2- To Isolate and identify *Borrelia anserina* from infected birds.

3- To study the effect of temperature and different storage conditions on viability of *Borrelia anserina.*

4-To Prepare an antigen and develop a practical simple test(s) for diagnosing the disease in the field.

5-To Study the antigenic relationship of field isolates.

Chapter one

Review of Literature

1.1 Spirocheatosis

Spirochaetosis is an important tick-borne disease of poultry transmitted by ticks and many biting arthropods(Barnes ,1997). The disease is characterized by marked illness, low morbidity and high mortality, increased culling rate and drop in egg and meat production (Wouda *et al*., 1975; DaMassa and Adler, 1979).It was first recognized in 1891 in Russia (Sakharoff, 1891), and in 1903 in Brazil (Marchoux and Salimbeni, 1903)and in the United States the disease was known since 1949 (Hoffman and Jackson 1949). Outbreaks were reported in southwestern United States in fowl, turkey, pheasants and in game birds (Cooper and Bickford, 1993). The disease is important in tropical and subtropical areas particularly arid and semiarid regions where fowl ticks are common.Spirochaetosis is common in free range husbandry system .The disease is uncommon in temperate areas and in intensively managed flocks (Kaschula, 1961).

1.2 The disease in Sudan

In the Sudan, the disease was known since1906 in fowl, 1925 in turkeys and pigeons and 1943 in ducks (Anon, 1923).Avian spirochetosis

is considered as a disease of an open system farms and backyard birds which represent the majority of poultry husbandry in Sudan.

It constitutes a problem to the small scale producer however, some outbreaks were reported in large well designed farms in Khartoum State.

The disease can be easily diagnosed and treated but according to the difficulties in prevention and eradication of the vector which colonizes the farms for as long as time, spirochetosis is considered an important tick-borne poultry disease. Knowledge of the epidemiology of the disease is still very scanty.

Reports from all over the country indicated that the disease occurred throughout the year and seasonality was not observed, but transmission was related directly to the activity of the tick *Argas persicus* which is the main vector of the disease (Cooper and Bickford., 1993).

Outbreaks of spirocheatosis diagnosed during 1999-2006 at the Department of Avian pathology and diagnosis ,Central Veterinary Research Laboratories (Soba) are shown in Fig(1).

1.3 Causative agent

The causative agent was first isolated from geese (Sakharoff, 1891). It was known at that time as *Spirochaeta anserina* .Other names *Spirochaeta gallinarum*, *Spirochaeta anatis* and *Treponema anserina*

were also suggested, (Barners, 1997). Breed *et al* .(1975) listed the organism as *Borrelia anserina*.

Pathogenic spirochetes belonging to the genus Borrelia are characterized by their obligatory biological transmission by blood feeding arthropods (Canale –Parola, 1984).

They are transmitted by different species of ticks and lice, causing many diseases in human and animals.

Tom *et al*.(2003) stated that pathogenic species of the genus *Borrelia* were divided into two major groups according to the following criteria:

1- Sequence of the disease produced.

2- Bacterial density in peripheral blood during parasitemia.

3- Tick vectors that transmit the disease.

The first group includes relapsing fever spirochetes and *Borrelia anserina* which causes fowl spirochetosis.The second group includes Lyme disease spirochetes. *Borrelia anserina* and relapsing fever spirochetes are both transmitted by fast feeding ticks (Argasidea) (Barbour and Hayyes, 1986; Dworkin *et al*., 1998). They attain cell density of 10^8or more per ml of blood which assist in diagnosis of the disease produced.

Lyme disease spirochetes also found in peripheral blood but according to Maraspin *et al*. (2001), Schwan *et al*.(1996) and Wormser(2000) the organism could be isolated from blood, but the cell density was not high enough to be detected microscopically.Differences in the level of bacterimia between the two groups suggest difference in the way these groups of spirocheate survive in the blood. Tom *et al*. (2003) reported that the main

difference is the presence or absence of the enzyme glycerophosphodiester phosphodiesterase which hydrolyzes deacylated phospholipids to an alcohol and glycerol 3 phosphate.Glycerol 3 phosphate (G3p) is an important intermediate for metabolism and phospholipids synthesis. Three pathways for acquisition of G3p exist among *Borrelia* species; reduction of dihydroxyacetone phosphate , uptake and phosphorylation of glycerol and hydrolysis of phospholipids .Experimental work based on enzyme assay, DNA sequences and southern blot analysis demonstrate that all species of Borrelia have the genetic potential to produce G3p via the first two ways , however *Borrelia anserina* and relapsing fever group have the unique ability to acquire G3p from hydrolysis of phospholipids this would conserve ATPS in the cell in contrast to its synthesis from the other two pathways(Tom *et al*., 2003).Phospholipids are found in mammalian erythrocytes and serum phosphatidylcholine (lecithin)and is the major phospholipids in Borrelia (Livermore *et al*,1978).

Comparisons of the 16s rRNA sequences demonstrated that spirochetes represented a monophyletic phylum within the bacteria spirocheates and were recently classified in the class Spirochaetes,order Spirochaetales ,family Spirochaetaceaes and genus *Borrelia* (Paster *et al*,1991;Paster and Dewhirst, 2000). *Borrelia anserina* is long and slender. The length of

organism varies from 6 to 30 μ and there is a wide variation in smears from the same bird due to division stage (McNeil *et al* ,1949). Under the electron-microscope the organism is regularly helical measuring about 9-21 μ long and 0.22-0.26 μ wide.

The cells are surrounded by a surface layer named the S-layer which is a triple layer in *Borrelia anserina* unlike other spirochetes. The cells divide by binary fission (Burgdofer and Schwan ., 1991).

The organism is highly motile and the type of motility and its organelles differ completely from other bacteria and are considered a unique feature of spirochetes. It has a rapidly drifting rotation associated with flexing undulating

movement (Breed *et al*;1975).

Flagella which are also known as axial fibrils are inserted at the ends of the cells in the periplasmic space under the outer cell membrane. Numbers of flagella differ between strains. Strains with 7 flagella were found to be pathogenic (Hovind-Hougen, 1995; Barnes, 1997).*Borrelia* species contain ornithine rather than diminopimelic acid in their cell wall peptidoglycan (Paster *et a*l.,1991) and their lipid components usually include cholesterol.

The genus *Borrelia* unlike other bacteria,are stained readily with different aniline dyes, and by Gemisa and Gram stains (Burgdorfer *et al*., 1991).

The organism is surrounded by two membranes, the outer one is more fluid and contains less protein than that of Gram-negative bacteria, *Borrelia anserina* are Gram negative and are lysed by ox bile (Challerjee and Sawhney, 1971).

Two species of the genus Borrelia; *Borrelia anserina* and *Borrelia burgdorferi* are known to infect birds (Anderson *et al*.,1986).The latter also infect mammals causing a multisystem severe disease(Steere *et al*.,1984) which was reported in dogs,cattle,sheep and horses (Kornblatt *et al*.,1985;Hormark *et al.*, 1986;Burgess *et al*.,1956).

Walker *et al*. (1989) found that there was antigenic cross reactivity between *Borrelia anserina* and *Borrelia burgdorferi*, they shared at least two flagellar epitopes. On the other hand David *et al.*(2000) demonstrated that *Borrelia anserina* lacked the direct *Borrelia* repeat related genes .

1.4 Pathogenesis

The organism gains entrance to the body through haemolymph of infected nymph. *Borrelia anserin*a multiplies in the tissues of the reticulo- endothelial system of the host. At the end of the incubation period, great number of organism invades the blood. Some of the organisms are destroyed by body defense elaborating endotoxins which cause fever and affect the blood vascular system resulting in spleen infarction and necrosis**.**

1.5. Borrelial genetic

Borrelial genome is unique among bacteria in that the structure and organization of *Borrelia* genome are similar to certain viruses. It is segmented and most genetic elements are linear with covalently closed termini or telomeres. It is composed of linear chromosomes and a series of linear and circular plasmids. Only 16% of these genes have predicted biological roles. Circular plasmids are stronger and contain much more information than the linear ones. the linear plasmids contain approximately 430 open reading frames and have a G/C content of about 23-32% which is low, hence they had a high A/T content. The entire isoelectric point of Borrelia organism is cationic (basic) this helps it binding and colonization of a wide variety of tissue on the basis of charge (Davis *et al.*, 2000).

Borrelia is able to tolerate remarkable genomic variability and diversity in the plasmid and the genes its carries (David *et al.*, 2000).This may illustrate the phenotypic diversity and environmental adaptation, of the organism in both ticks and vertebrates.

1.6 Cultivation of *Borrelia anserina*

Ordinary bacteriological media are not suitable for the growth of *Borrelia anserina* .It is one of the bacteria that are identified on morphological and serological grounds or by their pathogenicity for various animals (Barrow and Felthan, 1993). Already there are over 200

spirocheatal species of which more than half were found uncultivable (Paster and Dewhirst,2000).

Barbour-stoenner -Kelly liquid medium is used for the isolation of the organism but the organism losses its infectivity by the 12th serial passages (Levine *et al.*, 1990).

Fertile eggs were known since 1949 as the simple medium and more economical method for isolation and propagation of *Borrelia anserina* (McNeil *et al*., 1949) by different routes. McKercher (1950) stated that the yolk sac is the best route for isolation of *Borrelia anserine*,six-day-old embryonated eggs are used and embryos death occur 4-7 day post inoculation. Materials must be collected before embryonic death. Blood, Liver and chorioallantoic fluid contain large number of spirochetes (McKercher, 1950). Mathey and Siddle (1955) used chorioallantoic membrane and allantoic sac routes and obtained a good yield of the organism.

Inoculation of one day- old chick with infective blood or tissue was considered as the best method for isolation and resulted in high spirochaetamia which was present up to 21 days post inoculation (McKercher., 1950; Packchanian and. Smith, 1970).

1.7 Serotypes

Strain variation in *Borrelia anserina* was reported (Mehta and Muley,1968) and it is of significance from the prophylaxis point due to

strain specific immunity although partial protection was obtained when two strains shared a common antigen (Soni and Joshi,1980).

Three serologic types were described namely Surnevo, Pamoukchii and Venets (Dzhankov *et al*., 1975) ,but no formal serotype classification scheme has been developed. Antigenic analysis was carried out first by serologic methods which established the similarities and differences between strains. Coffey and Eveland (1967) were able to identify 4 major serotypes of *Borrelia hermsi* using the direct immuno-fluorescence technique. Other methods for antigenic analysis include gel diffusion, immunoelectophoresis and whole serum macroagglutination reaction (Dzhankov *et al*., 1975),agar-gel precipitation test (Al-Hilly and Abbood, 1969; ALattar and Johanly, 1974), plate and tube agglutination (Metha and Muley 1968; Challerjee and Sawhney, 1971) and spirochaete immobilization test (Verma and Malik, 1968) .These tests were used to identify different antigenic strains of *Borrelia anserina*. Several studies have demonstrated the presence of different antigenic types in a given area (Walker *et al*., 1989; Wouda *et al*., 1975; Soni and Joshi., 1980; Levine *et al*., 1990)

In Bulgaria 3 serotypes were identified by agglutination test namly Surnevo 1, Pamoukchii and Venets (Dzhankov *et al*., 1975).In USA different serologic and immunologic isolates were identified (Damassa and Adler., 1978). In India strains variation were identified in the same

area(Soni and Joshi,1980).Recently a Brazilian isolate was genetically studied and identified as strain PL (Ataliba *et al* ,2007) .Strains also differ in virulence (Damassa and Adler, 1978). Steinhaus and Hughes (1947)isolated a nonpathogenic spirochete in developing chick embryo.

1.8 Incubation period

The incubation period depends on virulence of the organism, route of infection, number of organism in the inoculm and the immune status of the bird (Abdu, 1987) .It ranged from 7-12 days after experimental inoculation and 4-7 days after infected tick bite (Khogali and Shommein, 1973). Different infectious materials were used experimentally and produced the disease with some variations, which was influenced by the material used dose and route of infection. Gorrie(1950) used fresh infected blood clot for oral route and produced the disease after 4-5 days post inoculation while intravenous injection of infected serum produced the disease within 2-3 days post infection. When citrated blood was injected intramuscularly the incubation period was 4-5 days (Wadalkar and Soni, 1982).

1.9 Clinical Signs

Many workers described the clinical signs of spirocheatosis (Rao *et al.*, 1958; Rokey and Snell 1961; Barnes., 1997). Acute, subacute and chronic forms of the disease exist.

In the acute form there is rise in temperature 43˚C, cynosis of comb and wattles and depression .On the 4th -5th day post infection soiled vent, weakness and or paralysis of legs and wings were also seen (McNeil *et al*., 1949; Roa, 1958). Death may occur due to the formation of emboli by the agglutinated organisms (Delamater and Saurion, 1952).

In sub acute and chronic forms anorexia, increased thirst, weakness of wings and legs, pale comb and wattles and greenish diarrhea were the main signs observed .Barnes (1997) described the clinical signs of the disease as a marked increase in body temperature, dehydration and rapid loss of weight during the first 4-5 day of illness. Paralysis and nervous signs were also seen and excess bile and urates were noticed in the droppings. Finally the birds were comatose with subnormal temperature (John and Davis, 1998).Infection with low virulent strains causes a mild infection or may pass unnoticed. Recovered birds do not become carriers (Dickie and Barrera, 1964).

The organism was detected in blood smears up to day 9 post infection and after 13 days in livers (Damassa and Adler, 1979). According to Dickie and Barrera (1964) *Borrelia anserina* disappeared completely from both blood and livers 28 days post infection. Spirochetemia were graded according to Dhawedker and Dhanesar (1983) classification (table 1).

1.10 Morbidity and Mortality

Morbidity and mortality are highly variable, lower mortalities occurred in flocks with constant exposure to infested ticks bites as immunity is passively transferred to the progeny. Higher mortalitiy rates occur when susceptible birds are mixed with bird infested with ticks, or placed in an environment infested with infected ticks (Barnes, 1997). Indigenous breeds in Sudan are more resistant compared to the imported foreign breeds (Khogali and Shommein, 1973). In some geographical areas morbidity may reach 100% and mortality 90% (Barnes and Swayne.,1998).

1. 11 Post mortem Lesions

1.11.1. Spleen

Enlargement with mottling appearance of spleen are characteristic lesions of spirochaetosis (Reddy *et al*., 1966; Shommein and Khogali, 1974). However in infections with low virulent strains or early in the disease, spleenomegaly may not be noticed (Cooper and Bickford .,1993). Large areas of hemorrhage and necrotic foci in the reticular cells with active proliferation of lymphatic cells were observed .Mottling is due to hyperplasia and fibrinoid necrosis of mononuclear phagocytes surrounding capillaries (Mc Neil *et al,* 1949.; Rokey and Snell, 1961).

Large areas of erythrophagocytosis and extra vascular haemolysis were also seen suggesting involvement of the immune system in the pathogenesis of spleenomegaly (Bandopadhyay and Vegad, 1983).

1. 11.2. Livers

Livers are usually swollen with focal areas of necrosis (Reddy *et a*l., 1966). Subcapsular hemorrhage and fatty degeneration were noticed by Rokey and Snell (1961). Hepatic cells showed cytoplasmic vacuolations and pyknotic nuclei. The intralobular connective tissue was accumulated with lymphocytes. Portal veins were thickened and engorged with erythrocytes , bile ducts were surrounded by wide zones of proliferated lymphocytes. Erythrophagocytosis was noticed which continued after the disappearance of spirochetes from the blood (Reddy *et al* 1966; Rokey and Snell, 1961). *Borrelia anserina* could be demonstrated in liver tissue (Bandopadhyay and Vegad 1983).

1.11.3. Kidneys.

Infected kidneys were enlarged with excess urates and the renal blood vessels and glomerular capillaries were slightly congested. The glomeruli were diffusely affected and the changes included necrosis of the capillary endothelium and proliferation of the epithelial lining cells of Bowmans capsules (McNeil, 1949; Ahmed *et al.,* 1965).

1. 11.4. Intestines

Greenish catarrhal enteritis is common there with slight congestion in the veins of the lamina propria and lymphocytic infiltration. Degeneration and desquamations of the epithelial cells lining the mucosa were common finding and some necrotic areas were noticed in the tips of the villi. Hemorrhages at the proventriculus junction were seen in some cases (Mcneil, 1949; Ahmed *et al*., 1965).

1.11.5. Lungs

Pulmonary veins were severely congested with blood and some arteries revealed the presence of thrombi. Lungs show haemosiderosis (McNeil, 1949). Spirochetes could be demonstrated in spleens, livers, kidneys and lungs even after disappearance from the blood (Bandopadhyay, 1983).

1.11.6 Brain

Changes in the brain were characterized by congestion of blood vessels in the cerebrum and cerebellum, swelling and fragmentation of neuroglial fibers, perineural oedema and neuronal damage (Ginawi, 1980).

1.12 Hematological changes

Marked alterations in blood parameters were reported during spirocheatosis which included a decrease in erythrocytes number, which

reached 1.26 by day 6 post infection compared to 2.72 in control birds (count x 10/ μ).Mean hemoglobin concentration reach 5.48 g/dl at day 6 post infection compared to 9.14 g/dl in control birds(Bandopadhyay ,1983). Despite severe anemia serum samples of infected birds were not tinged with hemoglobin at any stage of infection also there were no icterus or yellow dropping. Slight increase in leucocytes especially lymphocytes and decrease in percent of hetrophiles, eosinophiles and basophiles, with prolonged coagulation time (Nikolov, 1987) were detected. Changes in blood chemistry were also described.

1.13 Transmission

Spirocheatosis is transmitted directly from bird to bird when susceptible birds are exposed to infected blood, droppings or tissue from live diseased chicken . Ingestion of infected droppings and cannibalism during spirocheatemia are the main methods of transmission within the flock (Hoffman and Jakson, 1946; Dzhankov *et al*., 1968).This was supported by the finding of Djanko *et al* (1970) who isolated *Borrelia anserina* from mouth and cloaca during spirochaetemia.

Inanimate objects like syringes play an important role in transmission of the disease between birds in the flock (Kapur, 1940).

Experimentally the disease could be produced orally, intranasal, intravenously, intramuscularly, intraorbitaly and even through unbroken skin and mucous membranes of conjunctiva with virulent strains (Kapur,

1940). Intra peritoneal route produced the disease experimentally in day old chicks (Anderson *et al*., 1986).

Spirochetes are extracellular pathogens. Borreliae have complicated transmission cycles involving ticks, mammals and birds (Anderson *et al* 1989 .,Mignarelli *et al*,1992.) *Borrelia anserina* cycles between the vertebrate blood and blood sucking arthropods (Hoogstraal, 1973).It is mechanically transmitted by many blood sucking parasites that infest chicken which include lice, mite ,*Dermanysus gallinae*,*Ornithodorus moubats* and *Argus persicus* (ELDardiry, 1945;Henry, 1950; Robert,1961; Rubina *et al*.,1975) . Of all *Argus* species, *Argus persicus* has been incriminated in epizootics of spirochetosis in poultry (Willy, 1998).

Culex , Aedes and Anophiles mosquitoes also transmit the organism mechanically (Roberts, 1961., Zulzer, 1963; Suliman *et al*., 2001).

Spirochetosis is transmitted mainly by the soft tick *Argas persicus* which belongs to the class Arachnida order Metastigmata family Argasidae Genus argas .*Argas persicus* parasitizes a variety of economically important poultry species and spread to many parts of the world (Hoogstral, 1985).Heavy infestation leads to irritations, restlessness, drop in production (Roberts, 1961; ElKammah *et al*., 2002) and tick paralysis in fowl mainly with larvael infestation (Gothe *et al*. 1981b).

