

Experimental infection of three Nigerian breeds of sheep with the Zinga strain of the Rift Valley Fever virus

O.D. Olaleye^{1*} O. Tomori¹ J.L. Fajimi² H. Schmitz³

Key words

Sheep - Djallonké sheep - Yankasa sheep - Ouda sheep - Virus Rift Valley Fever - Strain - Septicaemia - Experimental infection - Nigeria.

Summary

Experimental infection of three indigenous breeds of sheep in Nigeria, namely the West African Dwarf (WAD), Yankasa and Ouda resulted in fatal disease with the Zinga Rift Valley Fever virus. Infected sheep of the three breeds responded by pyrexia within 24 h of infection, that lasted 6 to 7 days, but peaked between day 2 and 4 post-infection. Viraemia coincided with pyrexia and peaked (10^9 PFU/ml) 3 days p.i. in Yankasa and WAD sheep, but with highest titre ($10^{7.5}$ PFU/ml) in Ouda sheep. Zinga Rift Valley Fever virus infection of sheep was characterised by hyperactivity, watery and mucoid nasal discharges, projectiles and bloody diarrhoea, external haemorrhage and clinical manifestations of nervous disorders. Viraemia was followed by low level of antibody development in all the infected sheep. Haematological changes included a sharp fall in the PCV, Hb concentration and total RBC count during the course of the disease. These changes were most severe in the Yankasa, followed by WAD and Ouda breeds. There were thrombocytopenia, prolongation of prothrombin and clotting times in all the infected sheep. There was also progressive leucopenia associated with lymphopenia. The total protein and albumin levels were depressed, but the globulin level rose from day 5 p.i. The changes in the serum biochemical constituents included sharp and progressive increase in the level of alanine aminotransferase and aspartate aminotransferase. The sodium level decreased gradually while that of potassium was initially stable but later increased until the infected animals died. There was a significant increase in the level of blood urea nitrogen from day 3 p.i. that continued until the infected animals died. Gross and microscopic examinations of the carcasses of the infected sheep showed significant lesions in many organs, including disseminated intravascular coagulation.

INTRODUCTION

Rift Valley Fever (RVF) is an infectious arthropod-borne disease of ruminants and humans characterized by fever, hepatitis and abortion. Severe outbreaks have been reported in various parts of Africa, mainly South of the Sahara, where young animals,

especially lambs, are more susceptible and more severely affected than adults (10, 32, 46, 53). Although there has been no official report of RVF outbreak in Nigeria, virological and serological studies have indicated that the virus is circulating in the country (19, 26, 49, 50). The existence of predisposing factors for possibility of a severe outbreak of RVF in Nigeria was highlighted by Tomori (49). In addition, recent detection of specific RVF virus IgM in sera of humans and domestic animals (36, 37) calls for a reassessment of the disease in the country.

Because of the usually high morbidity of sheep and high mortality of lambs during RVF outbreaks, many experiments have been carried out using this species (16, 18, 48). This study compares the susceptibility of three breeds of sheep common in Nigeria - West African Dwarf, Yankasa and Ouda sheep - to experimental infection with the Zinga strain of the RVF virus.

1. Department of Virology, College of Medicine, University of Ibadan, University College Hospital, Ibadan, Nigeria

2. Department of chemical Pathology, University College Hospital, Ibadan, Nigeria

3. Department of Virology, Bernhard-Noch Institute of tropical Medicine and Hygiene, Bernhard-Noch Street, Hamburg, Federal Republic of Germany

* Address for correspondence

It is divided into five main sections:

- I. Clinical and immunological responses
- II. Haematology
- III. Biochemistry
- IV. Pathology - infectivity
- V. General conclusion

I. CLINICAL AND IMMUNOLOGICAL RESPONSES

■ MATERIALS AND METHODS

Preparation of the virus stock

The origin and the properties of the Zinga strain of the RVF virus have been described previously (35). The sixth mouse brain passage was used to inoculate the sheep. The virus was passed in mice 2 to 3 days old. Brain and serum materials were harvested from the sick mice for titration as the stock virus. A 10 % suspension of infected mouse brain in bovine albumin phosphate buffer solution (BAPS) containing 2000 IU/ml of penicillin and 60 µg/ml of streptomycin was centrifuged at 1000 g for 30 min. The supernatant from the brain suspension and the pooled serum from the mice were kept in aliquots at -70°C.

The contents of one ampoule of each of the two materials were titrated by 10-fold serial dilutions. A 0.02 ml aliquot of each dilution was inoculated i.c. into each of six 2-3 day old suckling mice which were observed daily for 14 days. End point titres were computed using the method proposed by Reed and Muench (40) and expressed as the logarithm to base 10 of the reciprocal of the 50 % end point. The infectivity titre of the brain material was $107.0 \log_{10} \text{LD}_{50}/0.02 \text{ ml}$, while that of the serum was $106.5 \log_{10} \text{LD}_{50}/0.02 \text{ ml}$.

Experimental animals

Six animals 7 to 11 months old from each breed and both sexes were used for the study. The three breeds of indigenous Nigerian sheep used were the West African Dwarf (WAD), Ouda and Yankasa. Their characteristics have been described by Oyenuga (38).

The WAD sheep were purchased from a village about 5 km North-East of Ibadan in one of the field stations previously used for other studies. It is known to be the only breed of sheep raised in the village. The Yankasa and Ouda sheep were purchased from a government farm at New Bussa in the middle belt of Nigeria. The WADs were aged by dentition; birth records were available for the Yankasa and Ouda. It is important to note that the animals were transported from the different sources to the laboratory by motor vehicle.

The animals weighed from 7 kg to 16 kg. They were kept in pens in fly-proof houses with complete partitions between the pens and zero-grazed throughout the experiment with *Panicum maximum* and *Centrosema pubescens* supplemented with dry groundnut and cowpea residues. Feed and water were supplied *ad libitum* but salt lick was not provided.

The animals were conditioned for two months before infection. During this period, they were checked for enteric and blood protozoan parasites and received a regimen of antibiotic, antihelminthic and anti-protozoan therapies. They were checked

again and found to be free of enteric and blood protozoan parasites. Blood from each animal was also inoculated i.c. into 2-3 day old suckling mice to check for subclinical or non-apparent infection with any arbovirus. All the animals were also negative for RVF virus HI antibodies.

