PREPARATION AND EVALUATION OF A BIVALENT *CLOSTRIDIUM PERFRINGENS* TYPES C AND D VACCINE FOR CONTROL OF SHEEP ENTEROTOXAEMIA IN **NIGERIA**

BY

*AUDU **EMMANUEL** ITODO*
D.V.M.. UNIVERSITY OF VETERINARY MEDICINE,
BUDAPEST, HUNGARY, 1977
M.Sc, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA, 1984.

A DISSERTATION SUBMITTED TO THE POSTGRADUATE SCHOOL **AHMADU** BELLO UNIVERSITY, ZARIA FOR THE DOCTOR OF PHILOSOPHY DEGREE IN THE DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, **ZARIA.**

1996

DECLARATION

I declare here that the study reported in this thesis was carried out by me, in the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria and at the National Veterinary Research Institute, Vom. The work was supervised by Professor J.U. Umoh, Professor J.O. Adekeye and by Dr. A.A. Adesiyun.

No part of the work reported here has been submitted for publication or for a degree elsewhere. The work of other investigators in relevant sections of this study is duly acknowledged and referred to.

DIGITIZED

Audu Emmanuel Itodo
AHMADU BELLO UNIVERSITY,
1996.

CERTIFICATION

The thesis entitiled: " Preparation and evaluation of a Bivalent *Clostridium perfringens* Types C and D Vaccine for control of sheep Enterotoxaemia in Nigeria" by Audu Emmanuel Itodo meets the regulations governing the award of the Doctor of Philosophy Degree of Ahmadu Bello University and is approved for its contributions to knowledge and literary presentation.

External Examiner

Prof. T.J.T. Princewill
Dept. of Microbiology
Rivers State University of Scince & Technology
Port-Harcourt, Rivers state

Internal Examiners
Prof. J.U. Umoh (Major Supervisor)
Department of Veterinary Public Health and Preventive Medicine
Ahmadu Bello University
Zaria

Dean Postgraduate School
Ahmadu Bello University
Zaria

Prof. J.O. Adekeye
Department of Veterinary Pathology and Microbiology
Ahmadu Bello University
Zaria

Date: Jan 8, 1996

DEDICATION

I dedicate this work to Almighty God for His protection throughout my pursuit of this study and also to my family for their patience and tolerance during my absence.

ACKNOWLEDGEMENTS

My profound gratitude goes to my supervisors, Professors J.U. Umoh (Major Supervisor), J.O. Adekeye and Dr. A.A. Adesiyun whose constructive criticisms, guidance and encouragement at all times made this work a reality.

I thank Drs. E.N. Eze and A.A. Makinde of the National Veterinary Research Institute, Vom and Professor P.D. Walker of Peter Walker Associates (Biotechnology Consultants), Kent, for their invaluable assistance and professional advice throughout the course of this study.

My thanks also go to the private farm owners in Plateau State, who welcomed me at all times despite all the inconveniences that accompanied the work.

Finally, I thank the Director Dr. A.G. Lamorde, National Veterinary Research Institute, Vom, for approving the course of study and providing fund for it.

ABSTRACT

Clostridium perfringens **enterotoxaemia is the second most important clostridial disease after black quarter in Nigeria. There is no vaccine or vaccination programme to control it in Nigeria. In order to combat the disease problem, strains of *CI. perfringens,* types C and D, isolated in Nigeria, were used to prepare bivalent bacterins and toxoids in adjuvant systems (potash alum and sodium alginate) for vaccination of sheep. Five vaccines (bacterins and toxoids) viz: Anaculture (FC), a formalinized culture or bacterin; Anaculture + Potash alum (PaT); Anaculture + Sodium alginate (SaA); Toxoid + Potash alum (PaA); and Toxoid + Sodium alginate (SaT) were prepared. The vaccines were subjected to standard tests and used to inoculate sheep. The quality and quantity of the antibody response in sheep sera was determined by toxin-antitoxin neutralization tests in mice and the rocket immunoelectrophoresis methods. The efficacy and shelf-life of SaT were determined by challenge and field tests, and by periodical testing of the vaccine's (SaT, stored at 4°C and 24-26°C) ability to protect guinea pigs challenged with *Clostridium perfringens* toxins (beta and epsilon) respectively.**

The estimated beta (type C) and epsilon (type D) toxin content in reinforced clostridial medium culture of *Cl. perfringens* used for vaccine preparation was 2500 and 4000 units (mouse minimal lethal dose) per ml respectively.