All developmental stages larvae, nymphs and adults feed on chicken. Adult argasidea mate off the host and the females feed several times and produce small batches of eggs after each meal. Soft ticks have several nymph stages and nymphs are always parasitic (Jongejan and Uilenberg, 2004). Adults feed for 30 minutes at night and hide in cracks during the day.

Females oviposits after each feeding in batches of 25-100 egg. Larvae (seed tick), with 6 legs, take 4-5 days to complete their feeding then moult. Nymphs, with 8 legs, feed, for 30 minutes at night. They feed and moult several times before reaching the adult stage. The life cycle of Argas takes 45 days to complete (Russel *et al*, 1990).

Adult males and females feed monthly under natural condition, but all stages are able to survive 3-5 years without feeding.Ticks become infected 6-7 days after feeding on a heavily infected host and are able to transmit the disease at every feeding for as long as 488 days (Zaher *et al*, 1977).

Argas persicus and *Argas miniatus* are known as vectors of *Borrelia anserina* since 1903 (Marchoux and Salimbeni, 1903) Recently it has been found that *Argas persicus* is the main reservoir of *Borrelia anserina* and is able to transmit the organism transtadial and transovarial ((Gothe and chrecke.,1972; Zaher *et al.,* 1977).

Borrelia anserina is found in large numbers in the gut lumen ,then decreases in number and becomes immobile with time and die at day 20

post feeding. Within 2 hours of feeding ,the organism penetrates the gut wall and enters the haemolymph and begins to multiply. After 7 days post infection *Borrelia anserina* is found in the central nervous system, salivary glands and gonads of ticks and it remain alive for 60 days (Diab and Soliman,1977 ; Barnes, 1997).

Infected ticks are able to transmit spirochetosis through eggs (Petney *et al*, 2004). *Borrelia anserina* easily penetrates developing oocytes but not the mature eggs because the former are separated from haemolymph by cellular tunica propria while mature eggs have dense cuticular chorion membranes (Bertram, 1960) .Some chemical factors in the ovaries may affect transovarial transmission (Zaher *et al*, 1977) demonstrated that the infection rate in larvae from infected adult female ticks was 100%.

Borrelia anserina has the ability to survive in *Argas persicus* from one developmental stage to the other. Many workers found that certain muscle groups of the ticks undergo histolysis during molting, while cells of midget and malpigian tubules which are the predilection sites of *Borrelia anserina,* were replaced gradually throughout the tick life cycle.

Some scientists observed that salivary gland alveoli were completely replaced during tick moulting and they suggested that repeated invasion of these glands after each molt is necessary for transmission through the saliva, (Hoogstraal, 1973; Diab and Soliman, 1977).Culex

,Aedes and Anopheles mosquitoes transmit the organism mechanically(Roberts,1961., Zulzer, 1963., Willy,1998., Suliman *et* al.,2001)

1.14 Methods of Diagnosis

Geographic areas, seasons, presence of ticks in birds and their environment are suggestive of spirocheatosis (Barnes, 1998). Demonstration of *Borrelia anserina* in stained blood smears by ordinary microscope or in wet smears by dark field microscope, during spirochetemia are the best methods for diagnosing the disease (Snoeyenbos, 1965; Barnes, 1997).

Examination of buffy coat smears where the organism is concentrated is of value in early infection and during epidemiological studies (Higgins, 1986).

1.14.1 Serology

Fowl spirochetes after the lapse of spirochetemic phase can be diagnosed only by serology. Different tests were applied efficiently since 1914(Aoki, 1914; Chatterjee and Sawhney, 1971;Djankov *et al*., 1972). Different antigens preparations were used each for a specific test.

1.14.1.1 Agglutination test

Plate and tube agglutination tests were performed for diagnosis of spirochetosis they were also used to ascertain the immune status of the flock (Metha and Muley, 1968; Verma and Malik, 1968; Chatterjeeand Sawheny, 1971).

Specific agglutinins appeared in the blood by the 7th day (Chatterjee and Sawhney, 1971). *Borrelia anserina* separated from infected citrated blood by centrifugation then suspended in 0.5% phenol saline colored with 1% alcoholic solution of crystal violet constituted the whole cell dead antigen for plate agglutination test (Mehta and Muley, 1968; Verma and Malik, 1968).

1.14.1.2 Spirochaete Immobilization test

It is a simple accurate test in which spirochete are immobilized within seconds .It could be used to determine the antigenic variations between strains and for evaluation of the immune status of the flock. The antigen used is live organism separated from infected citrated blood by centrifugation and the sedimented organism was resuspended in normal chicken serum (Mehta and Muley, 1968; Verma and Malik, 1968).

Chatterjee and Sawhney (1971) suggested that the same antibodies may be responsible for both agglutination and immobilization reactions, and they might be closely related to each other. Immobilization antibodies

were demonstrated in treated and recovered birds (Levaditi *et al.* 1952).

1.14.1.3 Agar gel precipitation test

The test was described by many investigators as a simple, reliable, accurate and effective test for diagnosing the disease caused by antigenically different strains (Verma and Malik, 1968.,Chatterjee and Sawheny, 1971;).The test was recommended for routine laboratory diagnosis in dead birds using visceral organs as a source of antigen. Different preparations were used which included: antigen prepared by autoclaving, alternate freezing and thawing and phenol extraction antigen (Sumrov *et al.*, 1967, Verma and Malik, 1968).

Ox-bile treated antigen was found to be the best as it remained stable and gave satisfactory results for 3 month after preparation (Chatterjee and Sawhney, 1971) stated that the appearance of non specific lines of precipitation was not fully explained but it was not difficult to identify them because of their early appearance and their disappearance within 72 hours.

1. 14.1.4 direct fluorescent antibody test

The direct fluorescent antibody test (Prudovsky *et al.*, 1978) is also used

successfully for diagnosing the disease.Immunodiffussion agar gel test (Jaafir *et al*., 1969) and immunofluorescence tests (Felsenfold, 1971) are both used after the disappearance of *Borrelia anserina* from the blood of infected birds.Spirochetal antibodies can readily be detected in yolk of eggs laid by immune hens using immobilization and immunodiffusion tests (Alhilly,1971).

1.15. Control of the disease

Control of fowl spirochetosis is a difficult procedure because of the hidden behaviors of the ticks. The disease is best prevented in endemic areas by not introducing tick infested birds into clean flocks nor introducing susceptible birds into infested flocks or housing were infected birds had been previously kept. Important is the control of the vectors that transmit the disease, hence some measures might help in controlling outbreaks and reducing mortality but not in eradicating the disease.

Eradication is a difficult measures , but larval ticks can be controlled in birds by dipping them in 0.5 % malathion (Barnes ,1997).Spraying of houses and the environment including cracks, crevices and other places where ticks hide at monthly interval help in keeping ticks at low

level.Insectside paints and pastes when used intensively might help in controlling ticks(Rodey and Soni.,1977).

1.15.1 Vaccines

Vaccination is considered a suitable mean to control spirochetosis.

For the production and application of a vaccine against spirochaetosis two points must be considered:

1- The vaccination must be practiced in areas where a spirocheatosis is prevalent.

2- The vaccine must be polyvalent containing all serotypes existing in a particular area due to type specific immunity (Wouda *et al*., 1975; Sambri *et al* 1999).

Several trials have been carried out to produce suitable effective vaccines using different protocols of preparation. Gorrie (1950) reported production of egg adapted yolk sac vaccine which gave a high degree of immunity throughout the laying period .Vaccines prepared from carbolized dilutions of citrated blood or emulsions of livers or spleens of infected fowl were found equally protective producing a solid immunity which lasted for 6 month after injection (Morcos *et al.,* 1946).

Phenolized and formalized embryo vaccines were produced. They were prepared from infected blood or tissues usually liver and spleen and of embryos which were inactivated by phenol or formalin (Sreenvasan and Sankaranarayan, 1943). They were all found effective, giving a solid immunity lasting for at least six months (Morcos *et al*., 1946). In a comparative trail a formalized vaccines was found better than phenolized vaccine it consisted of whole chick embryos which had been inoculated with the organism then emulsified and inactivated with formalin and used as a vaccine (Nobrega and Ries, 1947).

Recently workers developed different types of vaccines, which are given to birds at 2-3months of age and the immunity produced lasted for as long as 8 month. These types are egg-propagated vaccine which is either a yolk material containing the spirochete or whole egg propagated bacterins, both types were used with success in India (Roa and Soni, 1982)and Bulgaria trivalent vaccine was produced against the serotypes established in the country .The vaccine was prepared according to Reshetnyaks method (Packchanian and Smith,1970), the organisms were obtained either from infected blood ,organs of infected fowl or infected embryonated eggs. They were then inactivated with 0.1% formalin and 1% phenol and subjected to 50˚C for 15-30 minutes or 20,000 R of

Gamma ray . The vaccine was administered S/C or I/M and produced good long lasting immunity (Packchanian and Smith 1970., Bozhilov *et al*., 1978).

1.16 Preservation of *Borrelia anserina*

The organism is not resistant outside the host, therefore several attempts have been made to find a suitable method for preserving *Borrelia anserina* live and infective for a long time. Maintenance of *Borrelia anserina* is important to manufacturing a vaccine. There are several problems associated with the maintenance of the organism in the laboratory .As with all blood parasites the degree with which these organisms undergo mutation in the vertebrate hosts must be considered.

Ordinary bacteriological media are not suitable because of the bacterial fastidious nutritional requirements of the organism(Merchant and Packer, 1965., Holt *et al*.,1997) .Although the organism was grown successfully in the liquid media Barbour- stoenner-kelly(BSK), but by the 12 serial passage infectivity of the organism for chicks was lost (Levine *et al* 1990).

Infected *Argas persicus* colonies, the main vector of the organism were used for preserving the organism .The organism keeps its viability

and pathogenicity unchanged but it needs continuous supply of chicken flock with antibiotic free diet and special housing for tick feeding.

EL Dardiry (1945) maintained *Borrelia anserina* separated from clotted blood of infected chicks live and virulent at +4º C for 30 days. Infected spleen, heart and liver stored at 32ºC retained their infectivity for 31 days (McNeil *et al* 1949).

Lotfy *et al*. (1966) kept *Borrelia anserina* in citrated blood live and virulent at +4ºC for 113 days. In centrifuged citrated infective blood the organism remained motile and virulent at +4ºC for 4 weeks when red blood cells were added weekly (Al-Hilly,1971).Sumorov(1967) reported the survival of *Borrelia anserina* in citrated blood for 120 days in liquid nitrogen .It also survived for 150 days when 10% glycerol was added (Hart,1963).

Labruna *et al*. (1999) maintained the organism in serum with glycerol or dimethyl sulfoxide (DMSO) live and infective for 15 months with uninterrupted storage in liquid nitrogen.

Materials and methods

2.1 Experimental Birds

Chicks were brought as day-old from Coral Commercial Farm and reared in special cages until they reached the required age. Adult chicken were obtained from well designed commercial layers farms. Koral farm is considered as one of the biggest poultry farms in Khartoum state. It is well designed applying both closed and semi closed husbandary systems and adopting biosafety and biosecurity measures .Spirochetosis was not reported in the farm and vaccine against the disease dosenot practiced. Local breed chickens were obtained from Khartoum north market. All experimental birds were fed commercial rations according to their ages.

2.2 Field Isolates

Borrelia anserine isolates used in this study were recovered from naturally infected birds which were either submitted to the Department of Avian Pathology and Diagnosis (CVRL) for routine diagnosis or collected during field investigation.

Infected chicken were of different age and breeds. Birds showed clinical signs which included dullness, ruffled feather, pale comb and wattles and greenish diarrhea. Hemorrhagic spots were seen in the wing and thigh region of these birds. Nymphs were seen aggregated in large

numbers in the wing and abdominal area in some birds. Post mortem examination showed enlarged mottled spleens of infected birds. Isolation attempts were made from blood, spleen, liver and intestine of infected bird, isolated organisms were considered as *Borrelia anserina* according to their morphology in stained smears, pathogenicity to susceptible chicken and reisolation from experimentally infected ones. Ten *Borrelia anserina* isolates were recovered during this study (Table 2).

Isolates used in this study were selected to represent the main localities of open system poultry farms in Khartoum State. With the exception of Omdurman where no farms met our criteria. The four isolates used were Soba1 representing southern area of Khartoum State, Taiba isolate from an area were large number of open system farms exist .Alkadaro isolate was the only isolate obtained from northern area. Butri isolate was selected (in addition to the criteria of chosen isolates) due to the acute and high mortality rate during an outbreak in layer farm.

2.3 **Embryonated eggs**

These were obtained from the hatcheries of the Vaccine Production Department at the Central Veterinary Research Laboratories (CVRL) and from Coral Commercial Poultry Farm as 6-10 day- old-embryos.

2.4 Housing of experimental Chicken

Special cages were designed to prevent exposure of bird to the external blood sucking parasite. It was made of fine wire with four long stands. Cages were cleaned, burned and sprayed with insecticide before

use. Stand of all cages were dipped in containers filled with oil throughout the experimental work.

2.5 Preparation of Reagents

2.5.1 Sodium chloride

Sodium chloride 0.7 g

Distilled water 1000ml

2.5.2 Anticoagulant

2.5.2.1. Ethylenediamintetraacitec acid EDTA

EDTA**(**supplied by BDH chemicals Ltd England) was prepared by adding 3.0 g to 100ml (0.7%) sodium chloride and was used at the rate of 1/10ml of blood.

2.5.2.2 Sodium citrate (2%):

It**(**supplied by Hopkin andWilliams England) was prepared by adding 2g of sodium citrate to 100ml distilled water,used at the rate of 1ml in 10 ml blood.

2.5.2.3 Sodium citrate (1%):

It was prepared by adding 1g of sodium citrate to 100ml normal saline and used at the rate of 1ml/10 blood.

2.5.2.4. Citrate saline

It was prepared by adding 50ml of 1% sodium citrate to 50ml normal saline

and used at the rate of 1ml/10blood.

2.5.3. Phosphate buffer

Solution A:

Na_2HPO_4 (anhydrous) 14.2 g

Distilled water. 1000 ml

Solution B:

NaH_2PO_4 $2H_2O$ 15.6 g

Distilled water 1000 ml.

2.5.4. Preparation of Stains

2.5.4.1. Giemsa solution

Giemsa stock solution(Supplied by H.D.UK) 1 ml

Phosphate buffer 9ml

(PH 7.0 - 7.2).

2.5.4.2 Crystal violet

Crystal violet:(supplied byRiedel-DE Haen AG-Seelze-Hannover (Germany) 10g

Distilled water 100ml

2.5.4.3. Carbol fuchsin

Basic fuchsin 1g

Absolute ethanol 10ml

Distilled water 100ml

Phenol.

5g

2.5.5 Methods of staining

2.5.5.1 Giemsa staining

Smears were fixed with methanol for 3-5 minute. The slides were placed in 10% Giemsa solution, for 20 -30 minutes, Rinsed in water or buffer and blotted dry.

2.5.5.2 Grams staining

Smears were fixed by flaming, stained with 0.5% crystal violet for 2 minutes and Rinsed with water the smears were then flooded with Lugols iodine and decolorized with absolute ethanol for 15 seconds and rinsed with tap water. The smears were counter stained with carbol fuchsin for 15 minute washed and blotted dry.

2.5.5.3 Crystal violet staining

Smears were fixed with methanol for 5 seconds. The slides were then stained with 10% crystal violet for 15 minutes, rinsed in tap water and blotted dry.

2.6 Preparation of blood smears

2.6.1 Stained smears

Smears were made on clean grease free microscopic slides cleaned by immersing in spirit and rinsed in water then wiped with soft cloth or

tissue paper.Two thin blood smears were made from the wing vein of each bird and air dried .

2.6.2 Wet smears

Drops of peripheral blood from the wing veins of infected birds were placed on cover slips which were inverted gently on a glass slide.

2.7. Sterilization

2.7.1. Glassware equipment

All glassware were boiled and washed with soap and water then soaked over night in distilled water. They were sterilized by autoclaving for 30 minutes at 15 pounds pressure/sq inch or in the hot air oven at 160˚C for 90 minutes.

2.7.2. Dimethylsulphoxide

It was filtered by 0.22 μ Millipore filter.

2.7.3 Glycerol.

This was sterilized by autoclave for 30 minute at 15 pounds/ sq inch

2.7.4 Sodium citrate

It was sterilized by autoclaving for 30 minutes at 15 pounds/sq inch.

2.8. Preparation of Infected materials

2.8.1. Infected blood

Infected birds were aseptically bled at the peak of spirochetemia by cardiac

puncture using10 ml sterile disposable syringes. Blood was collected in

tubes containing EDTA and sodium citrate respectively. The tubes were shaked gently to ensure proper mixing with the anticoagulant. Blood was then distributed in 1 ml aliquot sterile tubes and stored at 4˚C till used or in liquid nitrogen for long time storage.

2.8.2. Infected serum.

Infected birds were aseptically bled at the peak of spirochetemia by cardiac puncture using 20 ml sterile disposable syringes. The blood was immediately delivered into one or more sterile test tubes and allowed to clot at room temperature .After 30 minutes, the firm clots was separated from the walls of the tubes with sterile Pasture pipettes. The tubes were kept for 12 hours in the refrigerator before sera were collected in sterile containers in aliquot of 1 ml tubes and stored at 4˚C and in liquid nitrogen till required.

2.8.3. Infected blood clot.

Infected birds were aseptically bled at peak of the disease by cardiac puncture using 20ml sterile disposable syringe. The blood was collected into tubes allowed to clot at room temperature for 30 minutes then the firm clots were separated from the wall of the tubes with sterile Pasteur pipettes.The clots and sera were placed in sterile Petri dish then cut transversely into discs about 0.2 cm thick with sharp sterile scissors and each disc was cut in a halves through its depth and stored at 4˚C till

used. Each half of blood clot produced in this manner provided an approximate amount of 0.5 g of infected material (Gorrie 1950).

2.8.4 Infected plasma

Infected plasma were prepared by centrifugation of infected citrated blood at 500 rpm for 15 minutes. Plasma were pipetted out in sterile containers and stored at 4˚C and used as inoculums.

2.8.5 Infected ticks

Ticks used in this study were collected from farms with previously diagnosed spirochetosis.

2.8.6 Tick homogenate

Ticks were crushed in a small amount of normal saline in a pestle and mortar, centrifuged at 1500 for 10 minutes and the supernatants were then collected and used.

2.8.7 Intestinal content

Fecal samples were collected from the cloacae of infected birds at the peak of infection in sterile vials and stored at 4˚ C.

2.9 Pathogenicity of *Borrelia anserina*

Four local isolates (Soba1, Alkadaro, Taiba and Butri), 3 experiments were conducted to study pathogenicity of *Borrelia anserina* in the three different age groups of chicken ,chicks (0-1 month),grower (2-5month) and adults (5-9months). Different

parameters were recorded which were incubation period, clinical signs, spirocheteamia, histopathological changes and postmortem lesions . Isolates were maintained in 8 weeks old apperantly health susceptible chicken .At the peak of spirochetemia, as detected by blood smears, blood was collected aseptically from the heart. Four types of inocula were prepared from each isolate, and used for different groups. Each inoculum was injected by at least four different routes in birds of the same group**.** Infected materials and doses were determined according to Gorrie (1950) Perk.*, et al* (1966), WadDalkar and Soni, (1982) Hovind –Hougen k (1995). Types and doses of inoculums and routes for the three age groups were illustrated in table (3,4 and 5).

Each field isolate were inoculated in a different group of birds in separate cage at specific time while other isolates where not in use in order to avoid cross infection. Control groups were kept in separate cages. Each age group was kept in a separate cage. All experimental birds were fed a commercial ration according to their age.