Experimental design and infection with RVF virus

For each breed, the animals were randomly divided into two groups : 4 animals to be infected (tagged as 1, 2, 3 and 4) and 2 animals for control (tagged as 5 and 6). The animals from the three breeds assigned for virus inoculation were kept in three different pens (four animals per pen), while all the control animals were kept in two pens distant (separated by two pens) from the infected animals.

Of the four animals infected from each breed, two were inoculated s.c. with 0.1 ml RVF virus infective material, while the other two were inoculated with 0.15 ml of infective serum, giving approximately $5 \times 10^5 \text{ SMICLD}_{50}$ per infected animal. One of the two control animals in each breed was inoculated with a similar amount of supernatant from normal mouse brain suspension prepared in the same way as the stock virus, while the other control animal in each group received normal mouse serum.

Following the inoculation, rectal temperatures were taken daily between 8:00 and 8:30 a.m. Similarly, a 1:5 dilution of the blood was prepared in BAPS and inoculated i.c. into a 2-3 day old suckling mouse. The inoculated mice and animals were observed daily for signs of illness or death. The specificity of the virus isolated from the sheep experimentally infected using suckling mice was confirmed by a complement fixation test.

Routine bleeding procedures

Between 8:30 and 9:30 a.m. each day for the first ten days post-inoculation, 0.5 ml of jugular blood was collected from each animal into a plain sterile *bijou* bottle and kept at -70°C for viraemic assay. 2.5 ml of the same blood was dispensed into a plastic container with crystals of ethylene diamine tetra-acetate-EDTA (Seward, U.K.) as the anticoagulant. Another 1 ml of blood from each animal was placed in a *bijou* bottle containing 0.1 ml of double oxalate mixture for the determination of the clotting factors. Finally, another 5 ml of blood was collected into plain sterile universal bottles or vacutainer tubes for serology and biochemical analysis. Animals that survived beyond the first ten days were then bled weekly for serology and biochemical assays.

Virus assay

Viraemia in the infected animals was determined by plaque assay in Vero E6 cell monolayer cultures. Serial 10-fold dilutions of blood from all the infected and control animals were cultured using the Rovozzo method (42). The virus titres were determined and expressed in terms of plaque forming units PFU/ml of blood.

Serological assay

Following inoculation, antibody development by the infected sheep was measured weekly using the complement fixation (CF) test (45), the haemagglutination-inhibition (HI) test (5) and the plaque reduction neutralization test (NT) (by 80 % inhibition of plaques formed) in Vero E6 cell monolayers in 24-well microtitre plates (14).

Statistical analysis

The mean values of various parameters measured were compared between breeds with 95 % confidence using Student's t-test.

■ RESULTS

Infection and clinical findings

Infection with RVF virus was established in all the inoculated sheep. All of the animals inoculated with both brain and infective serum sources of the virus responded with elevated temperatures (40°C and above) within 24 h. The onset of fever was sharp, with a peak of 42°C between the 2nd and 4th day p.i. in different animals. The early stage of the disease was associated with hyperactivity which was quickly followed by anorexia and weakness in all the infected animals. Other clinical changes included watery and mucoid nasal discharges; watery, projectile and later bloody diarrhoea; increased respiratory rate, nervous signs which included abnormal gait, lateral flexion of the neck and sometimes posterior paralysis. These symptoms were most severe in the Yankasa breed.

The period of fever varied between and within breeds. It lasted between 6 and 7 days in infected WADs, 3-7 days in the Ouda and 3-4 days in the Yankasa. Furthermore, while all 4 Yankasa died during this febrile phase, most of the other WAD and Ouda animals died when the temperature was subsiding or had actually dropped to a state of hypothermia in some animals. During the febrile phase, the animals became dull, weak and had a rough hair coat. On the third day, the animals became inappetent but drank more water. This stage was followed by moderate to severe serous nasal discharges and laboured respiration becoming abdominal at the terminal stage in most of the animals.

Following this period, all the animals became diarrhoeic. The diarrhoea started as watery, projectile expulsion of highly fetid faecal materials, soiling the whole hind quarters. Within one or two days, the diarrhoea became severe and bloody. This was more marked in the Yankasa breed than in the other two. At about the same period, there were subcutaneous and conjunctival haemorrhages characterized by widespread areas of petechiation and ecchymoses, mostly seen in hairless areas.

All WADs and Oudas survived the early stage of pyrexia and widespread haemorrhage, but showed nervous signs which included unsteady gait, quickly followed by lateral flexion of the neck or extension of the neck, straddling of the legs and sometimes posterior paralysis. However, they later entered a state of hypothermia, sternal recumbency and brief coma before death.

The disease ran a peracute course in all the Yankasa sheep and they died on average after 5 days. The course of the disease was longer in the WAD and Ouda breeds, with the animals dying on average after 11 days and 9 days, respectively.

The clinical response and mortality patterns in the animal groups that were inoculated with either mouse infective serum or brain material were similar.

None of the control sheep from the three breeds showed fever or any clinical signs of disease throughout the experiment.

Viraemia pattern

The virus was detected by plaque assay in the blood 24 h p.i. The viraemia titre peaked on the 3rd day in most of the infected animals and on day 2 p.i. in the others. Peak titres of 10^9 PFU/ml were observed on day 3 p.i. in two Yankasa and one WAD sheep. Peak titres of $10^{7.5}$ PFU/ml and above were detected in all the other infected sheep.

There was a gradual clearance of the virus from the blood after the peak titre. The period of viraemia lasted 6 to 7 days in the WAD

and Ouda breeds, while all the Yankasa sheep died during the viraemic stage. Most of the animals died before total clearance of the virus from the blood had been achieved.

Comparison of the animals that received infective mouse serum and brain material did not show any difference in the viraemia titres within the different breeds.

Antibody conversions

Measurable levels of different types of antibodies (table I) showed that the pattern, as well as the levels of CF, HI and NT antibodies, were not affected by the fact that the inoculum was infective serum or brain material.

■ DISCUSSION

Subcutaneous inoculation of three breeds of Nigerian indigenous sheep resulted in fatal infection in all the animals. The short incubation period characteristic of natural and experimental RVF virus infection was observed in this study (1, 39, 54). Although there were slight variations in the course of the fever and viraemia between individual animals and breeds, they showed similar patterns. When the animals of the same breed which received either brain or serum infective inoculum were compared, there was no difference either in the onset or the severity of the fever.