Antibody production as a result of vaccination with these vaccines was at its peak, 4 weeks post vaccination. Potash alum toxoid (PaT) produced the highest level (8.33+1.45 i.u. per ml) of beta-antitoxin,which

was significantly higher (P<0.05) than that of any other vaccine. Epsilon-antitoxin level of 16.58±2.99 i.u. per ml produced by sodium alginate toxoid (SaT) was significantly higher (P<0.05) than that produced by any other vaccine.

Pairing the beta antibody mean values present in sheep sera post vaccination per duration,the level of beta antitoxin at 4 weeks (5.57 i.u. per ml) was significantly higher (P<0.05) than that (2.49 i.u. per ml) at 2 weeks, that at 12 weeks (3.05 i.u per ml) and that at 40 weeks (0.82 i.u per ml). The mean epsilon antitoxin titre (8.55 i.u. per ml) measured at 4 weeks was significantly higher (P<0.05) than that at 2 weeks (3.21 i.u per ml), 12 weeks (5.77 i.u per ml) and 40 weeks (3.20 i.u per ml). Compared to sheep,a relatively high serum antibody titre was induced in rabbits. The pattern of antibody production in rabbit was similar to that in sheep. Potash alum toxoid (PaT) produced the highest beta-antitoxin titre of 42.0 i.u. per ml while sodium alginate toxoid (SaT) produced the highest epsilon-antitoxin titre of 44.7 i.u. per ml. The lowest levels of both beta and epsilon antibodies, 10.0 and 12.0 i.u. per ml respectively, were produced by formol culture or the plain bacterin (FC) 2 weeks after booster dose.

Except FC which produced just about the minimum internationally acceptable antitoxic values, all other vaccines produced two to four times the acceptable levels of beta and epsilon antitoxins in rabbits. At 40 weeks, the mean antitoxic values, except for sheep vaccinated with FC, still remained well above the protective levels of 0.1 - 0.2 i.u per ml of epsilon antitoxin and twice that value for beta antitoxin.

Production of specific antibodies (IgG, IgM) after vaccination of sheep with SaT was quantified by the rocket-immunoelectrophoresis method. Peak production for IgM was about the 14th day while IgG reached its peak at about the 28^{th} day post-inoculation.

Chi-square analyses on associations in the survival rate of guinea pigs vaccinated with *Cl. perfringens* types C and D sodium alginate toxoid (SaT) vaccine stored at 4^0C and at 24–26^0C through a 12 month period, and challenged with epsilon toxin in one instance and beta toxin on the other were not significant ($P>0.05$). Although the storage temperature (24–26^0C) did not affect the vaccine efficacy significantly there was a corresponding decrease in protection with increase in temperature. A 10 month period is recommended as the shelf-life.

The results of the field test showed an average disease-specific mortality rate for vaccinated sheep to be 1.9% while that for unvaccinated sheep (controls) was 25.3%.The Chi-square analysis of the associations between vaccination and mortality rate, as observed on 3 farms, was significantly different [$P< 0.05$, x^1(cal.)=28.03, x^1(tab.)=5.99, df=2] between the vaccinates and the controls in the rate of survival as a result of vaccination. Unvaccinated sheep were 13.3 times more likely to be affected by the disease than vaccinated sheep. The calculated vaccine efficiency for SaT was 98.6%.

All the vaccines can be said to be highly immunogenic. Sodium alginate as an adjuvant system had a good depot effect, enhanced the vaccine's efficiency and durability. Results presented in this Thesis have demonstrated the suitability of vaccines developed from local isolates of *Clostridium perfringens* for protection of sheep against enterotoxaemia in Nigeria.