2.9.1 Experimental infection of chicks

One hundred and thirty (2- 4 week old) susceptible chicks were divided into five subgroups. Group 1 (S/C): Consisted of 30 chicks divided into3 subgroups each of 10 birds each subgroup was infected subcutaneously with citrated blood, serum and plasma. Group 2(I/M): Consisted of 30 chicks divided into 3 sub groups each of 10

birds each subgroup was infected intramuscularly into the pectoral muscles with citrated blood, serum and plasma.

Group 3(Intranasal): Consisted of 30 chicks divided into 3 subgroups each of 10 infected intranasally by dropping with citrated blood, serum and plasma.

Group 4(I/ocular): Consisted of 30 chicks which were divided into 3 subgroups each of 10 birds. each subgroup was infected ocularly by dropping with citrated blood, serum and plasma respectively. Group 5(C) consists of 10 chicks as uninfected controls. These protocols were applied for the two test isolates, Soba 1 and Butri.

All birds were examined daily for any clinical signs, presence of *Borrelia anserine* in the peripheral blood and mortality .

2.9.2 experital infection of growers

One hundred and ten(2- 5 month old) susceptible apparently healthy pretested growers were divided into five groups designated according to the route of infection as follows:Group 1 (S/C): Consisted of 30 growers divided into 3 subgroups each of 10 . Each subgroup was infected subcutaneously with citrated blood, serum or plasma.Group 2(I/M): Consisted of 30 growers divided into 3 subgroups of 10 each. Each subgroup was infected intramuscularly with citrated blood, serum or plasma. Group 3(Intravenous) consisted of 30 growers divided into 3 subgroups of

10 each. Birds in each subgroup were infected intravenously with citrated blood, serum or plasma.Group 4(orally) consisted of 10 growers infected orally with blood clots.Group 5(C) consisted of 10 growers and kept as uninfected controls.Two test isolates; Soba1 and Alkadaro were used to infect growers.

All birds were examined daily for clinical signs and presence of *Borrelia anserina* in peripheral blood and mortality.

2.9.3 Experimental infection of Adult Chicken

One hundred and ten(6- 8 month old)susceptible pretested birds were divided into five groups, designated according to the route of infection as follows.Group 1 (S/C) consisted of 30 birds divided into 3 subgroups of 10 each birds in this group were infected subcutaneously with citrated blood, serum or plasma.Group 2(I/M) consisted of 30 birds divided into 3 sub - groups of 10 each. Birds in this group were infected with citrated blood, serum or plasma in the pectoral muscles.Group 3(Intravenous) consisted of 30 birds divided into 3 subgroups of 10 each birds were infected intravenously in the wing vein with citrated blood, or serum and plasma .

Group 4(orally): Consisted of 10 birds, which were infected orally with infected blood clots.

Group 5(C) consisted of 10 birds which were kept as uninfected controls.

Infective materials of two isolates Soba 1 and Taiba were used to infect birds. All birds were examined daily for clinical signs, presence of *Borrelia anserina* in peripheral blood and mortality.Spirocheteamia was monitored by examination of fixed smears stained with crystal violet and wet smears from each bird from day 2 till day 10 post infection. At least 10 microscopic fields were examined before considering the bird negative for *Borrelia.*

Three days post infection two infected birds from each group and two controls birds were killed and postmortem was conducted any changes in Lungs, livers, spleens, intestines, brains and hearts were recorded and the infected organs were removed and fixed in 10 % formalin. Paraffin sections thickness (5μ) were made and slides were stained with haemotoxylin eosin. Analysis of variance (ANOVA) was performed to verify the results.

2.9.4 Experimental infection byTick feeding

Birds: A total of 40 susceptible chicks of different age were used in this experiment. Ten chicks, 10 growers and 10 adults, Ten more growers were kept as a control group. Stained blood

smears were prepared from each individual bird stained and examined to exclude positive cases.

Ticks: Starved adult ticks were selected for this experiment. They were collected from farms with recently or previously diagnosed spirochetosis. Species of ticks and their sex and stage of development were recorded.

Experimental plan: each subgroup of birds(chicks ,grower and adult) were placed in a tray in a dark room plastic tube open at both ends was fixed over the slightly stretched ventral side of a bird wing.

Ticks were then dropped inside the tube. After 10-15 minutes some of the ticks fixed themselves and began feeding which usually continued for 30 minute then drop off.

Birds were then kept in separate pens. Smears from the birds were examined daily for *Borrelia anserina* for 15 days post infection.

The birds were considered infected on the first appearance of the organism in blood smears.

2.10 Propagation and serial passage of *Borrelia anserina* field isolates

Passage of field isolates was performed in three different host system which included embryonated eggs, day- old chicks and 4-

6 month old chicken. The four field isolates, Soba 1, Alkadaro, Butri and Taiba were used in this study.

2.10.1. Embryonated eggs

Chicken embryos 5-13 day old were inoculated through different routes namely yolk sac, chorioallantoic membrane and allantoic cavity with infected materials. Eggs were injected with either 0.1 ml of infected serum of each isolate diluted with an equal volume of normal saline or with 0.2 ml of infected citrated blood (Gorrie, 1950., Hart, 1970). Inoculated embryos were observed for mortality and examined for gross lesions and histopathological change. The quality and quantity of the harvest was evaluated according to purity and number of live motile organism per microscopic field- using wet preparation in relation to the inoculums and route of inoculation.

2.10.1.1 Yolk sac route

Chickens embryos of two different age groups 3-5 day-old embryo and 11-13 day- old embryo were inoculated by this route(Dennis,1998)

Group (1) consisted of 30 egg embryo age 3-5 day which were divided into three subgroup. Subgroup A of twelve eggs was inoculated with infected serum

Subgroup B of 12 eggs were inoculated with infected citrated blood and subgroup C consisted of 6 eggs which were kept as uninfected controls.

Group (2) consisted of 30 egg embryos divided into 3 subgroups.

Subgroup A of 12 eggs were inoculated with infected serum, subgroup B of 12 eggs were inoculated with infected citrated blood and subgroup C consisted of six eggs were kept as uninfected controls.

All embryos were candled before inoculation and the site of inoculation

was marked. The area over the air sac was disinfected with 70% alcohol.

A small hole was made through the egg shell along the central axis at the top of the egg .22-gauge 1- inch needle was used for inoculation by inserting the needle vertically to its full length. The hole was then sealed with melted wax. The same plan was repeated for the four test isolates. Thirteen passages were performed for each isolate.

2.10.1.2 Chorioallantoic membrane route

Thirty egg embryos age 11-13 day-old were inoculated by this route(Dennis,1998). Eggs were divided into 3 subgroups

;subgroup (1) of 12 eggs was inoculated with infected serum , group (2) of 12 eggs was inoculated with infected citrated blood and group (3) of 6 eggs was kept as uninfected control.

Eggs embryos were candled before inoculation and the sites of inoculation were marked, each egg was disinfected with 70% alcohol and a false chorioallantoic membrane was prepared at a blood vesel free region at one side of the egg. A hole was made through the shell at the center of the air sac and another hole was drilled on the side of the egg. By applying gentle vacuum to the hole at the air sac a new false chorioallantoic membrane was formed. The hole over the air sac was then sealed. A 25 gauge 16 inch needle was used to deliver 0.1 – 0.3 ml of inoculum per egg.

2.10.1.3 Allantoic sac

Thirty egg embryos 8 – 11 day old were divided into 3subgroups;

subgroup (1) consisted of 12 eggs was inoculated with infected serum

subgroup (2) of 12 eggs was inoculated with infected citrated blood and

group (3) of 6 eggs was kept as uninfected control.

All eggs were candled before inoculation to exclude unfertile eggs. The sites of inoculation were marked over the air sac and

disinfected with 70 % alcohol. A small hole was made through the egg shell above the air sac. A 25- gauge ⅝ inch (16mm) needle was used for inoculation by inserting it vertically to its full length (Dennis,1998) . The same plan was repeated for the four isolates and thirteen passages were made for each test isolate. Inoculated eggs were then incubated at 37°C with humidity for 7-10 days. Non specific deaths which occurred within 24 hours of inoculation were discarded. Deaths due to infection occurred 5-9day post inoculation. Embryos that died during this period were stored at 4˚C till harvested.

2.10.1.4 Harvest

Seven days post inoculation all dead and live embryos were opened (For collecting samples to the next passage it is essential that embryos were still alive).*Borrelia anserina* isolates were subjected to 13 serial passages

The surface of the eggs was sterilized by swabbing with 70% alcohol and

flaming .The shells over the air sac was peeled off and the shell membrane was removed with sterile forceps.

Chorioallantoic membranes were then ruptured with sterile forceps. This caused bleeding of the vessels into the chorioallantoic fluid. After few seconds, the bloody

chorioallantoic fluid was aspirated using sterile disposable syringes. Embryos were consider infected according to presence of *Borrelia anserina* in the fluid and these were detected microscopically in stained and tested for viability in wet smears. Fluids containing 2 – 20 organisms per field were liquated in two a ml vials and stored till used undiluted for the next passage. Embryos were hooked around the neck and transferred to sterile Petri dishes for clinical and post mortem examination. The spleens, livers and kidneys were collected from each embryo and fixed in 10% formalin for histopathological examination.

2.10.2 Propagation of *Borrelia anserina* in Day-old-chicks.

Serial passages of *Borrelia anserina* isolates in day –old chicks were done as follows: Thirty one-day-old chicks were divided in three subgroups of 10 each. Chicks in subgroup (1) were inoculated subcutaneously with infected citrated blood prepared from Soba 1 field isolate. Chicks in subgroup (2) were infected s/c with citrated blood prepared from Taiba field isolate, and chicks in subgroup (3) were kept as uninfected controls.

Chicks were observed daily for clinical signs. Blood smears were examined daily from day two post infection till death. At the peak of spirochetemia blood were collected and used as inoculum

for the next passage. Ten serial passages were performed for each two isolate. Immediately after death of infected chicks post mortem examination were performed and organs were collected for histopathological examination.

2.10.3 Propagation of *Borrelia anserina* in Six – eight - month old birds

Two isolate were used in this study Soba1 and Butri isolates. Seventy birds were used for each isolate in this experiment. Ten chickens were used for each passage; the first group of 10 chickens was infected with citrated blood prepared from the two isolates. Chicken were observed daily for clinical signs and blood smears prepared from each individual bird were examined. At the peak of spirochetosis blood was collected aseptically, examined for presence of the organism and used as inoculum for the next passage.

2.11 Infectivity of *Borrelia anserina* in chicken after passage in chick embryo

This experiment was conducted to determine the effect of serial passage on the virulence of the organism. It was performed by infecting growers with infected bloody chorioallantoic fluid collected from passage 3, 7 and 13 of Soba 1 and Alkadaro isolates in egg embryos. Each passage was injected s/c into a

group of 10 growers. Birds were examined for clinical signs, parasitemia, mortality and post mortem lesions.

2.12 Preservation of *Borrelia anserina*

The organism was preserved in 6 preparations in this study. Those are serum, citrated blood, plasma, buffy coat, embryonic materials and organs.

2.12.1 Serum

About 15 ml of blood were collected aseptically by cardiac puncture from experimentally infected birds at the peak of spirochetemia. The blood was distributed in three different 10ml tubes, and allowed to clot at room temperature for two hours followed by 4 hours at 4˚C and one hour at 37˚C. Sera for each isolate were pooled and kept for 2 hours at 4˚C then transferred to fresh tubes and stored for 30 minutes at 4˚C.The method of (Labruna *et al*., 1999) was followed.Sera containing vigorously motile organisms were divided into 4 aliquots which were subjected to different procedures. Sterilize glycerol was added to give a dilution of 1:2 to the first aliquot which was labeled as GS, to the second aliquot an equal volume of 15% glycerol was added and labeled as 15%GS. The third aliquot were mixed with dimethylsulfoxide (DMSO) at the dilution of 1:10

and was labeled as 10%DS. The last aliquot was kept without a stabilizer and was

labeled S. Each preparation was distributed in 2ml in cryotubes and stored at 4˚C, -20˚C and -196˚C (liquid nitrogen).

After 3 month of uninterrupted storage two cryotubes of each preparation were removed, thawed in water bath at 25˚C and motility of *Borrelia anserina* was immediately observed in wet smears preparation.

The content of each tube was then mixed with 1 ml citrate saline and injected in both chicken and chick embryos. Three 5-8 month old chicken and three 11- 13 day -old embryos were used for each preparation.

2.12.2 Citrated blood

Blood was taken aseptically from experimentally infected chicken at the peak of spirochetemia , mixed with an equal volume of 1% citrate saline and kept for 3 hours at 4°C .It was then divided into two portions .To one portion an equal volume of sterile 10% glycerol was added gradually, then distributed in cryotubes ,labeled and stored at 4°C, -20˚C and in liquid nitrogen.

The second portion was kept without a stabilizer and was stored at 4˚C, -20˚C and in liquid nitrogen.It should be stated that the preparations preserved in liquid nitrogen were first kept

for 1 hour at room temperature then for 20 minutes at -20˚C then held in liquid nitrogen vapor, for 36 hours before being mersed in liquid nitrogen (Hart 1970).

At 3 month intervals, 2 cryotubes from each preparation were removed thawed under running tap water and examine microscopically for motility of the organism, both after staining and as wet smears.

2.12.3 Preparation of plasma

Citrated blood was collected by cardiac puncture in sterile test tubes and centrifuged at 1500 rpm for 15 minute. Plasma was pipetted off and checked for the presence of motile organism.

The plasma was divided into two portions; one was kept without stabilizer and the other was mixed with DMSO to give a dilution of 1:10 before being divided into aliquots which were labeled and stored in 4˚C,-20˚C and in liquid nitrogen.

After 3 months of uninterrupted storage, 2 cryotubes of each preparation were removed, thawed and examined for motility by wet smear.

2.12.4 Buffy coat

The buffy coat was collected from fresh blood of infected birds and was centrifuged at 1500 for 15 minute. It was aspirated

and diluted with normal chicken serum to give a dilution of 1:5 and stored at 4˚C,-20˚C and in liquid nitrogen without a stabilizer.

2.12.5 Yolk and chorioallantoic fluids

Infected yolk and chorioallantoic fluids were collected from inoculated egg embryos 7 days post inoculation and were stored at 4˚C, -20°C and in liquid nitrogen.

2.12.6 Organs

Spleens**,** livers and intestines were collected at post mortem from experimentally infected birds and stored at 4°C, - 20° and in liquid nitrogen.

2.13 Experimental infection of local breeds

Thirty apperantly healthy adult chickens of local breeds were used in this experiment. The chickens were divided into 3 subgroups each of 10 birds. The birds were infected with citrated blood prepared from Soba 1 field isolate.Birds in subgroup 1 were infected subcutaneously, those in subgroup2 were infected I/M and birds in subgroup 3 were kept as uninfected control.

Infected birds were kept in separate pens and clinical signs of spirochetosis and presences of *Borrelia anserina* in the peripheral blood were monitored daily.

2.14 Experimental infection of pigeons

Soba 1and Butri field isolates were used to infect pigeons. Thirty adult pigeons were used for each isolate. They were obtained from Khartoum North market and were divided into three subgroups each of 10 pigeons birds in subgroup1 were infected with citrated blood S/C. Subgroup2 were infected with citrated blood I/M . Pigeons in subgroups 3 were kept as uninfected control.

Clinical signs and presence of *Borrelia anserina* in peripheral blood were monitored daily from day 2 post infection for 15 days.

2.15 Cultivation of *Borrelia anserina* in bacteriological media

Attempts to grow *Borrelia anserina* in artificial media were made using blood agar base with 10 % defibrinated chicken blood and rabbit serum.

2.16 Oral infection using tick homogenate.

Homogenate of infected ticks which had been fed in infected chicken were used to infect ten chicks, 10 growers and 10 adults. Infected ticks were crushed in small amount of normal saline in a pestle and mortar, and then distributed in Petri dishes in chicken pens.

2.17 Comparative studies on spleen weights

Spleens of naturally and experimentally infected chicken, collected 5-6 days post infection in addition spleens from noninfected chicken were used as a control,Spleens were collected after post mortem examination and weighted.statistical analysis was performed to verify the results.

2.18 Passage of *Borrelia anserina* field isolate

Continuous passage of Butri field isolate was made in 3 chickens 2-8 months old per passage. Passage was made s/c every 5 days using infected citrated blood. Birds were examined daily for clinical signs and spirochetemia .

2. 19 Serology

Serological tests plate and tube agglutination test and immobilization lysis test were performed to identify antigenically different isolates and for diagnosis of the disease after disappearance of the organism from blood. Four field The isolate were used (Soba1, Butri, Taiba and alkadaro).

2.19.1. Preparation of agglutination antigen

According to Mehta and Muley (1968), Verma and Malik (1968) blood was collected from experimentally infected birds on day 4-5 post infection in sterile 2% citrate saline. It was diluted in 1:2 with normal saline, mixed and centrifuged at 500 rpm for

5 minute. The supernatant which contained the organism was collected in clean sterile tubes. The sedimented red blood cells were resuspended in normal saline and centrifuged at the same speed and time 4-5 times to collect more organisms. All supernatants were pooled centrifuged at 3500 for 15 minutes and deposit was resuspended. 0.5% phenol saline was added gradually to adjust the opacity of the suspension to match Mc Farlands opacity tube No3 (Chatterjee and Sawhney 1971) for plate test antigen, antigens without visible floccules were used. On the other hand McFarland's tube No 6 was used for the tube agglutination test. Finally the plate agglutination antigen was colored with 1% alcoholic solution of crystal violet to give the final concentration 0.03% of crystal violets.

The whole dead cell antigens, for the 4 field isolates were prepared separately as above.

2.19.2 Preparation of immobilization antigen

The antigen was separated from citrated blood from infected chicken by centrifugation and the sedimented organism was resuspended in normal chicken serum .This contained live organism which constituted the immobilization antigen (Mehta and Muley; 1968). Antigen for each of the 4 isolate was prepared separately.

2.19.3. Preparation of hyperimmune sera

Antiserum against each isolate was prepared by injection of 0.2ml of infected citrated blood into ten 4-6 month old chicken intramuscularly. Four days post infection, birds were injected with 20.000 units of penicillin and on the following day no live spirochetes were seen in wet smears. Two booster injections of 1 ml each of infected blood were given on days 19 and 21 with the respective field isolate. Birds were bled on day 31 and 32 and 35 for the immune serum (Chatterrjee and Sawhney 1971).Hyperimmune sera were prepared against the 4 field isolate Soba1, Butri, Taiba and alkadaro.

2.19.4 Preparation of Convalescent serum

Twenty adult and 20 chicks were injected I/M with infected citrated blood and 3 days post infection, 20.000.units of penicillin was given to all birds. Twenty one days post infection the chickens were bled and the convalescent sera were separated .Convalescent sera were prepared against the 4 field isolates Soba1, Butri, Taiba and Alkadaro separately.

2.19.5 Plate agglutination test

One hundred and thirty sera were tested which consisted of 20 convalescent and 10 hyperimmune sera against each isolate.

The test was performed by mixing 2 drops of the stained dead antigen with one drop each of homologous antisera, heterologous antisera and negative control serum. The plates were then rotated for seconds in a circular manner; agglutination was observed in oblique light and recorded as positive when definite clumping occurred. Positive reaction appeared within 10-15 second in the form of distinct clump, the mixture remained uniformly cloud in negative reaction.

2.19.6 Tube agglutination test

A total of one hundred and seventy sera were tested, which consisted of 20 convalescent sera, 20 antisera aganist each isolate and 10 negative control sera. In addition the first appearance of agglutination after infection was determined in a group of 20 adult birds. Sera were collected at day 6,8,11 post experimental infection.