The clinical changes observed in RVF virus-infected sheep were similar to earlier findings in both experimental and natural infections. However, different clinical patterns were observed in the three breeds. The course of the disease in the Yankasa sheep was very short, lasting 5 days on average. Following the peaks of fever and viraemia, the animals became diarrhoeic. The diarrhoea in the Yankasa sheep was marked by severe haemorrhage which continued until death. Bloody diarrhoea was less severe in Ouda and WAD sheep. Furthermore, there were petechiations and ecchymoses on the visible mucous membranes and hairless parts of the skin of the Yankasa sheep which were not observed in the other breeds. Dickson (11) and Henning (22) reported bloody diarrhoea in sheep naturally infected with the RVF virus. In addition, RVF virus-infected Yankasa sheep had severe respiratory distress associated with epistaxis, commencing from day 2 p.i. until death. As far as we can establish it, this syndrome has not been described before in either naturally or experimentally RVF virus-infected sheep. Using the classification of the disease's symptomatology proposed by Dickson (11), all the infected animals of the three breeds suffered the acute form of RVF.

II. HAEMATOLOGY

The pathogenesis of RVF, particularly the events leading to the different complications such as the haemorrhagic syndrome, is still not properly understood. Experimental infection of rats showed prolonged prothrombin time later in the course of the diseases (1), but the severe haemorrhage described in humans in the acute phase cannot be explained by this finding alone (30, 31). We sought to evaluate and compare the changes associated with certain haematological parameters in the three breeds experimentally infected with the Zinga strain of the RVF virus.

■ MATERIALS AND METHODS

Between 8:30 and 9:30 a.m. each day for the first 10 days, 2.5 ml of blood from each sheep was collected into a plastic container

with ethylene diamine tetra-acetate-EDTA (Seward, U.K.) as the anticoagulant. This sample was used for the determination of the packed cell volume, haemoglobin concentration, total red and white blood cells, thrombocytes, plasma fibrinogen and differential leukocytes. Another 1 ml of blood from each animal was placed in a *bijou* bottle containing 0.1 ml of double oxalate mixture (2) to determine the clotting factors.

Laboratory techniques and tests

The packed cell volume (PCV) was determined by the microhaematocrit method (43) using the Hawksley Microhaematocrit Centrifuge (Hawksley and Sons, London) at 1200 g for 5 min.

Haemoglobin (Hb) concentration: the oxyhaemoglobin method (43) was used.

Red blood cell (RBC) and white blood cell (WBC) counts: these were performed by the haemocytometer method, using the improved Hawksley haemocytometer (G. Gurr Ltd., London). The diluting fluid was 0.89 % saline for the RBC count and 1 % acetic acid coloured with gentian violet for the WBC.

Thrombocyte counts: the thrombocytes were enumerated by the Rees-Ecker direct method (52).

Plasma fibrinogen: this was quantified using a TS hand refractometer (American Optical Scientific Instruments Division, Buffalo, N.Y.).

Clotting time: this was determined as described by Kaneko and Cornelius (24).

Prothrombin time: this was estimated by the routine technique (9).

Differential leukocyte counts: thin blood films were prepared on clean microscope slides from EDTA-preserved blood soon after collection. They were stained by the Giemsa technique as described by Lynch *et al.* (27), using either commercially-prepared Giemsa solution or a laboratory solution prepared from powdered Giemsa stain (Sigma, Deisenhofen, Germany). The films were used to obtain WBC differential counts for which 200 cells were counted and classified.

Statistical analysis

The differences between the mean values of various parameters in the three breeds were compared using Student's *t*-test.

RESULTS

Red blood cell values

The mean PCV decreased from the pre-infection values of 29.3 %, 30.0 % and 30.3 % to 19.3 %, 17.5 % and 21.0 % on day 5 p.i. for WAD, Yankasa and Ouda breeds, respectively. The mean PCV of the two Oudas that survived up to day 9 was 16.5 %. By the time each of the infected animals of each breed died, its PCV had declined to about 50 % or more of the pre-infection value. In the WAD and Ouda breeds that survived beyond day 5 p.i., blood loss became very severe by day 7 p.i., after which animals in all the groups succumbed to infection with the RVF virus. When the red cell indices of infected and control animals of the same breed were compared at different stages of infection, the results showed lower PCV, Hb concentration and total red cell counts from day 3 p.i. in the Yankasa and, from day 5 p.i., in WAD and Ouda. Generally, slight variations occurred in the responses of individual animals within each breed. No alterations were found in the red blood cell parameters of control sheep of the three breeds throughout the period of the experiment.

White blood cell values

Slight variations occurred in the white blood cell changes between the breeds. The values declined gradually but were not significantly different from the pre-infection level ($t_{df4}=1.3$, $P>0.50$) or between control and infected ($t_{df4}=1.4$, $P>0.50$) WAD sheep. However, the total leukocyte counts in the infected Yankasa and Ouda sheep were significantly ($t_{df4}=4.9$, $P<0.001$ and $t_{df4}=5.5$, $P<0.005$) lower than the values for the control sheep of the same breed by day 5 p.i. and 7 p.i., respectively. The neutrophilic responses of the three breeds were different. West African Dwarf sheep showed marked neutrophilia from 3 p.i. until day 9 p.i., when 3 of the 4 infected WAD sheep died. Similarly, the Ouda sheep showed initial neutrophilia which progressed until day 7 p.i., followed by severe neutropaenia from day 9 p.i. until all the sheep in the group had died. Conversely, the Yankasa sheep had consistent neutropaenia throughout the period of infection with the virus. By day 5 p.i., the mean neutrophil value in the Yankasa sheep had dropped by 28.2 % (from 489.4 ± 46.8 to $352.0\pm 235.3/\mu\text{l}$). The lymphocyte counts declined to severely low levels before the death of the animals in all three breeds. The level was significantly lower in the infected sheep than in the control sheep by day 3 p.i. in the Yankasa, day 5 p.i. in the Ouda and day 9 p.i. in the WAD breed ($t_{df4}=5.5$, $P<0.005$ for Yankasa and $t_{df4}=54.4$, $P<0.001$ for Ouda).

There was no significant difference in the eosinophil and basophil values of the infected and control sheep from each breed. In addition, the white blood cell series of the control sheep from the three breeds showed no significant variation throughout the period of this study.