TABLE OF CONTENTS

TITLE PAGE i

DECLARATION ii

CERTIFICATION iii

DEDICATION iv

ACKNOWLEDGEMENTS v

ABSTRACT vi

TABLE OF CONTENTS ix

LIST OF TABLES xii

LIST OF PLATES xiv

LIST OF FIGURES xv

LIST OF APPENDICES xvi

CHAPTER ONE

INTRODUCTION 1

CHAPTER TWO

2.0 REVIEW OF LITERATURE 4

2.1 The Organism 4

2.2. Antigenic Characteristics - Toxins 5

2.2.1 Alpha Toxin 5

2.2.2 Beta Toxin 6

2.2.3 Epsilon Toxin 7

2.2.4 Iota Toxin 8

2.3. Biological Assay of *Clostridium perfringens* Toxins (Toxicity) 8

2.4. Enterotoxaemia Disease 9

2.4.1 Pathogenesis of Disease 12

2.4.2. Type D 12

2.4.3.	Type C	14
2.4.4.	Type B	15
2.4.5.	Type A	16
2.4.6.	Differential Diagnosis of Disease	16
2.4.7.	Control and Prevention of *Cl. perfringens* Enterotoxaemia	17
2.5.	Vaccines	18
2.5.1.	Immune Response of Host Animals	19

CHAPTER THREE

EXPERIMENT 3.1.	PREPARATION AND TESTING OF *CLOSTRIDIUM PERFRINGENS* TYPES C AND D BACTERINS AND TOXOIDS	22
3.1.1.	ABSTRACT	22
3.1.2.	INTRODUCTION	23
3.1.3.	MATERIALS AND METHODS	24
3.1.4	RESULTS	33
3.1.5	DISCUSSION	37
EXPERIMENT 3.2	ESTIMATION OF IgG AND IgM LEVELS IN POOLED SERUM SAMPLES OF SHEEP VACCINATED WITH SODIUM ALGINATE VACCINE (SaT) BY THE ROCKET IMMUNOELECTROPHORESIS METHODS.	59
3.2.1	ABSTRACT	59
3.2.2	INTRODUCTION	59
3.2.3	MATERIALS AND METHODS	60
3.2.4.	RESULTS	65
3.2.4.	DISCUSSION	66
EXPERIMENT 3.3	PRELIMINARY FIELD TRIAL WITH SODIUM ALGINATE CLOSTRIDIUM PERFRINGENS TOXOID AGAINST ENTEROTOXAEMIA IN SHEEP	73
3.3.1	ABSTRACT	73
3.3.2	INTRODUCTION	74
3.3.3	MATERIALS AND METHODS	75

3.3.4.	RESULTS	77
3.3.5.	DISCUSSION	89
EXPERIMENT 3.4	DETERMINATION OF SHELF-LIFE OF THE SODIUM ALGINATE TOXOID(SaT) VACCINE	91
3.4.1	ABSTRACT	91
3.4.2.	INTRODUCTION	91
3.4.3	MATERIALS AND METHODS	92
3.4.4.	RESULTS	93
3.4.5.	DISCUSSION	93