To determine the antibody titers serial 2 fold dilutions for tested sera were made in normal saline from 1:2 to 1:1024 One drop of sera of each dilution was placed on a glass plate using pasteur pipette mixed with an equal amount of antigen. Agglutination reaction with normal sera and normal saline were observed throughout the experiment as a control. The end point

was taken as the highest dilution showing positive reaction. Agglutination was observed in oblique light.

2.19.7 Immobilization lysis test

The test was done by mixing two drops of live antigen and one drop of homologous or hetrologus serum in separate slides. Cover slips were placed over the mixture and the slides were examined using the dark field microscope for death, clumping and lysis of the organism.

2.20 Cross Protection Study of the four field isolates in susceptible chicken

The 4 field isolates were used in this experiment. Twenty chickens 4 month old were infected with each isolate with 0.2ml of infected blood intramuscularly. Birds were treated with penicillin 3-4 days post infection to kill the spirochetes. Recovered birds infected with isolates were divided into 4 groups. The second exposure was performed 10 weeks after the first infection.

Birds infected with Butri isolate were divided into subgroups each of 4 birds each then challenged after 2 month with 0.2 ml infected blood of each Butri, Soba1, Taiba and Alkadaro isolates. Those infected with Soba1 isolate were challenged with 0.2ml of infected blood of each Soba1, Butri, Taiba and Alkadaro isolates,

birds initially infected with Taiba isolate were challenged with 0.2 ml of infected blood of Taiba, Butri, Soba1 and Alkadaro field isolates on the other hand Alkadaro recovered birds were challenged with 0.2ml of infected blood of Alkadaro, Soba1, Butri and Taiba field isolate. Table (6). Twenty grower Chickens were used as uninfected control group. Cross protection against challenge was evaluated during an observation period of ten days by recording motility,spirochetemia and post mortem lesions.

Chapter **3**

Results

Results of the parameters assessed the pathogenicity of *Borrelia anserina*

3.1 Incubation period

Four field isolates, five different routes of inoculation and four inoculums were used in this experiment. The incubation period (IP) was determined by the first detection of *Borrelia anserina* in peripheral blood as a mean in days (Table 7). Three age group were treated with the different isolates. Soba 1 isolate gave the shortest (IP); 1.3 days when given by I/V route in growers (Table 7) as where the longest IP was 9.3days when given by the oral route in adults (Table 8). The shorter (IP) for Butri isolate was 2.5 days when given by S/C route and the longest was 4.8 when given by I/O route in chicks (Table 9). For Alkadaro isolate, the I/V route gave the shortest (IP) which was 3.1 days and the longest was 8.6 days when given orally(Table 7) .For Taiba isolate the shorter IP was 2.1 days when given by I/V route and the longest was 6 days after I/M injection (Table 7).

On the different age groups, the shortest and longest IP were observed among adults (2.6 and 9.3 days) respectively. The shortest I P (2.6day) was observed when blood was used as inoculums and the longest (9.2days) when blood clot was used (Table 8). Of the different routes of administration I/ route gave the shortest incubation 1.9day whereas the longest (9.3days) was recorded after oral infection (Table 9).

The IP was variable in various age groups for the different isolate.

3.1.1 Soba1 isolate

Chicks

Among this age group three inoculums were used citrated blood, serum and plasma via 4 routes of inoculation. The IP was found to range from 2-3 days post infection when s/c route was used. The period ranged from 2-5 days and 3-5 for I/M and I/O route respectively. The shortest I P was recorded with citrated blood (2.3) days compared to serum (5 days) and plasma (3 days) of the same infected bird sample. Means of the I P were recorded in Table (9).Experimental infection via nasal route failed to produce the disease (Table 10).

Growers

The same inoculum was used for this age group, but the oral and I/V routes were used in stead of I/O and I/N routes that were used in chicks. The mean of IP was 2.4 days after inoculation via S/C route, 4 days, and 1.3 day for I/M and I/V routes respectively. The oral route gave the longest IP (9days).

Regarding the inoculums, citrated blood showed the shortest period (1.3) days, while 2.5 day and 1.7 day were recorded for serum and plasma respectively. Blood clot gave the longest IP (9 days) post infection (Table 11).

Adult

The same routes of inoculation were used as in the growers .The mean of IP was 1day for I/V route and the longest was 9.3 for oral route. Citrated blood gave the shortest IP which was 1.4 day while the longest was 9.3 days (Table 12) produced by oral route .

3.1.2 Butri isolate

The IP of this isolate was studied in chicks using different inoculums and various routes for administration as those used for Soba1 isolate. The period ranged from 2.5 days by S/c route,and 3 and 4.8 day by I/M, I/O routes respectively. Citrated blood had the shortest I P (2.5) days , while adults inoculated with infected serum and plasma showed a longer IP 4.5 and 3.3 days (Table 10).

3.1.3Alkadaro isolate

The same route of administration and inoculums were used but in grower chicken . The IP was ranged from 3.1-8.5 days .It from 5.5 and 5-8 days for I/M, 5.3 days for s/c and 8.5days by oral route .Citrated blood and plasma had a similar short IP which was 3 days. Blood clot showed the longest IP (8.5 days). Means of (IP) were recorded in Table (11).

3.1.4Taiba Isolate

As with the above isolates same routes and inoculums were used, but in adult chicken. by S/c route the I P was 3 days, by I/M and I/V the period was 4 and 2 days respectively. Citrated blood gave the shortest period of incubation ;2 days followed by plasma 2.7 days and finally serum 3.3 days (Table 12).

Comparison between the means of (IP) after infection with the four local field isolates showed statistical significant difference ($p<0.01$) between Alkadaro and the 3 other isolate. When the effect of the different inoculums was studied significant difference ($p<0.05$)was found between the blood clot and serum, plasma and blood clot, blood and blood clot .No significant difference was found between plasma and citrated blood. Regarding routes of administration No significant difference was found between S/C, I/M and I/O, but they all significantly differed ($p<0.05$) from I/V and oral route (Table 13).

3.2Clinical signs

The clinical signs were investigated after infection of the three age group with the 4 field isolates .The least percentage of birds showing clinical signs were recorded in chicks infected with Soba1 15/120(12.5%) followed by Butri 16/120(13.3%), while 72/100(72%) of growers infected with the Soba1 and 58/100 (58%) with Alkadaro isolate showed clinical signs . The numbers of adult chicken showing clinical signs after infection with Soba1 and Taiba isolate were 80/100(80%) and 52/100 (52%) respectively (Fig 2). Chicks infected with Soba1 isolate showed no rise in temperature while the growers started showing slight rise in temperature during the course of the disease which ranged from 2-6 days.

On the other hand, adults infected with both soba1 and Taiba field isolate showed marked rise in body temperature 44˚C, which increased gradually to reach the peak on day 4-5 post infection. Regarding the other clinical signs, adult and growers showed various symptoms which included dullness, greenish diarrhea, drowsiness, ruffled feathers and paleness of the comb. Aggregates of nymphs ticks were usually found (fig 3) .Terminally infected birds showed disease nervous signs such as paralysis of wings and lateral recumbancy (fig4).The maximum mortality occurred in the late phase of the disease. Soba1 isolate infection in adult and growers gave severe clinical signs. Greenish diarrhea was observed in both age groups from day4 post infection (Table 14).Number of birds showing clinical signs after experimental infection were shown in appendix(1-6).

3.3 Post mortem lesions

In all experimentally infected birds the characteristic pathological lesion observed was enlargement and mottling of the spleen with observable difference between age group infected with different field isolate (Fig 5, 6).

Fragile liver, pale carcasses, pale kidney and enteritis were also observed among growers and adults (Table 14).

3.4 Histopathological changes

Result were shown in Fig7-10 spleens of experimentally infected chicken with *Borrelia anserina* isolates (4-5 days post infection)showed areas of necrosis and lymphatic infiltration (Fig 7). In advance cases necrotic foci were increased in number and size resulting into typical mottling appearance. Vascular system of the organ showed thickens of the capillary walls and infiltration with inflammatory cells.Splenic pulps were densely packed with lymphocytes (Fig 8).

Histopathology of the brain tissue showed infiltration of mononuclear cells in the subventricular area (Fig 9). Intestine of experimentally infected birds showed catarrhal enteritis, slight congestion in the vein of lamina propria With slight lymphocytic infiltration, and desquamation of the epithelial cells lining the mucosa were common findings. The submucosa showed a generalized lymphocyticic infiltration (Fig10).

3.5 Spirochetemia

The presence of *Borrelia anserina* in the peripheral blood was detected by examination of stained blood smears under the microscope fig(11).*Borrelia anserina* appeared in peripheral blood 2 -3 days post infection then the number of organism increased gradually and the peak of spirochetemia was detected 3-5 days. Clumping and disappearance of *Borrelia anserina* from the circulation were detected 5-12 days post infection. Duration of each phase was variable in birds of different age groups. Numbers of organisms in peripheral blood of birds infected with the four field isolate were recorded (Appendix18-23) which was calculated as a mean number of organisms per10 microscopic fields.

Phases of spirochetemia apperance(peak, clumping, lysis and complete disappearance from the blood) when studied in chicks, growers and adults infected with the 4 filed isolates revealed variable results.

During this study the highest countable number (900 organisms) was detected in adults infected with Soba1 isolate, 4days post infection. The results were illustrated in figures assuming numbers for the four phase as follows (2000) uncountable, (1000) clumping, (500) lysis and (100) for complete disappearance of organism from the circulation.

3.5.1 Spirochetemia in chicks

Chicks were experimentally infected with Soba1 and Butri isolates. The organism appeared in peripheral blood 48 hours post infection in chicks infected with both isolates. Peak of spirochetemia was recorded on day 3-7 post infection where the organism reached uncountable numbers under the microscope. This stage persisted for 4-6 days. Clumping began from day 8 onwards, but some actively motile organisms remained in the

circulation up to 10 days post infection. Both Soba1 and Butri isolates produced extensive spirochetemia even in apparently healthy chicks, which persisted for a long time in peripheral blood. Complete disappearance of *Borrelia anserina* from blood was noticed on day 15 post infection(Fig 12).

3.5.2 Spirochetemia in growers

Growers were experimentally infected with Soba1 and Alkadaro isolates. Birds showed a marked rise in body temperature during spirochetemia.*Borrelia anserina* appeared in the peripheral blood 2 days post infection and number of organisms increased gradually reaching a peak at day 3-4 for Soba1 and Alkadaro isolates. This phase lasted for 1-2 days then clumping appeared at day 5-6. Lysis and complete disappearance of *Borrelia anserina* from the circulation occurred 6-8 day post infection.Soba1 field isolate showed extensive multiplication of organism compared to Alkadaro isolate as shown in Fig 13.

3.5.3 Spirochetemia in adults

Phases of spirochetemia in this age group were similar to those seen in growers .Adult chicken infected with Soba1 and Taiba isolates showed a marked rise in body temperature. The rise of temperature was concurrent with *Borrelia anserina* concentration in peripheral blood. The organism appeared in the peripheral blood 2-3 days post infection and the number increased gradually reaching a peak at day 4 for soba1 isolate. Clumping of organisms appeared 5 days post infection in those infected with Soba1 isolate whereas lysis of the organism was observed on day 6 on word .On the other hand the number of *Borrelia anserina* at peak of spirochetemia in birds infected with Taiba isolate was the lowest among all birds examined. Lysis began at day 5 post infection; complete disappearance from the circulation was detected 7 days post infection (Fig 14).

Comparison of spirochetemia in the three age groups infected with Soba1 field isolate showed that rapid multiplication of *Borrelia anserina* appeared in the first 3 days of infection in all groups. Chicks showed delay in clump formation and long spirochetemia (Fig 15).

3.6 Propagation of *Borrelia anserina*

3.6.1 Propagation in chick embryos

The growth and infectivity of the 4 field isolates in chick embryos were studied using infected citrated blood and serum inoculated via 4 different routes (Fig 16).Growth and infectivity of the organism was determined by the presence of numerous motile viable organisms in embryonic fluids and membranes and the numbers of dead in shell embryos. Mean of mortalities were recorded in Table (15).

Borrelia anserina grew equally well when inoculated via yolk sac and allantoic cavity .Usually the organism was numerous in the embryos and embryonic membranes few organisms were found in the extra embryonic fluids. Congested blood vessels of the CAM with large numbers of organisms were detected. Inoculated embryos showed petechial hemorrhage, retarded growth and bad feathering (Fig 17). Splenomegaly was seen in all embryos inoculated with Soba 1 and Butri isolates. Embryos inoculated through dropped CAM showed edematous congested areas at site of inoculation and the organisms were detected in CAM vessels. Splenomegaly was not observed in embryos infected with Taiba and Alkadaro isolates. Control embryos showed no lesions on the CAM or enlargement of spleens.

Butri and soba1 isolate grew equally well when inoculated via the 4 different routes in chick embryos and it was observed that number of dead in shell embryos increased by passage.

Alkadaro and Taiba isolates were not fully adapted to grow in chick embryos as they showed low mortalities, absences of embryonic lesions and low multiplication of organism as shown in appendix 7-14(analysis of variance was performed to verify these results). Results showed that no significant difference was detected between the two inoculums (serum and blood) on the infectivity and mortality of chick embryos when inoculated via the 4 different routes. Both Soba1 and Butri isolates produced significant ($P<0.01$) increased in embryo mortality, than Alkadaro and Taiba isolates (Table16). Inoculation through Yolk sac (2), allantoic cavity and yolk sac (1) route showed significant increased mortality ($P<0.01$) than the CAM route.Althought significant difference ($P<0.05$) was detected between the 3 routes (Table, 16).

3.6.2 **Propagation of *Borrelia anserina* in one day old chicks**

Variable results were obtained when Soba1 and Taiba field isolates were inoculated in one day old chicks .Those inoculated with Soba1 isolate showed spirochetemia for as long as 2 weeks. The organism was usually found in large numbers in peripheral blood in the first 1-2 days post infection. The organism appeared thinner and shorter. Mild clinical signs were observed, chicks appeared weak with ruffled feathers and dropping wings in some birds. At necropsy slightly congested liver and enlarged spleens were seen. The number of birds showing spirochetemia remained constant during passages, reached (50%) in chicks infected with Soba1 isolate. Chick infected with Taiba isolate developed transient spirochetemia and *Borrelia anserina* appeared in the peripheral blood 2-3 days post infection. Spirochetemic chicks did not show clinical signs other than dullness and the number of chicks showing spirochetemia was lower (10%).Some infected birds recovered from spirochetemia in a week. The only lesion observed at necropsy was slight enlargement of the spleens.

3.6.3 Propagation in six-eight month old chicken

After the 22nd passage of Soba1 and Butri isolates in 6-8 month old adults, they produced an acute form of the disease and clinical signs and spirochetemia appeared 2-3 days post infection. Typical lesions of spirochetosis were observed including enlarged mottled spleens,anaemic carcasses and congested fragile livers. Both soba1 and Butri isolates were found equally potent and produced the disease in its acute form. Severe lesions in spleens were observed in birds infected with bacteria from the 3rd and 22nd passage (Fig, 18&19). Mortality of birds is shown in Fig (20).

3.6.4 Growth of *Borrelia anserina* in chicken after passage in chick embryos

The test was performed to evaluate the virulence of 2 field isolates (Soba1 and Alkadaro) after passage in chick embryos. Growers were infected with bloody chorioallantoic fluids collected from the 3^{rd} ,7^{th} and13th passage . Number of Growers infected with soba1 isolate that showed clinical signs were 8/10 (80%)for passage 3^{rd} , 9/10(90%) and 8/10 (80%) for the 7^{th} ,13^{th} passage respectively .Those infected with bloody fluid collected from 3^{rd} ,7^{th} and 13^{th} passage of Alkadaro isolate showed milder clinical signs the percentage were as follows 30%, 35% and 32% respectively.

3 7. Comparative study on spleen weight

Spleens were collected 5-6 days post infection from naturally and experimentally infected birds. In chicks comparisons were studied between non infected and experimentally infected ones as no natural outbreaks in chicks was recorded during this study.

Spleens weight of grower chicken infected with Soba1 and Alkadaro isolates are shown in table(17). Spleen weights increased significantly ($p<0.01$) on birds infected with soba1 isolate. Also a significant difference ($p<0.01$) was detected between infected experimental and natural infected birds and noninfected control (table 17a).

Significant difference was found between chicks and the other two groups (Grower and adults) infected with soba1 isolate with a higher mean among the adults. No statistical difference was found between naturally and experimentally infected ones, but there was a significant difference between noninfected and birds that were infected either naturally or experimentally.

Splenomegaly was observed in adult chicken infected with the three different field isolates Soba1, Butri and Taiba (Table 18). A significant increase in weight ($p<0.01$) was detected in spleens of birds infected with Butri isolate and those infected with both Soba1 and Taiba isolate. There was no significant difference between those infected with soba1 and Taiba isolate. When spleens of infected adult chicken were compared with those from non infected ones,there was statistical difference ($P< 0.05$) between spleens of naturally and experimentally infected one and non infected control, but no significant difference was found between spleens of naturally and experimentally infected adults chicken (Table 18 a). Spleen weights was studied in the three age group infected with Soba1 isolate fig (21) both adults and growers showed a significant increase ($P< 0.01$) than chicks. Significant difference ($P< 0.01$) was also detected between naturally and experimentally infected and non infected control adults and growers (Table 19 and 19 a).

3.8Tick feeding

This experiment was done to detect the ability of the soft ticks *Argas persicus* to transmit *Borrelia anserina.* The percentages of birds exhibiting clinical signs and /or spirochetemia were as follows 80 %(8/10) of chicks20% (2/10) of growers and 30% (3/10) of adults (Fig 22). The (IP) varied according to age from 5-9 days in chicks to 6-7 days in growers and 5-6 in adults.

The course of the disease in experimentally infected birds was similar to that in natural infected ones but the (IP) was long.

3.9 **Experimental infection in local chicken breed**s

The 30 birds used in this study were examined for 21 days post infection for the presence of *Borrelia anserina* in peripheral blood .None of them had detectable organisms in their blood or buffy coat,and no clinical signs or mortality were observed.

3.10 Experimental infection in pigeons

All inoculated pigeons were examined for 20 days post infection for the presence of *Borrelia anserina* in peripheral blood, none of the 60 pigeons had detectable organism in their blood or buffy coat,and no clinical signs or mortality were observed.

3.11 Preservation of *Borrelia anserina*

3.11.1 Liquid nitrogen

3.11.1.1 Infected serum

Borrelia anserina remained viable for 15 months in spirochetemic chicken serum stored in liquid nitrogen (Table, 20).Numerous organisms were observed under the dark field microscope immediately after thawing. When three chickens, 6-8 months old, were inoculated intramuscularly with the preserved materials, they developed typical spirochetosis and the organism was seen in peripheral blood (Fig 22).The organism was also seen in films prepared from embryonated eggs inoculated with the same material. Infected 10%GS (serum with 10% glycerol) showed zero motility when examined after 6 months of uninterrupted storage in liquid nitrogen. For confirmation of viability infectivity test were performed in 3 chicken, 6-8 months old. Only one bird showed transient spirochetemia (+) with few *Borrelia anserina* in the

peripheral blood 4 days post infection. Spirochete preserved in 50% glycerol(serum in 50% glycerol) showed zero motility when examined after months of storage in liquid nitrogen. Chicken inoculated with this preparation were negative to spirochetemia till 10 days post infection. Spirochete preserved in 10% DMSO (serum in 10% DMSO) retained viability and infectivity of *Borrelia anserina* after 9 months of uninterrupted storage in liquid nitrogen. Chicken used for infectivity tests developed the disease 3 days post infection (Fig 24).

3.11.1.2 Infected citrated blood

Infected citrated blood remained infective after 23 months of uninterrupted storage in liquid nitrogen and produced the disease in susceptible chicken with high spirochetemia(Fig 25).The organism remained viable and infective up to 6 months in citrated blood with 10% glycerol and 12 month in 10%DMSO (Table 20).