Coagulation factors

The infection led to thrombocytopenia which was progressive in the WAD and Ouda breeds but rapid in the Yankasa breed. While the thrombocyte values increased again on day 9 p.i. in the WAD sheep, the fall progressed in the Yankasa and Ouda breeds until the sheep died. There was no significant difference ($t_{df4}=14.29$, $P=0.6<P<0.7$) between the infected and control WAD sheep throughout the period of the infection. The difference in the thrombocyte levels in the infected and control Yankasa and Ouda breeds became significant ($t_{df4}=2.93$, $P<0.05$ and $t_{df4}=2.9$, $P<0.05$, respectively) from day 5 post-infection.

Plasma fibrinogen fluctuated slightly throughout the period of infection but was not different from the control sheep of the same breed. The prothrombin time (PT) increased progressively in the Yankasa and Ouda breeds from day 3 p.i. to a high level on day 5 p.i. and continued until all the sheep of both breeds died. The PT also increased in the infected WAD sheep compared with the controls.

The clotting time was progressively prolonged in all the infected animals but remained relatively stable in the controls. As with the other coagulation factors, the clotting time was most prolonged in the Yankasa, reaching twice the zero-day value by day 3 p.i. The second most prolonged clotting time was found in the Ouda breed. It was initially only moderately prolonged in the WAD sheep but also became severely prolonged by day 16 p.i.

DISCUSSION

There is very little information on haematological disorders associated with RVF virus infection in humans and domestic animals. An investigation of the human haemorrhagic form of

RVF during the 1977/78 epizootic by Boctor (4) and Laughlin *et al.* (25) showed certain haematological disorders in affected humans. Easterday (15) also reported alterations in the blood parameters of naturally infected animals. Under experimental conditions, Peters *et al.* (39) reported prolonged PT, disseminated intravascular coagulation, leukopaenia, thrombocytopenia and microangiopathic haemolytic anaemia in rhesus macaque monkeys experimentally infected with the ZH-501 strain of the RVF virus.

Studies of the mechanism of anaemia in RVF have indicated that many factors may be involved, although the anaemia is essentially haemorrhagic. In a study by Peters *et al.* (39), 3 of 15 rhesus macaque monkeys inoculated intravenously with the ZH-501 strain of the RVF virus had haemorrhagic fever syndrome. The workers suggested the involvement of the vascular endothelium and liver damage in the pathogenesis of haemorrhage in the infected monkeys. In this study, all the Yankasa sheep died during the early phase of the disease as a result of severe haemorrhage leading probably to hypovolaemic shock. Haemorrhage was moderate but also progressed till death in the WAD and Ouda breeds. The present results show that a haemorrhagic syndrome in animals can follow infection with certain strains of the RVF virus even by the subcutaneous route of infection. Earlier experimental infection of WAD sheep with other strains of the RVF virus (18, 48) did not result in the haemorrhagic syndrome observed in this study. Haemorrhage in the infected sheep probably resulted from a combination of factors. There was direct damage of the vascular endothelium in many organs, as evidenced by severe external bleeding in Yankasa sheep and intravascular coagulation and haemorrhage found in many organs (see Pathology). The animals also exhibited severe thrombocytopenia, prolonged prothrombin time (PT) and consequently the prolonged bleeding time found. Prolonged PT probably resulted from liver necrosis. It is therefore reasonable to suggest that the haemorrhage which occurred in the animals was initiated by destruction of the vascular endothelium and precipitated by an inability of the blood to clot due to severe thrombocytopenia and poor supply of prothrombin as a consequence of severe hepatic necrosis.

Leukocyte response

The leukocytic response reported by other workers in humans (25) and animals (15) included initial leukocytosis followed by lymphocytic leukopaenia. The results of this study follow a similar pattern. In all the infected sheep, leukocytosis occurred 3 days p.i. At this stage, neutrophilia occurred as the initial response of the animals to the virus infection because of tissue damage under inflammatory conditions or as a general response to stress conditions (13). A similar situation was observed at the onset of experimental disease in the case of infection of WAD goats with *peste des petits ruminants* virus (PPR) (34). The prolonged neutrophilia found in WAD sheep compared with the other breeds in this study may be a breed-specific reaction, because none of the animals showed any evidence of bacterial infection clinically or during post-mortem examination.

The exact mechanism of lymphopaenia is not clearly understood. However, the results of microscopic examination of the lymphoid tissues of the animals in this study show necrosis of the lymphocytes which indicates cytolytic destruction of the lymphocytes by the virus. Lymphopaenia could also result in limited antibody response to the virus by the animals, as reported earlier (see part I). It is not known if there are specific receptors for the RVF virus on sheep lymphocytes, but a similar phenomenon was reported concerning bovine rinderpest (3) and *peste des petits ruminants* (33, 34).

III. BIOCHEMISTRY

■ MATERIALS AND METHODS

Sampling methods have been described previously.

Laboratory analysis

Protein: total plasma protein was determined by a modified Biuret reaction (41) and albumin (12) using a sequential multiple analyzer 12/60 (Technicon Instruments Co., New York, USA). The plasma globulin was estimated by the difference between the total protein concentration and the albumin concentration.

Creatinine: the level was determined by a modified method using Laffe's alkaline picrate (51).

Sodium and potassium: the value was determined by flame photometry using an Elvi 655 photometer (13).

The other parameters were determined using a sequential multiple analyzer 12/60, as follows: carbon dioxide (bicarbonate concentration) and chloride were measured as described by Skeggs and Hochstrasse (47), calcium was estimated by the modified method of Gilelman (21), inorganic phosphate as described by Horst (23) and urea nitrogen as described by Marsh (28).

Liver enzymes: serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by the colorimetric method (51).

Statistical analysis

The mean values of different parameters in infected and control sheep of each breed were compared with a 95 % degree of confidence using Student's t-test.

■ RESULTS

Changes in protein levels

There was an initial sharp fall to less than 50 % of the pre-infection values of total protein, albumin and globulin by day 3 p.i. in all breeds. The value of the proteins was lower in infected sheep than control sheep of each breed by day 3 p.i. The albumin level dropped progressively until day 5 p.i. in the Yankasa sheep, day 9 p.i. in the WAD sheep and terminally in the Ouda sheep. However, the globulin level increased steadily from day 5 p.i. until death in all the infected animals (table I). By the time each animal had died, the globulin value was higher than the pre-infection value or the value in the WAD and Ouda control sheep. The infected WAD and Ouda sheep survived beyond day 5 p.i.