CHAPTER FOUR

4.0	DISCUSSION AND CONCLUSION	96
	REFERENCES	99

LIST OF TABLES

Table 2.4.1:	Enterotoxaemias, major toxins, and related disease conditions caused by *Cl. perfringens*	20
Table 2.4.2	Pathogenesis pathways of enterotoxaemias caused by *Cl. perfringens* types C and D	21
Table 3.1.1.	Constructed dose scheme for the determination of anti-beta and anti-epsilon toxin levels in serum of vaccinated sheep and rabbits.	41
Table 3.1.2.	Constructed dose scheme for the determination of anti-beta and anti-epsilon toxin levels in serum of vaccinated sheep and rabbits.	42
Table 3.1.3.	Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks post vaccination in sheep vaccinated with *Clostridium perfringens* type C and D formalinized culture (FC).	43
Table 3.1.4.	Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks post vaccination in sheep vaccinated with *Clostridium perfringens* type C and D bacterin in potash alum adjuvant(PaA).	44
Table 3.1.5.	Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks post vaccination. in sheep vaccinated with *Clostridium perfringens* type C and D bacterin in sodium alginate adjuvant(SaA).	45
Table 3.1.6.	Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks post vaccination in sheep vaccinated with *Clostridium perfringens* type C and D toxoid in potash alum adjuvant(PaT).	46
Table 3.1.7.	Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks post vaccination in sheep vaccinated with *Clostridium perfringens* type C and D toxoid in sodium alginate adjuvant(SaT)	47
Table 3.1.8	Summary of means of beta anti-toxin values*, for 2 weeks, 4 weeks, 12 weeks and 40 weeks after initial vaccination of sheep with the vaccines.	48

Table 3.1.9: Summary of means of epsilon anti-toxin values*, for 2 weeks, 4 weeks, 12 weeks and 40 weeks after initial vaccination with the vaccines. . . . 49

Table 3.1.10: Antitoxin (Beta and Epsilon) values (i.u per ml) of rabbit sera 14 days after second injection with vaccines. 50

Table 3.2.1: Results of Rocket immuno-electrophoresis quantitation of pre- and post vaccination serum IgG in a group of sheep 1,7,14,28 and 42 days after vaccination with *Cl. perfringens* sodium alginate toxoid. 68

Table 3.2.2: Results of Rocket immuno-electrophoresis quantitation of pre- and post vaccination serum IgM in a group of sheep 1,7,14,28 and 42 days after vaccination with *Cl. perfringens* sodium alginate toxoid. 69

Table 3.3.1: Results of natural exposure of sheep vaccinated (A) with Sodium alginate toxoid (SaT) and unvaccinated controls (B) to enterotoxaemia. 82

Table 3.4.1: Survival rate of guinea pigs challenged* with epsilon (E group) and beta (B group) toxins after vaccination wth sodium alginate toxoid (SaT) stored at 4^0 C and 24 -26^0 C room temperature. 95

LIST OF PLATES

Plate 3.1.1: Colonies of *Cl. perfringens* surrounded by zones of haemolysis 51

Plate 3.1.2: Culture of *Cl. welchii* and *Cl. bifermentans* at 48 h on half-antitoxin egg yolk agar. 52

Plate 3.1.3: Culture of *Cl. welchii* type A at 24 h on half-antitoxin egg yolk agar. *Cl. welchii* type A-antitoxin on right-half of the plate. Thediffuse lecithinase C opacity is inhibited by alpha-antitoxin. 53

Plate 3.2.1: The rocket heights ofIgG concentrations measured before vaccination with *Cl.perfringens* toxoid (SaT). 70

Plate 3.2.2: The rocket heights of IgG concentrations measured 7, 14, 28 and 42 days after vaccination with *Cl.perfringens* toxoid (SaT). . 70

Plate 3.2.3: Rocket heights of IgM concentrations measured before vaccination with *Cl.perfringens* toxoid (SaT). . 71

Plate 3.2.4: Rocket heights of IgM concentrations measured 7, 14, 28 and 42 days after vaccination with *Cl.perfringens* toxoid (SaT). 71

Plate 3.3.1: The cortex and medullary regions of kidney of unvaccinated sheep which died during natural exposure to clostridial enterotoxaemia, showing areas of coagulative necrosis with slight cellular infiltration (HE-Stain). 83

Plate 3.3.2: Kidney section from a sick unvaccinated sheep killed in extrimis,after exposure to clostridial enterotoxaemia, showing haemorrhages, oedema and congestion with dilation of the blood vessels and fibrin deposits in the interstitial spaces (HE-Stain). 84

Plate 3.3.3: Kidney section from vaccinated (normal) sheep showing no significant lesions, after exposure to clostridial enterotoxaemia(HE-Stain). 85

LIST OF FIGURES

Figure 3.1.1: Overall comparison of mean serum beta and epsilon antitoxin titres produced by vaccines (FC, PaA, SaA, PaT and SaT) in sheep 2, 4, 12 and 40 weeks post-vaccination. 54