3.11.1.3 Infected plasma

Infected plasma frozen in liquid nitrogen remained viable and virulent for 6 months when 10% DMSO was added as preservative; 10% Glycerol maintained the organism for 3 month at liquid nitrogen , (Table20).

3.11.1.4 Infected embryonic materials

Infected chorioallantoic membrane kept *Borrelia anserina* live and infective for up to 6 month at liquid nitrogen .No motile organisms were observed after the first three months of storage of yolk and allantoic fluid in liquid nitrogen (Table, 21).

3.11.1.5 Infected tissue

Livers and spleens homogenates were found infective up to 3 months of storage at liquid nitrogen (Table ,21).

3.11.2 Cold preservation

3.11.2.1 Infected serum

At 4˚C the organisms were live and virulent after 3 months of storage in serum. After another 3 months no motile organisms were detected (Table 22).When infected sera were preserved at -20˚C, no motile organism were found when examined after the first 3 month of storage and none of the chicken inoculated showed signs of the disease.

3.11.2.2 Infected citrated blood

At 4˚C temperature spirochetes in citrated blood remained viable and infective up to 6 months (Tale, 22).Some of the red blood cells retained their shape and the majority of *Borrelia anserina* remained actively motile.

3.11.2.3 Infected plasma and buffy coat

Chilling at 4˚C of plasma and buffy coat preserved the viability and infectivity of the organism up to one month (Tables, 22 & 23).

3.11.2.4 Infected embryonic materials

Infected chorioallantoic membrane kept *Borrelia anserina* live and infective for one month at 4˚C. No motile organisms were obsereved after the first 3 months of storage of yolk and allantoic fluid in liquid nitrogen, but *Borrelia anserina* were viable for 2 weeks only when yolk and allantoic fluid were kept at 4˚C (Table 23).

3.11.2.5 Infected Tissues

Livers and spleens were found infective for up to 2 weeks when stored at 4˚C (Table, 23).

3.12 Serological Tests

3.12.1 Plate agglutination test

All the 20 adult convalescent and the 10 hyperimmune sera prepared from Soba1 isolate showed strong positive agglutination with the homologus antigen. Agglutination was also observed when the heterologus antigens of Butri, Taiba and Alkadaro isolates were examined against Soba1 sera separately. Both convalescent and hyperimmune sera to Butri isolate showed agglutination with the homologus antigen. Agglutination was also seen with Soba1, Taiba and Alkadaro antigen. All convalescent and hyperimmune sera prepared from Taiba isolate showed agglutination with the homologus antigen and when examined with the other 3 heterologus antigens of Butri, Soba1 and Alkadaro they gave similar results. Both convalescent and hyperimmune sera of Alkadaro isolate showed weak reaction with both homologus and heterologus antigens.Agglutinin appeared on the 6^{th} day post infection in 2 out of 20 birds as indicated by weak agglutination .On day 10 post infection of sera all birds gave strong positive reactions.

3.12.2 Tube agglutination test

Twenty hyperimmune and 20 convalescent sera against soba1, Butri, Taiba and Alkadaro field isolates were tested with their homologus antigens only. Ten percent hyperimmune sera of Soba1 gave a titer of 1:1024 ,75% sera gave a titer of 1:512 and the remaining 15% sera gave a titer of 1:256. On the other hand 65% of the convalescent sera gave a titer of 1:128 with the homologus antigen.

Ninety percent of sera of Butri isolate gave a titer 1:1024 and the remaining 10% gave a titer of 1:512. Convalescent sera showed lower titers than the hyperimmune sera; 50% gave a titer of 1:512 and 50% gave a titer of 1:256.

All hyperimune sera of Taiba isolate gave a titer of 1:256 while 60% of convalescent sera gave a titer of 1: 16 and 20% gave a titer of 1:64with the homologus antigen.

Fifteen percentage of Alkadaro hyperimmune sera gave a titer of 1:128 , 40% gave a titer of 1:64 an the remaining 45% gave titer of 1:32 . Convalescent sera of this isolate gave titers of 1:16 in10% of the tested sera.

Butri hyperimmune sera gave the highest titer of 1:1024 while Alkadaro convalescent sera gave the lowest titer 1: 16.Agglutinins appeared on the 6^{th} day post infection in one of the 15 birds examined, weak positive reaction, by day 8 all 15 birds showed strong positive agglutination reaction.

3.12.3 Spirochete immobilization test

The test was performed on slides with 20 convalescents and 10 hyperimmune sera prepared separately against each of the four fields isolate. Sera were examined with the homologous and heterologus live antigens. Ten sera from normal chicken were used as negative controls.

Both convalescent and hyperimmune sera against Soba1 isolate produced complete immobilization of Soba1 live antigen within few seconds, and also completely immobilized the heterologus live antigen of Butri, Taiba and Alkadaro isolates.

Both convalescent and hyperimmune sera against Butri isolate produced complete immobilization of Butri live antigen within few seconds,and also completely immobilized the heterologus live antigens of Soba1, Taiba and Alkadaro isolates.

Both convalescent and hyperimmune sera against Taiba isolate produced complete immobilization of Taiba live antigen within few seconds, also completely immobilized the heterologus live antigens of Soba1, Butri and Alkadaro isolates.

Both convalescent and hyperimmune sera against Alkadaro isolate produced complete immobilization of Alkadaro live antigen but a longer time has been taken (5-10minutes) to incompletely immobilize the heterologus live antigens of Soba1, Butri and Taiba isolates as detected by the presences of live organisms after 1 hour.

None of the normal sera were able to immobilize *Borrelia anserina* live antigens of the four field isolates.

3.13 Cross protection tests

The mortality rate (percentage) in birds infected and cross challenged and the control uninfected challenge birds with the four field isolates were recorded. Twenty five percent mortalities were detected in birds immunized by Alkadaro isolate and challenged with either Butri , Soba1 and Taiba isolates.Birds immunized with Taiba isolate when challenged with Soba1 or Butri isolates showed the same mortality rate (25%) .On the other hand no mortalities were recorded in birds immunized with Soba1 r Butri isolates when challenged with either of the four isolates. Deaths in birds challenged with any of the four field isolate were recorded only in Soba1 (12.5%) and Butri isolates (12.5%)(Table, 24).

Extensive spirochetemia (4+) was found in the control birds after challenge with Butri isolate. While (3+) spirochetemia was observed in control birds infected with Soba1 isolate. In birds immunized with Taiba or Alkadaro isolate (2+) spirochetemia was observed after challenged with soba1 and Butri isolate. In birds immunized with Alkadoro isolate 1+ spirochetemia was noticed after challenged with Taiba isolate and in the control group +1 spirochetemia occurred after challenge with Alkadaro isolate (table 24).

Splenic lesions were considered positive when enlargement and/or mottling were observed.Changes were observed in the uninfected control birds when infected with the four field isolates, and in birds immunized with Taiba and Alkadaro isolate and challenged with Soba1 and Butri isolate spleenic lesions were also seen in birds immunize with Alkadaro isolate and challenge with Taiba isolate (table, 24).

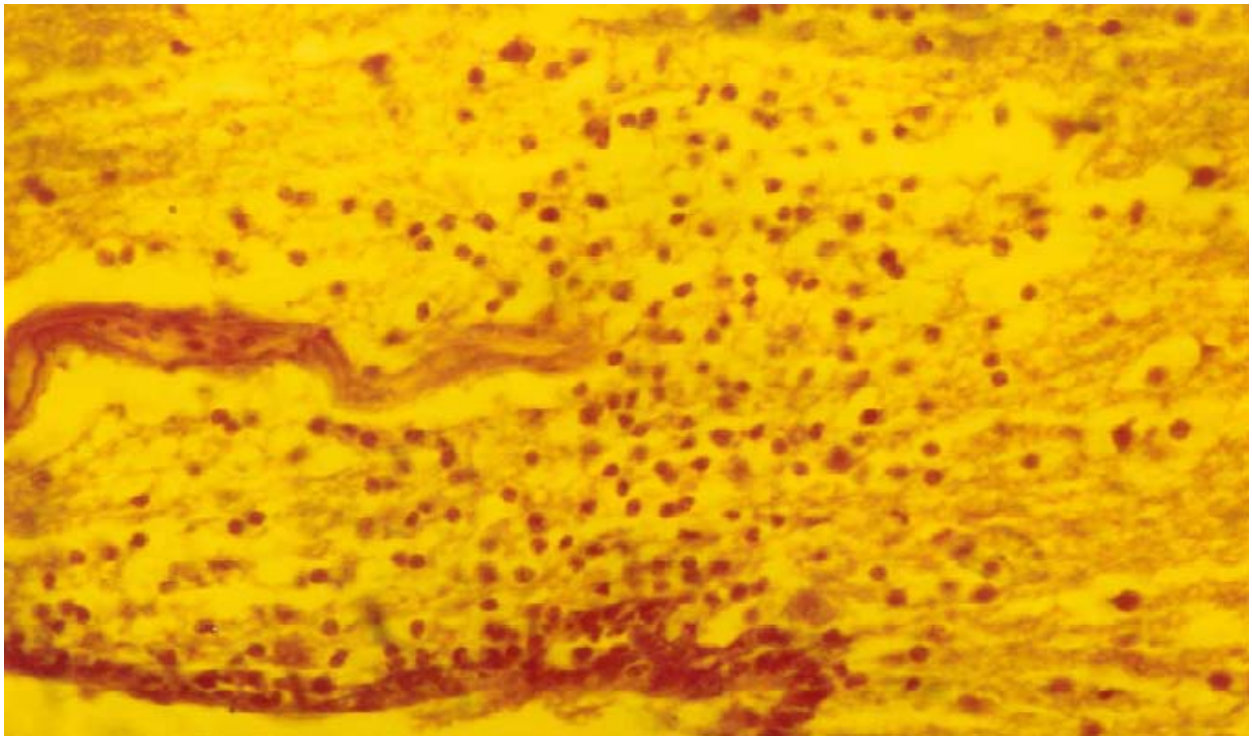

Fig (9). Brain of adult chicken experimentally infected showing infiltration with mononuclear cells in the subventricular area

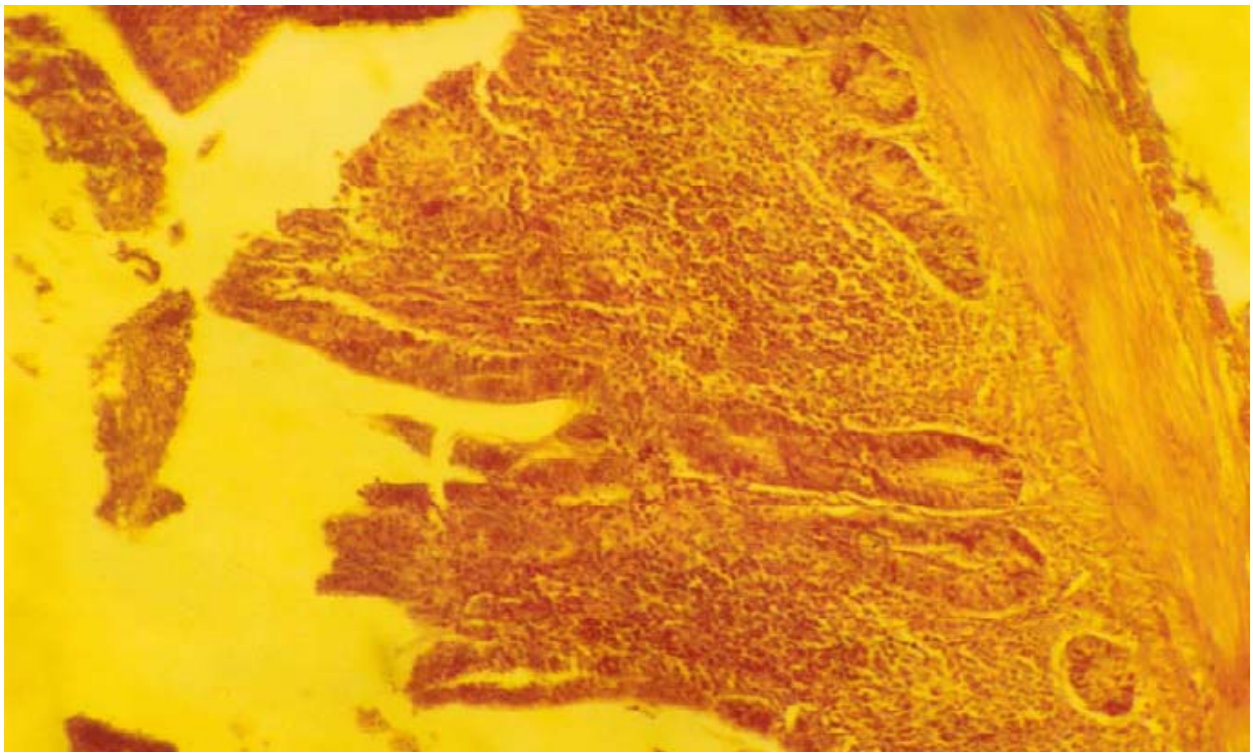

Fig (10). Intestine of eperimentally infected adult chicken showing epithelial desquamation

Fig (17) Chicken embryos inoculated with Butri isolate. Note retarted growth, poor feathering and petecial hemorrhage

Fig (18) Spleens of adult chicken collected 5-6 days after experimental infection with Butri isolate (passage 3)

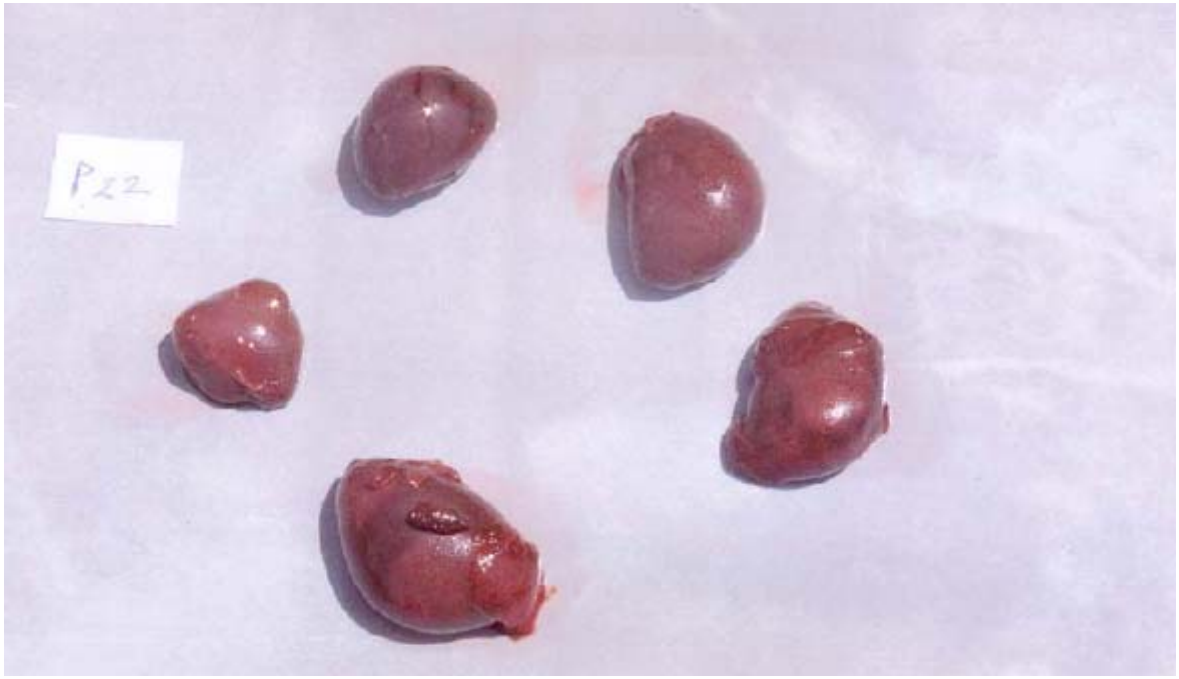

Fig (19) Spleens of adult chicken collected 5-6 days after experimental infection with Butri isolate (passage 22)

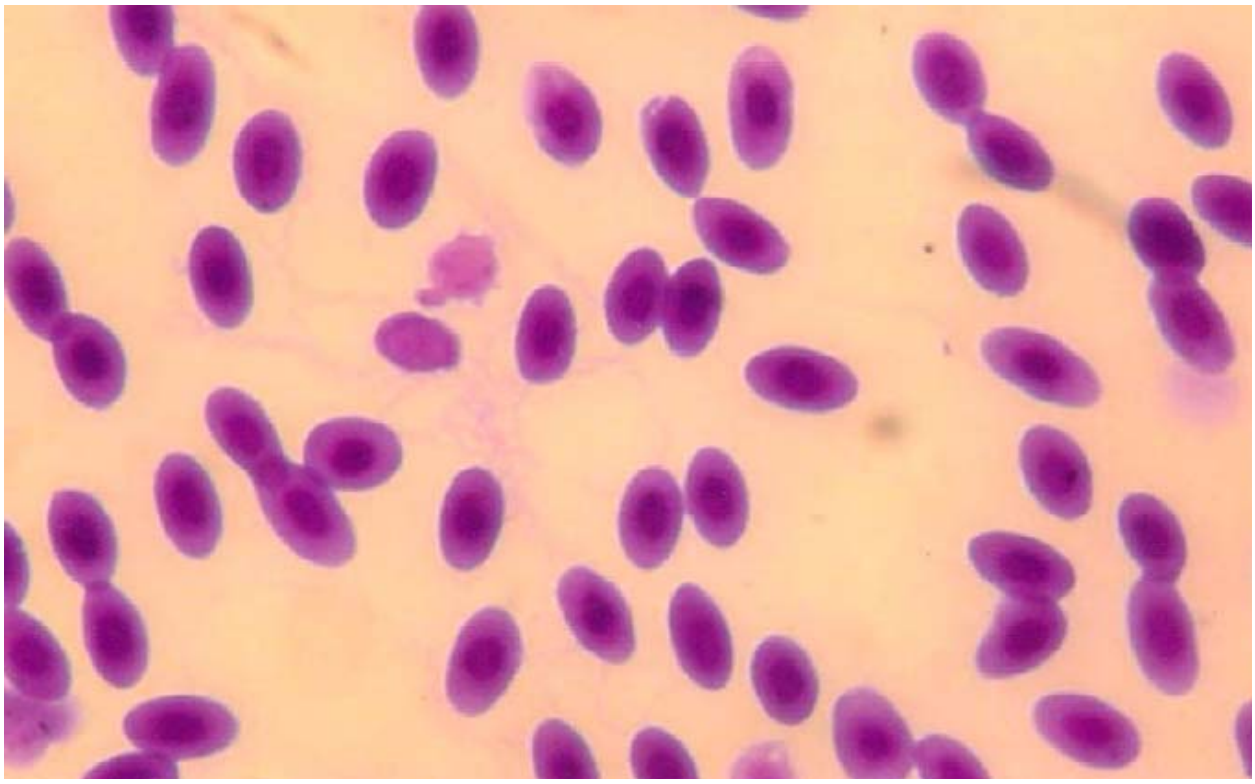

Fig (11). Blood smear stained with crystal violet from expermintally infected chicken

Fig (3). A grower naturally infected with spirochetosis. Note Pale comb and tick infectation

Fig (4). Adult chicken experimentally infected. Note Pale comb ruffled feather and paralysis of wings

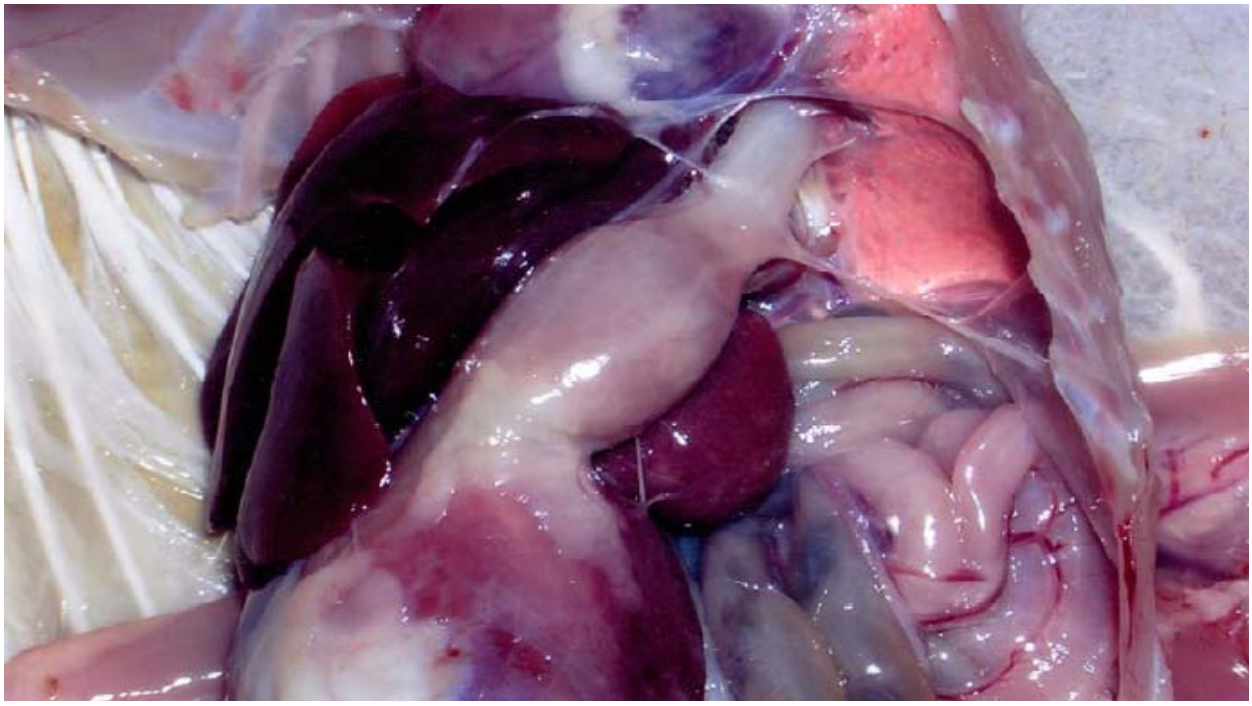

Fig (5) An adult chicken experimentally infected with *Borrelia anserine*. Note enlarged mottled spleen.