Creatinine and blood urea nitrogen

The creatinine levels of the three breeds only increased slightly throughout the course of the disease (table I). However, this was not significant. On the other hand, the BUN levels rose sharply and progressively following infection until the animals died. The increase in the BUN of infected sheep was significantly higher than the control sheep by day 5 p.i. in the Yankasa and Ouda breeds and similarly by day 7 p.i. in the WAD breed ($t_{df4}=7.6$, $P<0.005$; $t_{df4}=2.95$, $P<0.05$ and $t_{df4}=7.6$, $P<0.005$, respectively).

Serum electrolytes (Na⁺, K⁺, Cl⁻, HCO₃⁻)

The infection of sheep with the RVF virus was characterized by a gradual fall in the values of both electrolytes (table II). While the

TABLE I

Mean values of antibodies and some biochemical parameters
of three breeds of sheep experimentally infected with the Zinga strain of the RVF virus

Parameter (units)	W. AFR. DWARF				YANKASA				OUDA			
	Control		Infected		Control		Infected		Control		Infected	
	day 5	day 9	day 5	day 5	day 5	day 9	day 5	day 9	day 5	day 9	day 5	day 9
	n = 2	n = 2	n = 4	n = 3	n = 2	n = 2	n = 4	n = 1	n = 2	n = 2	n = 4	n = 2
CF antibody (titre)	0	0	4	32	0	0	4	4	0	0	8	16
HI antibody (titre)	0	0	10	128	0	0	0	10	0	0	10	40
NT antibody (titre)	0	0	0	10	0	0	0	0	0	0	10	20
Total protein (g/dl)	6.8	6.6	4.2	4.1	6.9	6.8	4.1	7.4	5.9	5.7	3.9	5.0
Albumin (g/dl)	5.3	5.5	2.5	1.6	5.2	5.4	3.0	2.0	4.5	4.6	2.5	2.1
Globulin (g/dl)	1.5	1.1	2.2	3.1	1.7	1.4	1.0	5.5	1.4	1.1	1.5	2.8
BUN (mg/dl)	27.6	28.2	29.9	15.6	29.4	30.1	32.9	10.8	24.5	22.7	43.9	22.4
Creatinine (mg/dl)	3.3	3.3	3.8	4.0	3.3	3.4	3.2	3.7	3.3	3.4	3.3	3.7
AST (SF unit/ml)	8.0	7.5	24.5	40.3	11.0	12.5	24.5	28.0	13.0	13.0	17.0	28.5
ALT (SF unit/ml)	20.5	20.5	44.8	40.1	22.5	23.0	38.0	52.0	24.0	23.0	37.3	41.0

TABLE II

Mean values of some serum electrolytes of three breeds of sheep infected with the Zinga strain of the RVF virus

Parameters (units)	W. AFR. DWARF				YANKASA				OUDA			
	Control		Infected		Control		Infected		Control		Infected	
	day 5	day 9	day 5	day 9	day 5	day 9	day 5	day 9	day 5	day 9	day 5	day 9
	n = 2	n = 2	n = 4	n = 3	n = 2	n = 4	n = 4	n = 1	n = 2	n = 4	n = 5	n = 2
Sodium (Meq/l)	142.0	143.0	125.0	110.3	137.0	138.0	123.8	112.0	141.5	143.0	127.3	112.5
Potassium (Meq/l)	6.8	6.7	6.8	13.4	6.8	6.6	9.6	12.0	8.1	8.2	6.8	12.5
Bicarbonate (Meq/l)	21.5	22.0	22.0	23.0	19.0	18.2	20.5	21.0	19.0	18.1	20.0	20.0
Chloride (Meq/l)	102.5	104.0	91.8	72.0	99.0	101.0	90.0	84.0	98.0	101.0	89.3	83.0
Calcium (mg/dl)	9.2	9.1	8.3	9.3	8.4	8.2	7.4	6.3	9.1	9.0	8.4	8.5
Phosphate (mg/dl)	6.9	6.8	7.1	7.0	5.2	5.4	5.5	6.7	6.3	6.4	6.1	6.0

sodium levels fell progressively until all the infected animals died, there was a slight rise in the potassium values on day 3 p.i. in Yankasa and on day 7 p.i. in the WAD and Ouda breeds. The level continued to rise progressively until the animals died.

There was a gradual decline in the level of chloride ions during the course of the disease while bicarbonate levels fluctuated slightly.

Serum calcium and phosphate

Throughout the period of the experiment, there was no appreciable difference in the calcium and phosphate values of RVF virus-infected and control sheep from all three breeds (table II).

Liver enzymes

Abnormalities in the liver function tests were detected from day 3 p.i. in all infected sheep of the three breeds (table I). The increase was highest in the WAD, followed by the Yankasa and Ouda, in that order. By day 5 p.i., the level of ALT in the serum of infected sheep was significantly ($t_{df4}=4.6$, $P=0.01$, and $t_{df4}=2.93$, $P<0.05$, respectively) higher than in control sheep in the WAD and Yankasa sheep. The same applied to Ouda sheep by day 9 p.i. ($t_{df4}=2.9$, $P<0.05$). The increase in the level of AST in the infected sheep was significantly higher by day 3 p.i. in WAD sheep, by day 5 p.i. in Yankasa sheep and by day 9 p.i. in Ouda sheep when compared with the levels in the controls of each breed over the same period ($t_{df4}=6.0$, $P<0.005$; $t_{df4}=7.0$, $P<0.005$ and $t_{df4}=12.9$, $P<0.01$, respectively).

■ DISCUSSION

The assessment of blood proteins during the course of RVF in experimentally infected sheep showed a similar pattern in the three breeds. The initial fall in the level of the proteins coincided with the stage of clinical haemorrhages and the external blood loss resulted in loss of the proteins into tissues. This is supported by the fact that the Yankasa breed, which suffered the worst haemorrhages, showed the most rapid fall in the level of total plasma protein. Hypoproteinaemia is a known characteristic of acute blood loss in animals (13). As a regenerative response, there was a gradual increase in the total plasma protein from day 5 p.i. in all the breeds. Apart from the Yankasa sheep which died within 5 days p.i., the globulin levels increased steadily until the animals died, but the albumin levels declined. The increased total protein observed was due to the increased globulin but the rate of albumin replacement could not compensate for the amount lost through bleeding vessels and damaged mucosal epithelial surfaces.

In this study, there was a progressive increase in the BUN levels. This shows that the kidneys were severely and progressively damaged by the virus, with up to 75 % of the nephrons becoming non-functional (3). Kidney damage in the infected animals was evident at post-mortem. Uraemia is an important complication of increased BUN levels. It may also be an important cause of death in animals or humans infected with the RVF virus.

Measurement of the electrolytes at different stages of RVF virus infection in sheep showed significant disturbances which could lead, either alone or in concert, to the death of the infected animals. Water and electrolyte disorders are important complications of liver and kidney diseases typical of the type seen in the animals infected with the RVF virus in this study. This hepatorenal syndrome occurred in the RVF virus-infected sheep. In addition, sodium depletion occurs in animals as a result of diarrhoea (24). Sodium depletion leads to extracellular hypovolaemia and, if it continues, leads to classical signs of dehydration, with an increase of the plasma urea concentration and haemoconcentration. A

build-up of plasma urea as a result of hyponataemia might have contributed to the high BUN levels found in the animals. The hyperkalaemia found in this study resulted from increased haemolysis leading to an excessive release of potassium from red blood cells into the extracellular fluid, mainly into the blood. The situation was compounded by the inability of the damaged kidneys to excrete potassium. Hyperkalaemia is a known complication of acute renal failure (13) and the major effect of hyperkalaemia is cardiac arrest (24). The hypochloridaemia encountered in this study may have been a direct consequence of hyponatraemia because chloride concentration is regulated passively by electrical gradients established by active sodium ion transport across cell membranes (13).

The two liver enzymes measured during the course of RVF in this study showed similar patterns in all the infected animals of the three breeds. The findings are consistent with earlier reports in natural human (46) and experimental animal (15) infection with the RVF virus. The period of elevation of both enzymes coincided with the period of elevated viraemia and, therefore, with the active replication of the virus in the liver. According to Duncan and Prasse (13), the magnitude of the increase is directly proportional to the number of hepatocytes affected. The fact that many hepatocytes looked normal microscopically (data not shown) at the stage when the Yankasa sheep died with concurrent high levels of liver enzymes in the serum showed that the early effects of the virus on hepatocytes may only be detected at the biochemical level (29). It also shows that an important effect of the RVF virus on cell structure is an alteration of the permeability of the cell membrane.

IV. PATHOLOGY - INFECTIVITY

■ MATERIALS AND METHODS

Necropsy of animals and histopathology

A complete post-mortem examination was performed on each animal as soon after death as possible and any gross lesions were recorded. The tissues were kept in sterile bottles and stored at -70°C for an infectivity assay and evaluation of the organ distribution of RVF virus complement fixing antigens (CF). Thin sections of tissues were fixed in a 10 % buffered neutral formalin solution. Standard paraffin sections were cut at 5 to 6 microns and stained with haematoxylin and eosin (8). The following special staining methods were also employed: Giemsa for inclusion bodies, Prussian blue for haemosiderin and Mallory's phosphotungstic acid haematoxylin method for fibrin (8).

Organ infectivity

The virus concentration in each organ was detected using 1 g of tissue previously frozen at -70°C in a plaque assay using Vero E6 cell monolayers in 24-well microtitre plates (42). The infectivity titre was calculated in terms of plaque forming units/g in each organ tested.

Organ distribution of RVF antigens

A 10 % suspension of each organ from infected animals, stored until then at -70°C , was made in medium 199. A 10-fold serial dilution of each organ suspension was made in microtitre u-plates and the titre of RVF virus CF antigen contained was determined by the method of Sever (45).

■ RESULTS

Gross lesions

The necropsy observations in all the infected sheep of the same breed were fairly uniform but varied in degree between the breeds. The Yankasa which ran a peracute course of the disease presented severe haemorrhagic manifestations, while the WAD and Ouda had severe hepatic necrosis with some degree of haemorrhagic manifestations.

Externally, the animals had their hind quarters soiled with blood-stained faecal material, petechiations and ecchymosis on the conjunctivae, oral mucosa, anal regions and other hairless parts of the body. All the animals had a serous oculonasal discharge and crusts on the nostrils, oral commissures and both canthuses of the eyes.

Respiratory lesions included hyperaemic turbinates and lesions of the nasal septum. Mucoid exudates filled the nasal cavities in the WAD and Ouda sheep. In all the Yankasa sheep, the nasal cavities, the trachea and the bronchi were completely occluded with blood clots. Blood-tinged frothy exudates in the WAD and Ouda sheep and frank blood in the Yankasa sheep oozed out from cut surfaces of the lungs after little or no pressure. The bronchial lymph nodes were highly oedematous and haemorrhagic in all the carcasses. The heart was very flabby and all the chambers of the heart were filled with blood clots which assumed the shape of each chamber in the infected animals. There were ecchymotic haemorrhages on the pericardial and endocardial surfaces of the heart and the myocardium appeared "cooked". In the Yankasa and Ouda sheep, the aorta and other large blood vessels contained blood clots. There was subcutaneous haemorrhage in all the carcasses. There were hyperaemia and focal haemorrhages along the gastrointestinal tract of all the animals and fibrin casts were found in the stomach and intestines of all the Yankasa sheep. The mesenteric lymph nodes were highly enlarged, oedematous and hyperaemic. There was marked hydroperitoneum in all the Yankasa sheep. The liver was severely congested, moist, heavy and slightly friable with pinpoint areas of necrosis on all the lobes and blood oozed out freely from cut surfaces in all the Yankasa sheep. The spleen was slightly enlarged in the Yankasa but shrunken and firm in the WAD and Ouda sheep. The pancreas was congested with prominent blood vessels in all the carcasses. The kidneys were pulpy and bulged on cut surfaces. There were petechiations on both the serosal and cut surfaces and the renal artery contained blood clots. The adrenal glands were enlarged and congested. The meningeal blood vessels of the brain were prominent and engorged with blood clots in all the carcasses. Cut surfaces of the brain also showed engorged blood vessels and petechiation. The cerebrospinal fluid was tinged with blood.