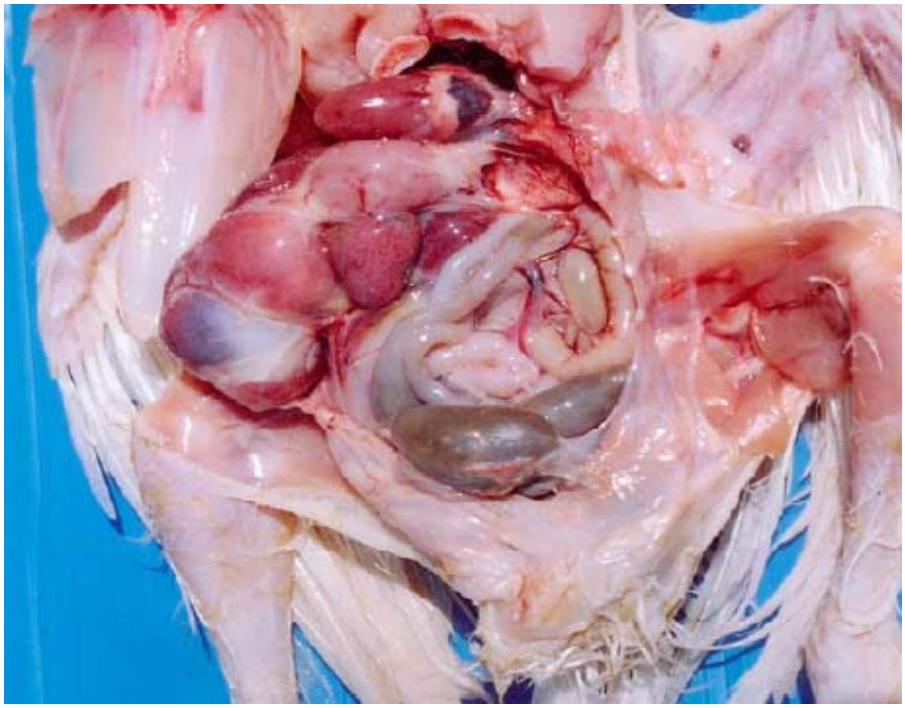

Fig (6). A grower chicken experimentally infected with *Borrelia anserine*. Note enlarged mottled spleen.

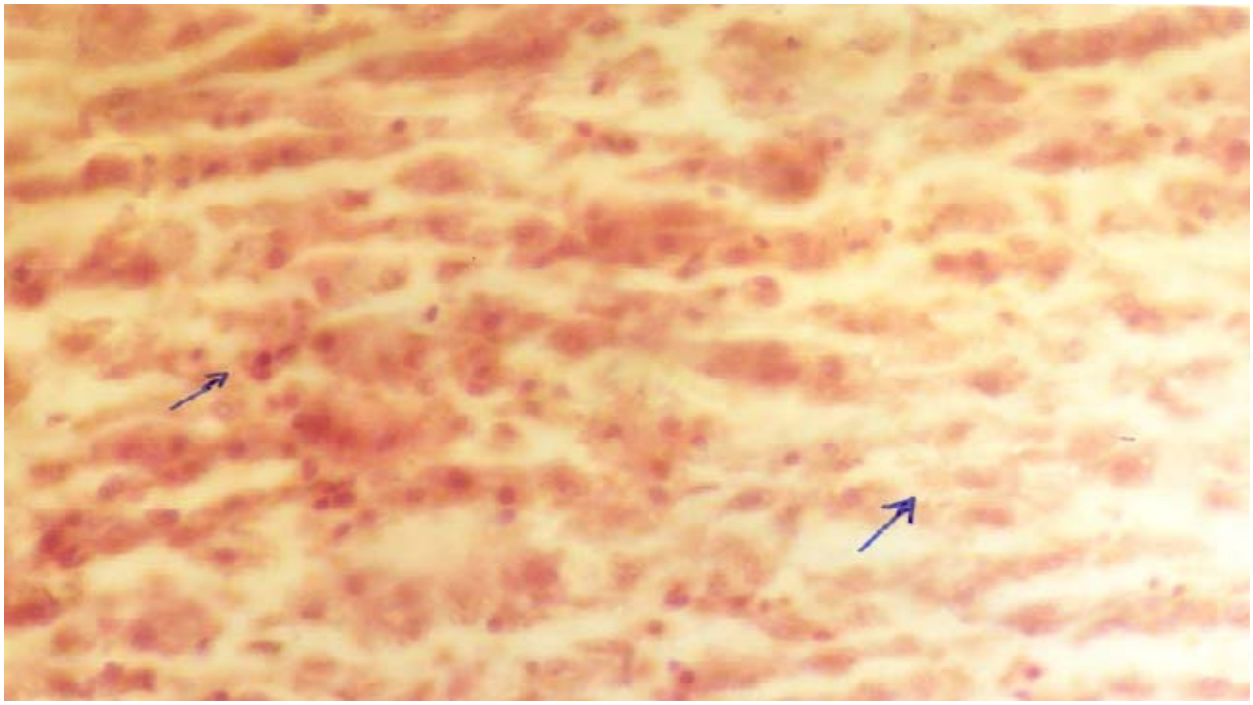

Fig (7). Spleen of an experimentally infected adult chicken showing lymphocitic infltration and necrosis

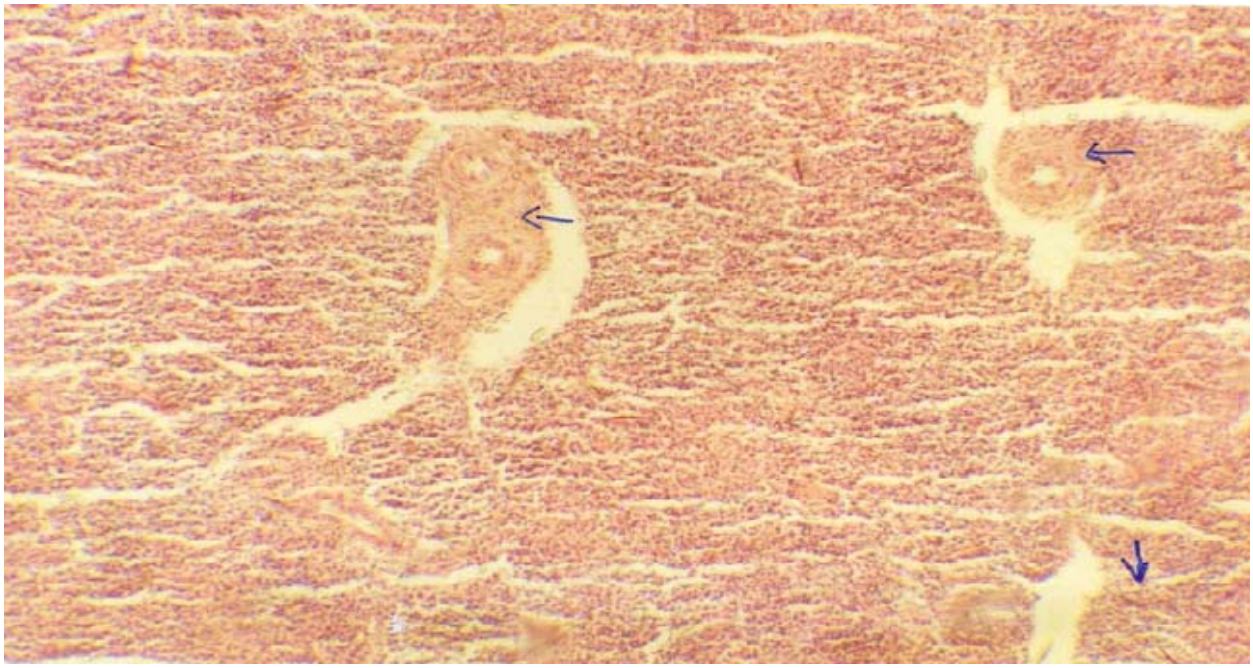

Fig (8). Spleen of an experimentally infected adult chicken showing thickness of capillary walls and mononuclear cells infiltration

Discussion

The present study was carried out to investigate the incidence and prevalence of spirochetosis in Khartoum state. During this work isolation of *Borrleia anserina* from different areas in Khartoum state was made. The isolated organisms were identified according to their morphology together with the clinical signs, characteristic splenic lesions in infected birds and presence of *Argas persicus* in both birds and its environment.Spirochetosis beside the backyard husbandry system, has been a problem in some large organized well managed open system farms in the studied area .Infection is prevalent throughout the year irrespective of seasonality as was observed during this study, this suggests the persistence of the organism in both chicken and arthropods vector, however Barnes (1997) reported that outbreaks are most common during the warm humid seasons which related directly to tick activity. In the present study ticks collected from infected farms , different stages were found ,nymph were always detected in birds while both nymph and adult were present in chicks pens.Ticks were identified as *Argas persicus* the main vector transmitting the disease, this confirmed the finding of Penty (2004) in Australia in addition he stated that adults and nymph are the most parasitic stages.Recent studies confirmed that nymph are more parasitic than the larvae and adults (Jongejanand Uilenbery,2004).Six *Argas* species occur in Sudan(Hoogestral,1973) birds and bats are the main host of most argas.

Ticks obtained from backyard flocks produce high infection rates than those from commercial flocks and male ticks are more infective than female tick, in addition *Argas persicus* were incriminated in the transmission

of some bacterial diseases such as *E.Coli*, *Staphylococcus aureus* and *Salmonella pullorum* (Buriro, 1979).

Natural outbreaks were recorded only in adults and growers, but chicks could be experimentally infected in this study this confirmed the finding of Choudhary and Roa(1985).The highest incidence of the disease was observed in adult chickens. Khartoum county showed the highest incidence of the disease (94.3%) during the period of study. All *Borrelia anserina* isolate used in the present work were isolated from the blood of naturally infected chicken.

Experimental work was carried out on various aspects of the disease with special reference to field isolate and age group .chicks showed marked differences in clinical signs, spirochetemia and duration of the disease compared to the other groups .Incubation period ranged from 2-5 days. The organisms remain in the circulation up to 17 days (Fig11). This delay in clumps formation as well as the disappearance of organism from the blood circulation suggest a reduce antibody production and probably the antibodies available were less in amount which were unable to remove and clear a rapidly multiplying organism and removed them from circulation after their lyses. These functions appeared to have improved with the advancement of age (Choudhary and Roa, 1985).

The characteristic gross lesion was the enlargement and mottling of the spleen which was detected from day 4-5 post infection even among apparently healthy chicks. The course of the disease was 11-15 days .These findings were in agreement with Choudhary and Roa(1985) in a similar study in India who stated that young chicks were more susceptible to the diseases but their clinical response to infection as compared to older one was low .Djankov *et al* (1974) in Bulgaria reported that amongst white leg horn birds, day old chick were the most susceptible group .Damassa and Adler (1979) stated that birds under three weeks of age were most sensitive to

spirochetosis than older one in USA.Soliman *et al* .(1966) reported considerable losses in young chicks in Egypt. The finding of other worker was found parallel with the present finding of these was William (1955) in USA, who stated that the response of chicks to *Borrelia anserina* infection was variable ranging between severe symptoms and deaths to only transient spirochetemia suggesting that the source of chicks was a factor. Chicks of vaccinated hens carried a passive immunity which is protective at early age and decline gradually with age. At 7-14 days maternal immunity had waned (Dutt *et al*,1977) ,however maternal antibodies are not considered a factor during this investigation because by now day- old- chicks used were either brought from a closed husbandry system local farms, where no history of spirochetosis or vaccination of the flocks, or exported ones. A possible reasons of the absences of natural outbreak in this age could be due to age susceptibility or the missed diagnosis of the disease in the field, in addition the misuse of antibiotics by poultry keepers specially in early chick live decreased the possibility of demonstrating the organism which is sensitive to wide ranged of antibiotics such as Penicillin,oxytetracyclin erythromycin and tylocin (Phulan *et al*., 1988).Clinical picture of the disease in both growers and adults were found nearly similar 70.5%of growers showed clinical signs while 66% of adults exhibited clinical signs after experimental infection with some of the field isolates as shown in Fig(2). Incubation period was variable according to isolate, inoculums and route of exposure ,it ranges from 1-9 days (Table 11and12). Birds showed typical clinical signs of spirochetosis (Barnes and Swayne, 1998) as shown in table (14). Nervous signs were frequently seen, *Borrleia anserina* were recovered in peripheral blood 2-3 days post infection, spirochetemia was less extensive in these groups. Peak of spirochetemia was recorded on day4-5 (Fig 13nd14) clumping appears from day 5 onward in most infected birds, two days latter the organism disappeared from the circulation.

As in chicks the characteristic gross lesion observed in these two groups was the enlargement and mottling of the spleen in addition to other lesions shown in table (14).The course of the disease in these age groups was shorter and severe (5-7) days. This finding was in agreement with Bandopadhyay and Vegad (1983) who recorded the same clinical signs and postmortem lesions in birds infected with virulent strains of *Borrelia anserina*.

Finding reported by Choudhary and Roa (1985) did not agree with the present results. They described gross lesions in both age group which was not observed during this investigation such as petecial hemorrhage on the liver and kidney, enlargement of gall bladder and linear hemorrhage in the inter glandular space of proventriculus , ictrus and muscle hemorrhage in older birds .

Splenomegaly as well as mottling is a characteristic of spirochetosis caused by highly virulent strains of *Borrelia anserina* and it may not be evident when birds were infected with low virulent strain or during the early stages of the disease (Barnes, 1997; Cooper and Bickford, 1993).During this study spleens of all infected chicken showed variable degree of enlargement and mottling. Significant increase in spleen weight was detected among growers and adults ($P < 0.01$) when compared to chicks but there was no significant difference between growers and adults.

Isolates showed great variation regarding splenomegaly in both naturally and experimentally infected birds with the four studied isolate. Butri showed the highest increased followed by Soba1 , Taiba and Alkadaro .A possible reason of the type of reaction could be due to age susceptibility and virulence of isolate. Enlargement and mottling of the spleen was described by many workers to be due to extensive phagocytosis of red blood cells and heamosiderosis (Bandpadhyaya and Vegad,1983) which is the consequence of erythrocyte destruction by reticuloendothilial cells, its

extend and distribution may be determined by the amount of reticuloendothilial tissues in the organ (Bennett,*et al*,1981).

During this study *Borrelia anserina* was demonstrated in the heart ,spleen,liver and lung as has also been reported byGross and Ball(1964) in the brain and bone marrow .The organism could not only be seen in and around the vascular system of these organs but it was also localized in the parenchyma of these organs making such tissues antigenic. The antigenicity of the tissue was partly due to the presence of spirochetes and partly due to the altered nature of infected organs to become antigenic .This part of observation was found similar to those observed in heamosporidian infections like malaria and babesiosis (Wadalker and Soni, 1982). During this study it was observed that number of *Borrelia anserina* capable of producing the disease (more than 50organism per microscopic field) is by far higher than the number recorded in previous studies (Buriro,1979) who reported that ten or fewer spirochete constituted an infective dose for susceptible chicken.

In another part of the study propagation and isolation of *Borrelia anserina* was carried out. Due to the difficulty associated with the primary isolation and propagation of spirochetes modification and evaluation of various cultural method have been tried in order to develop and improve the isolation rate Babour _stonner Kelly media was used successfully to isolate some strains of *Borrelia anserina* but the isolates lost thier infectivity by the 12 passage (Levine *et al* .,1990).

It was found that about half of the known spirochete were uncultivable (Paster, 2000).Strains of *Borrelia anserina* isolated from various countries differ in their ability to grew in artificial media of these are the Nigerian strains while other as in USA could be isolated (Hovin, 1995). Different methods were described by many workers to isolate and maintained the uncultivable strains such as the use of chick embryo or day-old-chicks;

therefore in this study a comprehensive first study was conducted in an attempt to test, use and evaluate three methods for isolation of the *Borrelia anserina* local isolates, namely chick embryo, day old chicks and adult chicken. The most reasonable criteria in comparing the efficacy and superiority of the method for isolation of *Borrelia anserina* from field materials are the recovery rate and mortalities of chick embryo in relation to the material from which they were recorded, also maintenance of virulence of the organism which determine by pathological changes in the embryo. The result showed that embryonated egg inoculated via various routes was superior to the other cultural media .The organism were obtained from all inoculation sites with some variation in number of organism and mortality rate, despite the fact that they all received equal volumes of inoculums from the same sample. Dead in shell embryos were significantly different at ($P<0.01$) between the four routes used .No significant difference was detected between yolk sac and allantoic cavity .But significant increase in mortality ($P<0.05$)was found between these and yolk sac 1 and CAM route. These results confirm the finding of McKercher (1950) and Gorrie (1950) who reported the abundant growth of organism by yolk sac inoculation at time before incubation up to 13 days. Hart (1963) in Australia emphasized that allantoic sac was the suitable route of inoculation for vaccine production yielding high infection rate with lower mortality, but these finding was not in agreement with William (1955) who stated that the organism grew equally well on CAM and allantoic sac. It was reported that CAM fluid exerting a deleterious effect on the spirochetes (McKercher, 1950).No significant difference was detected between inoculums (blood and serum).

During this investigation it was observed that 11-13 day-old embryos inoculated via yolk sac were the best for isolation of *Borrelia anserina* due to high recovery rate, and less bacterial contamination.

The second method for isolation was day- old chicks which gave a high concentration of organism with long persistence in blood circulation these results confirmed the finding of Barnes and Swayne(1998)who maintained the organism in one –day- old chicks by weekly passage. Result of propagation and serial passage of the four field isolate in embryonated eggs showed that the infectivity rate produce by both Soba1 and Butri isolate increased in succeeding passages, in case of embryo infected with Taiba and Alkadaro isolates infection rate was low During this study an attempt to produce the disease in local chicken breed was unsuccessful these were in agreement with (Ginawi, 1980; Khogali and shommein, 1973) this may be due to the continuous exposure to infected tick bites which render them challenging to reinfection .The work so far done seems to be confined to chicken only and the role of *Borrelia anserina* in other domestic poultry such as pigeon has not been given any attention.3.26% of urban pigeon in Italy were found to have *Borrelia anserina* antibodies (Fabbi,1995).During this investigation experimental infection of pigeon was unsuccessful It was found that among all *Argas* species only *argas reflexus* parasitized pigeon causes loss of blood (Jongejan,1996),while *argas persicus* which is the main vector of *Borrelia anserina* in its all stage is chiefly a parasite of chicken, ducks, geese and turkey(Hoogstral,1973).Some authors reported that pigeon and guinea fowl were relatively resistant to experimental infection (Fabbi,1995) .Negative results obtained during this study do not necessarily exclude these species as having a role in infecting ticks because spirochetosis in pigeons was previously reported in the country (Anon,1925).

There are several problems associated with the maintenance of *Borrelia anserina* in the laboratory. Propagation of the organism in chicken or ticks as described by many workers may be expensive both in term of labor and the cost of animal host. Storage of the organism has been described by many investigators which seems to require freezing the organism in their

arthropods host or freezing them in a high concentration of blood of vertebrate host (Schneider *et al.* 1968., Hart, 1970).During this study experiments were carried out to search for a suitable media to maintain *Borrelia anserina* over a period of months without changing their virulence .In citrated blood stored in liquid nitrogen the majority of organisms remained motile and all vials opened over a 23 month period were found to be infectious (Fig 25).

These result are in agreement with Hart (1970) ,Sumrov(1975) who reported the survival of organism in citrated blood for a period of 4-5 month in liquid nitrogen .The organism remain motile and infective in serum and plasma for a period of 15,6 month ,respectively .This study showed that the addition of glycerol 10-50% to the preparation reduced the life span of the organism to 6 month in both citrated blood and serum .This finding agrees with Dhawedkar and Dhanesar(1983) but did not agree with the findings of Labruna *et al*., (1999) in Brazil who stated that *Borrelia anserina* retained its viability and infectivity in 50% glycerol for as long as 15 months in liquid nitrogen. On the other hand 10% DMSO preparation kept the organism live and infective for as long as 12 month in citrated blood and 9 month in serum (Fig 24).Loss of motility and virulence were observed in 50% glycerol preparations after the first 3 month of storage .From the result obtained in this work DMSO was found to be superior to glycerol as a preservative in liquid nitrogen . Loss of infectivity in glycerol sample was assumed to be due to partial loss of *Borrelia anserina* virulence or inactivation of the organisms with glycerol which induced an immune response and chicken recovery (Labruna, 1999).*Borrelia anserina* was found extremely sensitive to a majority of chemical compounds including glycerol (Cottral,1978). Addition of glycerol or other nonelectrolytes reduces the gross deviations in salt concentration from normal during the freezing process .Great increase in salt concentration within cells seems to be responsible for a major part of damage of live cells

rather than formation of ice (Lovelock,1953) .During this work the suitable media for short term preservation as during experimental work was foud to be the citrated blood kept at 4°C which maintained infectivity and viability for 6 months. This finding agrees with other(Hart,1963.,Ahilly, ,1973 .,Ginawi and Shommien,1980).In this study bloody chorioallantoic fluid was found to be the only embryonic material in which the organism can be kept for a significant period which is 6 months at 4°C.

Outbreaks of spirochetosis are commonly reported but laboratory confirmation is a difficult since the bird is generally given antibiotic treatment immediately on the appearance of mortality in the flock. The organism is sensitive to wide range of currently used antibiotic including penicillin, tetracycline, streptomycin, kanamycin and tylocin (Phulan, 1988). These *Borrelia anserina* are not usually demonstrated in the blood after treatment. Difficulty in diagnosis is also experienced when the organism disappears from the blood after clumping in large aggregates as a result of the development of agglutination.

The main objective of this study as previously mentioned was to establish a simple tests for diagnosis of fowl spirochetosis after lapse of spirochetemia the result indicate that serological reactions such as plate agglutination and spirochete Immobilization test could be effectively used for the diagnosis of the disease. These tests are sensitive, specific and quick .This is in agreement with the finding of others (Verma and Malik ,1968; Mehta and Muly1963 and Chatterjee and swahney,1971).

The present investigation was undertaken to prepare an antigen for serological diagnosis and confirmation of the disease. During this study 2 antigens for serological tests were prepared according to Mehta and Muley (1968) namely agglutination and immobilization antigen .Plate agglutination and spirochete immobilization tests were used to test sera, both test were comparable and useful to monitor antibodies in chicken sera , however in

this investigation plate agglutination test was found superior as a field test due to its antigen stability which remain stable for as long as 3 month at room temperature unlike the immobilization test which needs a fresh prepared antigen. Which require an elaborate laboratory capacity.

At present study immunity against fowl spirochetosis was determined in experimentally infected chicken using the four field isolate by plate agglutination and immobilization tests .Convalescent and hyperimmune sera were examined .All of them showed positive reaction with the homologous antigen these results show that serological tests could effectively be used to ascertain the immune status of a flock. These results support the finding of others(Verma and Malik, 1968; Mehta and Muley, 1968; Chatterjee and Sawhney, 1971) in similar studies in India.

Specific agglutinins appeared in the blood in a few cases on the 6^{th} day post infection and in all of them by the 10^{th} day .The appearance of agglutinin and immobilisins in the serum suggest that the same antibody may be responsible for both the reactions or the two entities may be closely related to each other (Djankov;Sumrov,and Penev,.1966; Chatterjee and Sawhney, 1971) .Hyperimmune sera of the 4 isolates showed higher titer than corresponding convalescent sera.

strain variation in the spirochete *Borrelia anserina* has been reported by many workers (Mehta and Muley 1968; Mehta and Muley ,1972;Paratkar and Mehta,1973), this information is of significant importance from prophylaxis point of view .Several studies has demonstrated both the presence of different antigenic type (Soni and Joshi ,1980;Levine,1990) and serotype specificity (Damassa and Adler,1979;Sambri et al.,1999).

During this work an attempt was carried out to study antigenic variation, between isolates, depending on the finding stated by Soni and Joshi(1980) and Damassa and Adler(1979) using agglutination, immobilization and cross infection tests. Results obtained designated that serum with particular isolate

antibodies immobilized completely its homologous antigen ,cross agglutination between the 4 field isolate revealed that Soba1,Butri, and Taiba reacted similarly with the hetrologous antigens .Similar results were obtained by spirochete immobilization tests. On the other hand Alkadaro isolate showed weak positive reaction with both homologous and hetrologous antigen. Cross protection studies revealed that chicken recovered from an infection of Soba1 and Butri isolate were completely protected for challenge with Taiba and Alkadaro isolates.where as birds immunized by Taiba and Alkadaro isolates developed the disease after challenge with Soba1 and Butri isolate. Although agglutination and immobilization tests showed cross reaction between the four isolates suggesting some homogeneity, but there appear to be variations between the isolates. Therefore further studies should be made to investigate the antigenic variation between the isolates in the country. Some author have established immunogenic difference between individual strains of *Borrelia anserina* (Djankov and Soumrov,1970;Coffey and Eveland,1967).On previous studies for serotype identification of *Borrelia anserina* strains in Bulgaria using direct immunofluoresence method it was found that granules exists in the spirochete of all strains regardless of whether they belong to similar or different serotypes these granules were the cellular antigens found in the membrane of the cell body which represent the group protein.

another type specific antigen may also exist(Djankov *et al*,1972, according to the presence of definite and permanent antigenic components, they divided their strains into two clearly distinct serologic groups (serotypes)in stead of serovariants. .In this study attempts were made for molecular characterization of *Borrelia anserina* isolates using DNA extract and amplification as described by Sambri *et al*.(1999) but no results were obtained.

From the result obtained in this study that there was obvious differences in virulence, among the four field isolates, Soba1 and Butri could be classified as virulent isolates while Taiba and Alkadaro as a moderately virulent isolate.All field isolates was original and isolated from natural outbreak .They produce spirochetosis in chicken proving that they belong to the genus Borrelia ,these isolates showed great variation in the in virulence .The four field isolate had been used experimentally to infect chick embryos and chicken of various age groups.Soba1 isolate originated from southern area of Khartoum state , causes 63% mortality in chick embryo ,12.5% in chicks and 74% in the older birds . The disease produced after experimental infection with this isolate characterized by marked illness ,high intensive spirochetosis and marked enlargement in spleen and greenish diarrhea.Alkadaro isolate of *Borrelia anserina* which originated from northern area of Khartoum state Experimental infection with this isolate resulted in 36% mortality in chick embryos and 44.6% in chicken ,clinical picture of the disease was milder less spirochetemia ,slight enlargement of spleen .these signs were reported previously due to infection with low pathogenic strains (Higgins,1986).Butri isolate originated from southern Khartoum it causes 67% mortalities chick embryo ,13% chicks and 95% adult chicken , and the disease produced was the typical acute form according to (Barnes ,1997; Choudhary and Roa ,1985) marked clinical signs greenish diarrhea which is a feature of spirochetosis caused by virulent strain (Bandopadhyay and Vegad,1983) and infected chickens showed marked enlargement and mottling of the spleen.Taiba isolate originated from western area of Khartoum causes milder disease ,Characterized by mild clinical signs less spirochetemia and lower mortality rate 31% in chick embryos,10% in chicks and 47% in older chicken. In relevant studies by Damassa and Adler (1979) who stated that *Borrelia anserina* strain that caused more than 60% mortality in chicken was considered as virulent strain and that caused 30%

were designated as moderately virulent. Isolate that causes only slight enlargement of spleen were identified as milder strain. Specific pathogenicity determinants in *Borrelia anserina* have not yet been identified but depending on the finding of the importance of the outer surface protein of *Borrelia Burgdorfei* in determination of lyme disease, analysis of the surface composition of *Borrelia anserina* pathogenic strain in comparison with strain that lost its avian pathogenicity, protein profile of the strains showed major differences in 20-22KDa region with other minor difference in 64-66KDa region. Attenuated strain highly represents 20 and 22KDa on the hand 24 KDa was represented by the pathogenic strain (Sambri.1993).Therefore further studies on protein profile were needed to identify specific pathogenicity determinants in *Borrelia anserina* isolates in the country.

Conclusions and Recommendations

Further studies of prevalence of spirochetosis in other states should be done Epidemiological investigation was needed to determine the species of

soft tikcs present in the country and their role in transmission of fowl spirochetosis followed by effective control programme.

Reliable serological methods should be used for detecting *Borrelia anserin*a antibodies in chicken serum, of this plate agglutination test was recommended.

More recent methods are needed for studying and typing of field isolates for these molecular biology and use of mabs are mainly helpful.

Production of a polyvalent vaccine from field strains.

Further studies are needed to investigate the disease in local chicken breeds and pigeon in the country.

References

Abdu, P.A. (1987). Infectious bursal disease and spirochetes in pullet chicks. Avian D.; **31**(1):204-205

Ahmed, A.A.S; Bahgat, A.M; Mostafa. and Soliman, M.K (1965) . Studies of fowl spirochetosis in Fayumi chicken. The pathology of experimental spirochete infection. J Vet Sci., **2:**255 -29

Al-Attar, M.A and Jahanly, F.M. (1974).Demonstration by immunodiffusion agar-gel test of Borrelia anserina antigen in the organs of infected chicken. Avian Dis. **18**(3):464-

Al-Hilly, J.N.A. (1969).Immunodiffusion agar 466. gel test for demonstration of *Borrelia anserina* produced by liver of infected chickens.Am.J.Trop.Med.Hyg. **30**:1877-1880.

Al-Hilly, J.N.A (1971) Immobilization and Immunodiffussion test for determination of antibodies against spirochetosis in the yolk of convalescent fowl .Avian Dis. **15**:419-421.

Anderson, J.F.; Magnarelli, L.A; Burgdofer, W. and Barbour, E.G. (1983)**.**Spirochete in oxides domino and mammals from Connecticut.Am.J.Trop.Hyg.,32:818-824.

Anderson,J.F; Russel C, Johnson; Louis, A.,Magarelli., Fred w and Hyde.W (1986) Involvement of birds in the epidemiology of the Lyme disease agent *Borrelia burgdorferi.* Infect.Immun.**51**:394-396.

Anderson, J.f. (1989**).**Epeizootiology of Borrelia in ixodes tick vector and reservoir hosts. Rev.Infect Dis .**,11**:1451-1459.

Anon. (1923-1925) .Annual Reports Ministry of Animal Resources Khartoum Sudan.

Ataliba,A; Resende,J.;Yoshinari,N, andLabruna,M(2007) Isolation and molecular characterization of Brazilian strain of *Borrelia anserina* the agent of fowl spirochetosis .Res. Vet. Sci., **83**(2):

145-149.

Bandopadhyay, A.Cand Vegad, J.L. (1983).Observation on the pathology of experimental avian spirochetosis.Res.Vet Sci ., **35**(2):138-144.

Barnes, H.J. (1997). In .disease of poultry 10 edition edited b B.W.Calnek with H.JohnBarnes, C.W; Beard , ;McDougland .Y.M.Saif. Iowa state University press Ames, Iowa USA (1997).

Barnes,H.J and Swayne ,D.E.(1998).In. laboratpry manual for the isolation and identification of avian pathogens. Edited by David E Swayne ; John R Glisson ;Mark W Jackwood ;James E Pearson; Willie M.Reed .American Association of Avian Pathologist.Universityof Pennsylvania .Fourth edition 1998 .

Bennett, D.F; Finnett, S.L; Nash, A.S and Kirkham.D.(1981) Vet. Rec.**109**:150-153

Bozhilov,B.M;Petkov ,M; Stoianova,L.(1978) .Production of trivalent vaccine against avian spirochetosis.Vet .Med. Nauki **15**(3):32-35.

Breed, W.; Murray, E.C.D.; Smith, N.R. (1975).Bergeys manual of determinative bacteriology 7thed Williams and Wilkins Co; Baltimore P 898.

Burgdorfer, W and Schwan, T.G.(1991). Borrelia in manual of clinical microbiology, 5thed.A.Balows, W.J.; shadowy, H.J.eds. American Society of Microbiologist Washington, D.C.pp.560-566.

Burgess, E.C.;Gilette, D. and Dickett, J.P. (1956).Arthritis and Panuveitis as manifestation of *Borrelia burgdorferi* infection in a Wisconsin pony .J. Am.Vet.Med.Asso, **189**:1340-1342.

Buriro,S.N.(1979). Role of *Argas (persiargus)persicus* in transmission of spirochetosis.Pakistan. J.Zool,**11**:221-224

Canale-Parola, E.(1984).Order1.Spirochaetales Buchanan1917, 163AL, p.38-39 In N.R.Krieg(ed).Bergeys manual of systematic bacteriology .vol1.The Williams& WilliamsCo Baltimore,Md.

Chatterjee,A and Sawhney,A.N (1971) **.** Serological studies on experimental fowl spirochetosis .Indian J. Anim. Sci ,**41**(12):1151-1153.

Chatterjee, A and Sawhney, A.N. (1971). Diagnosis of fowl spirochetosis by agar gel precipitation test .Indian J.Anim.Sci.**41** (8):727-730.

Choudhary,C.R and Roa,K,N.P.(1985) Studies on clinicopathological changes in young chicks. Indian. Vet. J, **62**:465-468.

Cowan and Steel (1993) .In. manual for the identification of medical Bacteria 3rd edition edited by G.I;Barrow.R.K.A Felthan .Cambridge university press. Reprint 1999.

Cottral, G.C.Manual of Standardized methods for veterinary Microbiology. Ithaca Cornell University (1978) p 731.

Cooper, G.L and Bickford, A.A. (1993).Spirochetosis in California game chicken .Avian dis,**7**:1176-1171.

David, M.R.; Jason.A.C.; Micheal.T and Richard,T.M.(2000).The bdr gene families of the lyme disease and relapsing fever spirochetes : potential influence on biology ,pathogensis and evolution. Emerging Infect. Dis. **6**(2):110-121.

DaMassa, A.J and Adler, H.E. (1979)**.** Avian spirochetosis natural transmission by argas (persicargas) Sanchezi (ixodoidea: argasidas) and existence of different serological and immunological type of *Borrelia anserina* in the United state .Am.J.Vet.Res,**40**(1):154-157.

DaMassa, A.J and Adler, H.E. (1979). Avian spirochetes enhance recognition of mild strain of *Borrelia anserina* with bursectomized and dexamethasone treated chicken .J.Comp.Path.**89(3)**:413-420.

Delamater, E .D and Saurino, V.R. (1952). Studies on immunology of Spirochetes .Bac.Proc.52nd general meeting society of American Bacteriology .Boston 127-130 cited by Charles, **W**.d and James, B. (1963)**.**A study of carrier state of avian spirochetosis in the chicken. Avian Dis. **8**:191-195.

Dennis ,A.Senne.(1998).In.Laboratory Manual of isolatin and identification of Avian pathogen.Fourth ed. Published by The American association of Avian pathologists.

Dhawedkar, R.G and Dhanesar, N.S. (1983).Preservation of *Borrelia anserina* by liquid nitrogen refrigeration .Indian J Anim Sci. **53**, 1124-1127.

Diab, F.M.and Soliman, Z.R. (1977)**.**An experimental study of Borrelia anserina in four species of Argas ticks. Spirochete localization and densities.Z.Parasit,**53(2)**:201-212.

Dickie, C.W and Barrera, J. (1964).A study of the carrier state of avian spirochetes in the chicken .Avian dis, **8**:191-195.

Djankov, I.; Soumrov.I.; Lozeva, T and Penev, P. (1970). Persistence and excretion of treponema anserina Sakharoff (1891) in hens. Zbl. Vet Med Reihe B ,**17**:544-548.

Djankov, I; Soumrov, I and Lozeva, T.(1972)**.** Use of the Immuno-fluorescence method for the serotype identification of *Borrelia anserina* (Sakharoff, 1891) Strain .Zbl Vet Med B, **19**:221-225.

Djankov, I.; Sumrov, I and Stefanova, P. (1973).Age related sensitivity of chickens to spirochetosis of the genus *Borrelia anserine* Vet. Med Nauki.10(6):7-11 cited by Choudhary, C.R and Roa, Kn.P. (1985) Studies on clinicopathological changes in young chicks. Indian Vet J **62**:465- 468.

Dzhankov, J.; Sumrov, I.; Lozeva, T and Paner, P. (1968)**.**Found spirochete in the mouth and cloacae during spirochetemia.Vet Med Naki, **5**:33-37.

Dzhankov ,I.; Sumrov,I and Lozeva,I. (1975) .Study Of the antigenic makeup of strain of *Borrelia anserina* Sakharoff 1891, of the Surnevo and Pamoukochii serotypes .Vet Med Naki,**12**(4):29-33

Dutta, G.N.; Mehta, M.L and Muley,.A.R. (1977).Studies on immunity in fowl spirochetosis.Indian J Anim Sci,**47** (9):554-558.