Microscopic lesions

The Yankasa sheep that died within 5 days of infection essentially showed haemorrhagic lesions with mild pathology in other organs. The alveoli were filled with oedema fluid and sometimes haemorrhages. The blood vessels were markedly congested with sludging and fibrin thrombi. There were focal haemorrhages in the heart muscles and blood vessels were congested with areas of intravascular coagulation. The intestines showed necrotic debris and blood in the lumen. There was marked hyperplasia and desquamation of the intestinal epithelium and the lamina propria was heavily infiltrated by inflammatory cells. The lymph nodes showed vascular congestion, intravascular coagulation and severe haemorrhage. Similar lesions were found in the kidney and the brain.

Other animals showed more severe lesions as well as mild to moderate haemorrhage in almost all the organs. The lesions included intravascular coagulation in the blood vessels of the liver, marked coagulative hepatic necrosis with both intranuclear and intracytoplasmic inclusion bodies. There were perivascular lymphocytic cuffing and moderate to severe lymphoid depletion of the follicles of both the spleen and lymph nodes. There was necrosis of the lymphoid cells and the macrophages were heavily laden with necrotic debris, erythrocytes and haemosiderin pigments. The kidneys showed marked tubular degeneration characterized by desquamation of the tubular epithelium and proteinaceous casts in the lumen. The central nervous system lesions included vascular congestion, intravascular coagulation, mild gliosis, neuronal degeneration, neuronophagia and satellitosis. The spinal cord showed mild hyperplasia of the ependymal cells and the epithelium of the spinal canal. There was also demyelination of the neurones and the spinal canal contained protein casts. There were intracytoplasmic inclusion bodies in the neurones of the cerebrum, cerebellum, brain stem and spinal cord.

In all the organs, the inflammatory infiltrates in the tissues were mixed and mainly comprised of lymphoid cells, neutrophils and histiocytes. Histopathologic lesions were observed in the adrenal glands, salivary glands, pancreas, ovaries, testes and eyes, but were rarely significant.

Organ infectivity and complement fixing antigen

Titration of organ homogenates of sheep succumbing to RVF showed high virus titres in the liver, spleen, lungs, lymph nodes and kidneys. The highest virus titre was found in the organs of Yankasa sheep, followed by the titre in Ouda sheep and that in the WAD sheep. Generally, the amount of CF antigen in the different organs of each animal followed the pattern of the infectivity virus titres. However, CF antigens were detected in some organs without infective virus, even in those animals which died during the viraemic stage.

■ DISCUSSION

The gross and microscopic lesions found in infected sheep were similar to those previously described in natural (10, 20, 44) and experimental (6, 16, 17) RVF in sheep. However, unlike most earlier reports where the lesions were confined to the liver with secondary spread to other organs, varying degrees of pathological changes were found in many organs of the infected animals in this study. This observation shows that Zinga RVF is pantropic. Using Nigerian Smithburn and Lunyo strains of the RVF virus, Tomori (48) found hepatic necrosis in only one WAD sheep that died after abortion following infection with the RVF virus. The same breed of sheep had widespread lesions when inoculated with Zinga RVF virus in this study. Furthermore, the infection of sheep also produced severe pulmonary congestion and oedema unlike the slight congestion and oedema reported by Coetzer (6). On the other hand, Daubney *et al.* (10) did not observe such changes in the lungs of sheep examined during an RVF epizootic. Another significant finding in this study is the severe cardiac dilation and the presence of blood clots in the heart chambers when the animals died. These changes strongly suggest that there was cardiac failure shortly before the animals died. It is also interesting that all the animals examined showed evidence of intravascular coagulation in their organs. This finding confirms earlier speculation that an important mechanism of the haemorrhagic form of RVF is the formation of disseminated intravascular coagulation (7). This syndrome was reported in human RVF in Egypt (25) and more

recently by Peters *et al.* (39) in experimentally infected monkeys. The current observations of disseminated intravascular coagulation show that, with the appropriate strain of RVF and a susceptible host, the classic haemorrhagic form of the disease can occur. Out of the three breeds of sheep in this study, the Yankasa showed the severest haemorrhagic syndrome in both clinical and pathological terms. The breed can therefore serve as a useful model for studying the detailed mechanism of haemorrhage in RVF.

So far, there have been few reports concerning the pathology of the RVF virus in the central nervous system of sheep. Daubney *et al.* (10) and Easterday *et al.* (16) did not observe pathological changes in the brains of the sheep they examined. Other workers reported mild degenerative changes of the neurones (44) and congestion and oedema of the brain (6). The infection of sheep with the Zinga strain of the RVF virus in this study produced advanced central nervous system pathology. Although the lesions described were found in all the infected animals, they varied in degree and distribution, depending mainly on the duration of the disease before death. This observation shows that the type of pathology that will occur under natural conditions will depend largely on the stage of the disease reached before death.

The organ infectivity assay in all the animals showed the presence of high concentrations of the virus in many organs. Higher virus titres were found in the organs of animals that died early. This may be associated with the high level of viraemia which characterized the disease. However, high titres of virus were also found in the organs of other animals, including those that died after the viraemia stage. This shows that replication of the virus probably continued in some organs after viraemia and supports the observation of Yedloutschnig *et al.* (54) that sheep can appear to recover from RVF and can be clinically normal, but that the carcass still remains infective when slaughtered. The detection of higher virus titres in the brains of animals that died later in the course of the disease supports the speculation that brain involvement in RVF occurs as a delayed complication of the disease.

Except in the salivary and adrenal glands, where CF antigen was detected without infective virus, the amount of CF antigen found in the organs was closely related to the infectivity titre in the different organs. Many of the organs can therefore serve as sources of CF antigen for the identification of the RVF virus in the tissues.

V. GENERAL CONCLUSION

Experimental infection of three indigenous breeds of sheep in Nigeria, namely West African Dwarf (WAD), Yankasa and Ouda resulted in fatal disease with Zinga RVF virus. Infected sheep of the three breeds responded by pyrexia within 24 h of infection. Fever peaked between day 2-4 p.i. and lasted 6 to 7 days. Viraemia coincided with pyrexia and peaked (10^9 PFU/ml) 3 days p.i. in two Yankasa and one West African Dwarf sheep. Peak titre of $10^{7.5}$ PFU/ml and above was detected in all other infected sheep. Infected sheep showed characteristic clinical changes which included hyperactivity, watery and mucoid nasal discharges, watery projectile and bloody diarrhoea, external haemorrhage and manifestations of nervous disorders. Viraemia was followed by mild antibody development in all the infected sheep. There was severe external haemorrhage in the Yankasa sheep, but mild to moderate in the other breeds.