Dworkin,M.S.;Andeson ,D.E.; Schwan.T .G .;Shoemaker P.C.;Banerjee,S.N.;Kassen and Burgdorfer.W. (1998). Tickborne relapsing fever in the northwestern united Canada. Clin .infect.Dis, **26**:122- 131.

ElDardiry, A.H. (1945) Studies on avian spirochetes in Egypt. In Egypt Tech.Sci serv Bull, **243**:1-78.

El Kammah ,K.M.;Oyoum ,L.M.I and Gabr ,H.S. (2002) .Studies on the feeding effects of argas and *argas hermanni*(Acari:Argasidae) on chicken and pigeon blood and plasma protein .Int.J.Acaro,**28**:273-276.

Fabbi,M.;Sambri,V.;Marangoni,A.; Magnino,S.;Basano ,R.;Cevenini,R and Genchi ,C.(1995) .Borrelia in pigeons: no serological evidence of borrelia burgdorferi infection.J Vet Med {B}, **42**: 503-507.

Felsenfeld, O. (1971)**.**Borrelia strains vectors human and animal Borreliosis.Warren H.Green Inc.st.Louis.

Garg, R.R and Gautam, O.P. (1971)**.**Serological diagnosis of fowl spirochetes Avian Dis, **15**:1-6.

Ginawi,M.Aand Shommein,A.M. (1980). Preservation of *Borrelia anserina* at different temperature.Bull.anim Hlth prod Afr, **28**:221-223.

Ginawi, M.A. (1980)**.**Study of the pathogenicity of experimental Borrelia anserina infection in chicken.M.V.Sc thesis University of Khartoum.

Gothe, R and Schrecke, W. (1972)**.**Zur epizootiologischen von persicarges.Zecken der huhner in Transvaal.Berl Muench tierarztl Wochenschr 85:9-11.cited Barnes**,** H.J (1997). In .Disease of poultry 10 edition edited by B.W.Calnek with H.JohnBarnes, C.W; Beard, I.R.McDougland .Y.M.Saif. Iowa state University press Ames, Iowa USA (1997).

Gorrie, C, J.R. (1950)**.**Vaccination against spirochetosis in fowls. The Australian veterinary Journal, November 309-315. Determinative bacteriology 9.ed.Baltimore: Williamsand Wilkins 1994.787p.

Gothe R.**,** Bucheim. C and Schrecke, W. (1981b)**.** Zur parlyse – indutzierenden kapazitact wildstaemmiger argas (persicagus) persicus und argas (argas)africolumbe als natuerliche biologische uebertraeger von borrelia anserine und aegyptionella pullorum in obervolta. Berl Muench Tieraertzl Wochenscher 94:299-302' cited

by Barnes**,** H.J (1997). In .Disease of poultry 10 edition edited by B.W.Calnek with H.JohnBarnes, C.W; Beard,I.R.McDougland .Y.M.Saif. Iowa State University press Ames, Iowa USA (1997).

Gross, W.B. (1984). Spirochetosis.In: .Diseases of poultry 8th edition. Iowa state university press, Ames, IA, pp.278-281.

Gross, W.H.and Ball, M.R. (1964)**.**Use of flourscein in labeled antibody to study Borrelia anserina infection in chicken.Am.J vet Res,**25**:1734-1739.

Hart,L. (1963). Spirochetosis in fowls: Studies on immunity .Aust Vet J **,39**:187-191.

Hart,L (1970) .Freeze preservation of *Borrelia anserina*.Aust vet J 46) sep 455.

Henry ,J.N. (1950) **.**Vaccination against spirochetes in fowl. Aust. vet .J,:301-305.

Higgins A.R. (1986)**.** Demonstration of borrelia anserina "A can worm" Vet Rec , **2**:119-120.

Hoffman,H.A and Jakson,T.W.(1949)**.** Spirochetosis in turkeys.J.Am Vet Assoc, **109**:481-486.

Hoogstral .H. (1973)**.**Viruses and ticks. In: Viruses and invertebrates (A.J.Gibbs,ed).Amesterdam: North Holland Publishing Co.

Hoogstral ,H.(1985) .Argasid and nuttalliellid ticks as parasite and vector .Adv Parasitol, **24**:135-238.

Hovind- Hougen ,K. (1995)**.** A morphological characterization of *Borrelia anserina*.Microbiology ,**141** (Pt1):79-83.

Hovmark,A.;Asbrink,E.;Schwan,O.;Hederstedt,B.and Christensson,D.(1986)**.**Antibodies to Borrelia spirochete in sera from Swedish cattle and sheep.Acta.Vet Scand, **27**:479-485.

Holt,J.G;Krieg ,N.R and Sneath, P.H.A .Bergeys manual of determinative bacteriology 9 ed Baltimore; William and Wilkin (1997) p 27.

Iturri,G and Cox, H.W. (1969)**.**Glomerulo nephritis associated with hemosporidian infection .Milit.Med ,(**134**):1119-28..

Jaafair, N;Abbood and Al-hill,B.V.M.S.(1969)**.**Immunodiffusion agar gel test for demonstration of borrelia anserine antigen produced by liver of infected chicken. Am .J.Vet .Res. **30**: 10:1877- 1880.

Jongejan, F; Uilenberg. (2004)**.** The global importance of ticks .Parasitology**, 129**: 1-12.

Kaschula, V.R. (1961)**.**A comparison of the spectrum of disease in village and modern poultry flocks in Nigeria .Bull .Epiz. Dis.Afr, **9**:397-407.

Kapur, H.R. (1940).Transmission of Spirochetosis through agent other than *argas persicus*.Indian .J .Ve.t Sci. Anim. Husb,**10**:354- 366 cited by Barnes**,** H.J (1997). In. Disease of poultry 10 edition edited by B.W.Calnek with H.JohnBarnes, C.W; Beard, I.R.McDougland .Y.M.Saif. Iowa state University press Ames, Iowa USA (1997).

Khogali,A.R and Shommein,A.M.(1973)**.**Studies on spirochetosis in fowl in Sudan. Epizootiology and experimental transmission.Bull.epiz.dis.Afr,**22**:251-254.

Kligler,I.J.;Hermoni,D and Perek,M.(1938)**.**Studies on fowl spirochetosis ll.Presence of serological different type of spirochete.J.Comp. Patholo.Ther**, 51**:206-212.

Knowles, R.; Das Gupta, B.M and Basu, B.C. (1932)**.** Studies on avian spirochetosis .Indian Med. Res. Mem **.22**:100-113.

Kornblatt,A.N.;Urb,P.H.;and Steere,A.G.(1985)**.**Arthritis caused by *Borrelia burgdorferi* in dogs.J.Am.vet.Assoc,**186**:960-964.

Labruna,M.B.;Resande,J.S.; Martins,N.R.S and Jorga,M .A(1999) .Cryopreservation of an avian spirochete strain in liquid nitrogen.Arq.Bras.Med.Vet Zootec, **51**(16) 1-5.

Levaditi,G.;Vaisman,A and Hamelin **,**A(1952)**.**Inn.Inst.Pasteur , 83,260.Cited by Verma,K.C and Malik,B.S, Diagnosis of spirochetosis of poultry by slide agglutination and spirochete immobilization test .Current science (1968). **37**(61):170-171.

Levine ,J.F.; Dykstra,M.J.;Nicholson ,W.L.;Walker, R.L.;Massy,G and Barnes,H.J.(1990). Attenuation of

Borrelia anserina by serial passage in liquid media .Res .vet .Sci **,48**:64-69.

.

Livermore,B.P.; Bey, R.f and Johnson, R.C. (1978).lipid metabolism of *Borrelia hermsii* infect.immun,**20**:215-220.

Lovelock ,J.E (1953) .The mechanism of the protective action of glycerol against heamolysis by freezing and thawing .Biophys Acta 11:28-36 cited by Emma ,G.A.(1970).Preservation of *borrelia Kansas* and Plasmodium Berghei.App microbio.Aug:224-226.

Loomis,E.C(1953) Avian spirochetosi in California turkey .Am.J Vet. Res, **14**:612-615.

Marchoux,E and Salimbeni,A.(1903**).**La spirillose de poutes .Ann Inst Pasteur 17:569-580.Cited by disease of poultry 10 ed. By B.W.Calnek with H.John Barnes,C.W.Beard,L.R ;McDougland .Y.M.Saif.Iowa University press Ames Iowa USA .1997.

Mathey W.J. and Siddle P.J. (1955)**.** Spirochetosis in pheasants .J.Am.Vet.Med.Assoc ,**126**:123-126.

Marspin, V.; Ruzic Sabljie.; Cimperman, J.C .; Lotricfulan, S.; Jurca, T.; Pikan, R.N and Strie, F. (2001). Isolation of *Borrelia burgdorferi* sensus lato from blood of patient with erythrema migrans. Infection **,29**:65-70.

Magnarelli,L.A.;Stofford ,K.C and Bladen,V.C.(1992). *Borrelia burgdorferi* in ixodes domminis (Acari:Ixodidae) Feeding on birds in lyme Connecticut, Usa .Can .J .Zool:2322-2325.cited by Olsen,B; David,C.D; Thomas,G.T.;Gylfe; banned ,A.H and Bergestorm,S.(1995).Transhemispheric exchange of lyme

disease spirochete by sea birds .J . Clini .Microbio .**33**(12)3270-3274.

Mckercher,D.G.(1950) .The propagation of *Borrelia anserina* in embryonated egg employing the yolk Sac technique.J.Bacteriol **59**:446-447.

McNeil,E.;Hinshaw,W.R and Kissiling,R.E.(1949)**.**A study of *Borrelia anserina* infection spirochetosis in turkey. J.Bacteriology,**57**:191-206.

Mehta,M.L. and Muley,A.R.(1968)**.** In vitro agglutination and immobilization lysis for typing antigenically different strains of borrelia gallinarum .Indian .Vet. J**,45**:1059.

Merchant, I .A and Packer, R.A (1967) Veterinary Bacteriology and Virology 7th edition .Ames, Iowa the Iowa state university press.

Nikolov, N.D. (1987) Crisis and blood coagulation in chickens with experimental spirochetosis. Vet .Med. Nauki,**24** (3) 4-5.

Morcos, Z.; Zaki, O.A.; and Zaki, R. (1946).A concise investigation of fowl spirochetosis**.** J.Amer.Vet.Med.Ass **,109**:113

Mohamed, A .Z.; Soliman, Z.R and Diab,F.M (1977).An experimental study of *Borrelia anserina* in four species of Argas tick.Z parasitenk, **53**:213-223.

Noberga ,P and Reis (1947) .On the immunity of chicks and chicks embryo from hens vaccinated against Fowl spirochetosis.Refual Veterinarith **.17**:183-187 .

Paster ,B.J.; Dwhirst,F.E.;WeisburgW.G .;Tordc,L.A.; Stanton,T,B.; Zablen ,L and Mandelco,L .(1991).Phylogenetic analysis of the spirochete. J. Bacteriol, **173**(19)6101-6109.

Paster, B, J and Dewhirst ,F.E.(2000)**.** Phylogenetic Foundation of spirochete.J.Mol.Microbiol Biotechnol,**2**(4):341-344.

Paratkar,M.L and Mehta,M.L.(1973). Prevalence of antigenic variants of *Borrelia gallinarum* in Jabalpur.JHKVV Res J (7)269 cited by Soni,J.L and Joshi,A.G. (1981) .A note on strain variation in akola and jabalpur strain of *Borrelia anserina*.Zbl Vet Med B(27): 70-72.

Packchanian, A; Smith, J.B. (1970). Immunization of chicken against avian spirochetosis with vaccine of *Borrelia anserina*. Tex Rep.Biol. Med. **28**(3):287-301.

Petney, T.N.;Andrews, R. H.; Diarmid, L.A and Dixon ,B.R. (2004). *Argas persicus* sensu stricto does occur in Australia. Parasitol .res . **93**:296-299**.**

Perk,K and Hart ,I.(1966). Paper electrophpretic studies of the serum proteins of chicks during experimental spirochetosis .Avian Dis, **10**:208-215.

Phulan ,M.S .;Sokolov ,A.N;Burro,S.N ;Bhalli ,W.M;,Soomro,I.A (1988) .Formtion of immunity with Tylan against spirochetosis in poultry.Pakistan vet J **8**:42-43.

Prudovsky,S ;Hadani ,A.;Rubina,M and Sklais,A. (1978).The use of indirect fluoresecent antibody technique in avian spirochetosis.Avian Pathol.**7**:421-425.

Reddy ,M.V.; Ramachandran,P.K and Ramachandran ,S.(1966) . Histopathological studies on experimental avian spirochetosis in chicks.Indian J. vet. Sci. anim. Hus. **36**:1-1

Rokey,N.W and Snell,V.N .(1961).Avian spirochetosis *Borrelia anserina* Epizootics in Arizona poultry.JAVMA **,138**:648-652.

Roa.M.L.V.and J.L.Soni (1982)**.**Augmentation of heamotissue vaccine doses out-turn through blood transfusion in *Borrelia anserina* infected chicken. Zbl. Vet .Med. (B),,, **29**:408-410.

Roa, S.B.V. (1958)**.**Spirochetosis in poultry. Indian Council of Agricultural research new dalhi India. Research series No18

Roberts, J.A. (1961) Experimental transmission of *Borrelia anserina* (Sakhrroff1891) by Aedes aegyptic. Nature, 191:1225.

Rosenstein, M.(1976)**.** Paralysis of chickens caused by larvae of the poultry tick *argas persicus*. Avian Dis **20**:407-409.

Rodey.M.V. and J.L.Soni, (1977) epidemiology of spirochetosis in chickens, effective measures for control of ticks *argas persicus* Poult Giude **14**:35-37.

Rubina, M.; Braverman, Y and Malkinson, M. (1975)**.** The possible transmission of *Borrelia anserina* (Sakhrroff 1891) by mosquitoes' .Refu.Vet.**32**:16-18.

Russell, E.Wright.; Robert, W.;Barker and John bolte**,** .Oklahoma state university (1990)**.**Oklahoma cooperative extension service.

Sakharoff, M.N. (1891)**.***Spirochete anserina* et la septicemia des OIE Ann Inst Pastuer5:564-566.Cited by Barnes**,** H.J (1997). In B.W.Calnek with disease of poultry 10 edition edited by ;McDougland .Y.M.Saif. Iowa H.JohnBarnes, C.W; Beard ,I.R state University press Ames, Iowa USA (1997).

Sambri,v.;Selfanelli,C.;Rossoni,C.R.;La placa,M and Cevenini,R . (1993).Acylated proteins in *Borrelia Hermsii,Borrelia Parkin* and *Borrelia anserina* and *Borrelia coriaceae* .Appl.Enviro. Microbiol, **59**:3938-3940.

Sambri,V.;Marang,A.;Olmo,A.;Storni,E.;Montaqnani,M.;Fabbi,M and Cevenini,R.(1999). Specific antibodies reactive with 22 kilodalton major outer surface protein of *Borrelia anserine* Ni-Nl Protect chicken from infection. Infect and immunity **67**(5):2633-2637.

Schwan,T.G.; Schrump, M.E.; Hinnebusch, B.J.; Anderson, D.Eand Konkel, M.M. (1996).GLPQ: An antigen for serological discrimination between re–lapsing fever and lyme borreliosis. J.Clin.Microbiol.**34**:2483-2492.

Schneider, M.D and Johnson, D.L. (1968)**.**Survival time and retention of antimalarial resistance of malarial parasite in reposity in liquid nitrogen (-195˚C) .Appl Microbiol. **16**:1422-1423.

Shommein ,A,M and Khogali ,A. (1974) .Fowl spirochetosis hematological and histopathological studies .Bull. Epizoot. Dis. Afr 22(3):255-261.

Snoeyenbos,G.N.In. Disease of poultry edited by Biester ,H.E an Schwarte,L.H.(1965)5th edtion Iowa state university press .

Soni,J.L.and Joshi,A.G.(1980) A note in strain variation in Akola and Jabalpur strains of *Borrelia anserina*.Zlbl .Vet .Med (B)**27**:70-72.

Soni, J.L and Cox, H.W. (1975a)**.** Pathogenesis of acute avian malaria, anemia mediated by a cold active autoheamagglutinin from the blood of chicken with acute plasmodium gallinacieum infection in chickens' .Am.J.Trop. Med. Hyg ,**24**: 206-213.

Soliman ,M.K.;Ahmed,A.A. ;Elamrousi,S and Mostafa,H.(1966) Cytological and biological studies on the blood constituent of normal and spirochete-infected chickens. Avian Dis.**10**:394-400.

Sreenvasan, M.K and Sankaranarayan, N.S. (1943). Spirochetosis in fowl in India. British Vet. J **.99**:208-214.

Steere,A,C.;Malawista ,S.E.;Bartenhagen,N.H.;Spider.P.N.;NewmaN.J.H.;Rahn.D.W.; Huchinson,G.J.; Green,J.Snydaman,D.R.and Taylar,E.(1984). The clinical spectrum and treatment of lyme disease.Yale J.Biol.Med.**57**:453-461.

Steinhaus, E.A and Hughes, L.E. (1947) .Abst.In. Boil Abstr.21:2009

Suliman,T.A.; Mamoun,I.E and Abdel Nour,O.M.(2001).Primary observation on spirochetes described from mosquitoes in Khartoum .Sudan J.Vet.Sci. Anim Husb (**40**)1&2:153-156.

Sumrov, I; Dzhanov, I; Penev, P and Lozeva, I. (1967) Immunological studies on antigen of Treponema anserina .Vet Med Nauki **4**:34.

Tom,G.S .;Buttisti,J.M.;Porcella,S.F.;Raffel,S.J.;schrumpf, M.E.; Fischer, J.A.; Caroll, J.A.; Stewart, P.E.; Rosa, P and Somerville, A. (2003).Glycerol 6 phosphate acquisition in spirochete distribution and biological activity of glycerolphosphodiester phosphodiestrase (GLPQ) among Borrelia species. J . Bacteriol, **185**(4): 1346-1356.

Verma, K.G and Malik, B.S .(1968). Diagnosis of poultry by slide agglutination test .Curr Sci **37**:170.

Verma, R.K.and Malik, B.S (1968) **.** Diagnosis of spirochetosis of poultry by gel diffusion test .Indian vet.J.**45**:460-462.

Wadalkar, B.G and Soni, J.L. (1982)**.**Use of fluorescent antibody technique for detection of spirochete antigen in prepatent phase and post spirochetemic phase in organs .Indian J. Anim. Sci. **52**:776-781.

Walker,R.L.; Greene,R.T.;Nicholson.W.L and Levine, J.F. (1989)**.**Shared flagellar Epitopes of *Borrelia burgdorferi* and *Borrelia anserina*.Vet.Microiology.**19**:361-371.

William, J.M (1955) Spirochetosis in Pheasants. JAVMA Feb 123-125

Willy ,Bergdorf (1998). Conference abstract Newyork city 11th International scientific conference on Lyme disease and other spirochete and tick borne disorder.

Wouda, W; Schillhorn, T.W and Barnes, H.J. (1975). *Borrelia anserina* in chickens previously exposed to *Borrelia theileri* .Avian Dis,**19**:209-210.

Wormser ,G .P.;Bittker,S.;Cooper,D.;Nowakowski,J.;Nadelman,R.B and Pavia,C.(2000).Comparison of the yield of blood culture using serum or plasma from patient with early lyme disease .J .Clin.Micro **38**:1648-1650.

Zaher, M.A.;Soliman, Z.R and Diab,F.M.(1977)**.** An experimental study of *Borrelia anserina* in four species of argas ticks transstadial survival 55and transovarial transmission .Z Parasitenkd **53**:213-223.

Zulzer, M. (1963) Culex a new vector of *Spirocheata gallinarum*.