Haematological changes included a sharp fall in the packed cell volume, haemoglobin concentration and total red blood cell count during the course of the disease. There were thrombocytopenia, prolongation of the prothrombin and clotting times in all the infected sheep. Infected animals also showed progressive leucopenia, which was associated with severe lymphopenia. Total protein dropped sharply during the early phase of the disease, but rose gradually from day 5 p.i. The rise was associated with gradual increase in the globulin level from day 5 p.i. On the other hand, the albumin level decreased progressively until the animals died. There was a significant increase in the level of blood urea nitrogen in all the infected animals from day 3 p.i., that continued until death.

Gross and microscopic examinations of the carcasses of all the infected animals showed significant lesions in many organs, which demonstrates that the Zinga RVF virus is pantropic for sheep and can also cause disseminated intravascular coagulation in this species.

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Résumé

Olaleye O.D., Tomori O., Fajimi J.L., Schmitz H. Infection expérimentale de trois races de moutons nigériens avec la souche Zinga du virus de la fièvre de la Vallée du Rift

L'infection expérimentale de trois races de mouton du Nigeria, les moutons Djallonké, Yankasa et Oudah, provoqua la maladie mortelle causée par la souche Zinga du virus de la fièvre de la Vallée du Rift. Dans les 24 h qui suivirent l'infection, les animaux infectés eurent de la fièvre qui dura 6 à 7 jours. Elle atteint son degré le plus élevé entre le 2ème et le 4ème jour post-infection (p.i.). Une virémie apparut avec la fièvre et atteint son niveau maximum (10^9 PFU/ml) 3 jours p.i. chez les moutons Yankasa et Djallonké mais avec les titres les plus élevés ($10^{7,5}$ PFU/ml) chez les moutons Oudah. L'infection des moutons par la souche Zinga du virus de la fièvre de la Vallée du Rift fut caractérisée par une hyperactivité, des écoulements nasaux séro-muqueux, une diarrhée sanglante et très liquide, une hémorragie externe et des manifestations cliniques de troubles nerveux. La virémie fut suivie d'un faible taux de développement des anticorps chez tous les moutons infectés. Les transformations hématologiques comportèrent une forte baisse de l'hématocrite, de la concentration d'hémoglobine et du taux des globules rouges pendant l'évolution de la maladie. Ces transformations furent les plus graves chez les moutons Yankasa, puis chez les moutons Djallonké et Oudah. La thrombocytopenie, les prolongations du temps de la prothrombine et de la coagulation furent observées chez tous les moutons infectés, ainsi qu'une leucopénie progressive associée à une lymphocytopenie. Les teneurs en protéines et en albumine baissèrent mais celle en globulines augmenta à partir du 5ème jour p.i. Les transformations dans les composants biochimiques du sérum comportèrent une forte augmentation progressive du niveau de l'alanine aminotransférase et de l'aspartate aminotransférase. Les taux de sodium baissèrent graduellement, mais ceux de potassium, bien que stables au début, augmentèrent par la suite jusqu'à la mort des animaux infectés. Il y eut une augmentation importante de l'azote uréique à partir du 3ème jour p.i. qui se poursuivit jusqu'à la mort des animaux infectés. L'examen macroscopique et microscopique des carcasses a montré des lésions importantes dans beaucoup d'organes, y compris des coagulations intravasculaires disséminées.

Mots-clés : Ovin - Mouton Djallonké - Mouton Yankasa - Mouton Oudah - Virus fièvre de la vallée du Rift - Souche - Septicémie - Infection expérimentale - Nigeria.

Resumen

Olaleye O.D., Tomori O., Fajimi J.L., Schmitz H. Infección experimental de tres razas de ovejas nigerianas con la cepa Zinga de la Fiebre del Valle del Rift

El resultado de una infección experimental con el virus de la Fiebre del Valle del Rift Zinga, en tres razas autóctonas de ovejas nigerianas, la Enana del Oeste Africano (WAD), Yankansa y Ouda, fue fatal. Las ovejas infectadas de las tres razas presentaron pirexia en las 24 h post-infección (p.i.), la cual duró de 6 a 7 días, con picos entre el día 2 y 4 p.i. La viremia coincidió con la pirexia, con un pico (10^9 PFU/ml) 3 días p.i. en las Yankansa y las WAD, siendo más elevado ($10^{7,5}$ PFU/ml) en las ovejas Ouda. La infección de las ovejas con el virus de la Fiebre del Valle del Rift Zinga, se caracterizó por una hiperactividad, descargas nasales mucosas y acuosas, diarreas sanguinolentas y acuosas, en proyectil, hemorragia externa y manifestaciones clínicas de alteraciones nerviosas. Todos los animales infectados presentaron, después de la viremia, un nivel bajo de desarrollo de anticuerpos. Los cambios hematológicos incluyeron una caída dramática en el hematocrito, concentración de Hb y del conteo de glóbulos rojos durante el curso de la enfermedad. Estos cambios fueron más severos en la raza Yankansa, seguidos por las WAD y las Ouda. En todos los animales se observó trombocitopenia, prolongación del tiempo de protrombina y de coagulación. Se observó también leucopenia progresiva asociada a la linfopenia. Los niveles totales de proteína y de albumina se encontraron reducidos, pero el nivel de globulina aumentó a partir del día 5 p.i. Los cambios bioquímicos en los componentes serológicos incluyeron un aumento dramático y progresivo del nivel de alanina aminotransferasa y de aspartato aminotransferasa. El nivel de sodio disminuyó gradualmente mientras que el de potasio fue inicialmente estable y luego aumentó hasta la muerte de los animales infectados. Se observó un aumento significativo en el nivel sanguíneo de nitrógeno ureico a partir del día 3 p.i., el cual continuó hasta la muerte. El examen macro y microscópico de las carcasses de los animales infectados, mostró lesiones significativas en varios órganos, incluyendo coagulación intravascular diseminada.

Palabras clave : Ovino - Ovino Djallonké - Ovino Yankasa - Ovino Ouda - Virus fiebre del Valle del Rift - Cepa - Septicemia - Infección experimental - Nigeria.