Figure 3.2.1: IgG and IgM concentrations (mg/ml), and beta and epsilon antitoxin titres measured 7 to 42 days after vaccination of sheep with sodium alginate toxoid (SaT). 72

LIST OF APPENDICES

Appendix 3.1.1. 55

Appendix 3.1.2 57

Appendix 3. 3.1 86

Appendix 3.3.2 87

Appendix 3.3.3. 88

Appendix 3.3.4. 89

Appendix 3.3.5. 90

CHAPTER ONE

INTRODUCTION

The genus *Clostridium* comprises a large number of toxigenic species, some of which are known to produce toxins, extracellular enzymes and other factors important in veterinary medicine (Sterne 1981). *Clostridium perfringens (welchii)*, a member of the genus produces at least 12 different soluble antigens referred to as toxins. It is, however, well known that four major lethal antigens are produced by *Cl. perfringens* (McDonel, 1980). These lethal toxins viz alpha, beta, epsilon and iota are used to group the species into five toxigenic types (A,B,C,D and E) which are responsible for specific disease conditions in animals (Brooks et al., 1957; Hauschild, 1970; Smith, 1975; McDonel, 1980, Sterne, 1981, Itodo and Ike, 1990; Blackwell and Butler, 1992).

The importance of the toxins particularly B, C and D in enterotoxaemic conditions resulting in deaths of sheep have been documented worldwide including Nigeria (MacAdam and Zwart, 1967; Hart and Hooper, 1967; Sterne, 1981; Princewill, 1983; Niilo, 1986; Itodo et al., 1987; Itodo and Ike, 1990; Itodo, 1991). In Nigeria, the disease affects sheep, goats, pigs and occasionally cattle (MacAdams and Zwart, 1967; Schillhorn van Veen, 1973; Itodo et al., 1983; Ugochukwu and Nwaneri, 1984).

The economic losses suffered by Nigerian farmers through enterotoxaemia in sheep are unknown but this may amount to several million naira per year. Of this amount, losses due to enterotoxaemia-like conditions caused by *Cl. perfringens* group of organisms account for a considerable portion (Njoku and Chineme, 1983; Princewill, 1983;

Ugochukwu and Nwaneri, 1984; Itodo et al., 1986a). The sporadic nature of *Cl. perfringens* enterotoxaemia in its incidence and mortality in herds and flocks makes it the second most important clostridial disease of sheep after black quarter in Nigeria (Eze, 1975; Seifert, 1975; Itodo et al., 1986a; Itodo, 1991).

The systematic study of the clostridia over many years resulted in the recognition of lethal toxins in culture filtrates which are the basis on which highly successful prophylactic vaccines are made. Multivalent vaccines have been developed comprising products derived from cultures of *Cl. perfringens* types B, C and D; *Cl. novyi, Cl. chauvoei* and *Cl. tetani* (Thompson and Batty, 1953; Thompson et al., 1969; Cygan and Buczek, 1989). When these vaccines are combined with certain adjuvants and injected by appropriate route, they confer a very high degree of immunity (particularly to sheep) against several clinically identifiable but separate diseases (Thompson et al.,1969). The treatment of enterotoxaemic conditions caused by *Cl. perfringens* is of limited value, neither antibiotics nor chemotherapeutic agents being very effective (Sterne, 1981, Blackwell and Butler, 1992). In addition, in most cases, the course of the disease can be so rapid that the animal is moribund or dead before treatment can be considered. However, while treatment is generally unsuccessful, all the conditions can be prevented by immunization (Jansen, 1967a, 1967b, 1967c).

Bacterins and toxoids (vaccines) have been prepared from toxigenic strains of *Cl. perfringens* for the control and prevention of enterotoxaemia of sheep in various countries (Bullen and Batty, 1957; Simos, 1982). In Nigeria, however, there is no vaccination programme to

contain the situation. Prompted by the above, the author embarked on isolation and typing of toxigenic *Cl. perfringens* types in Nigerian sheep and their environments (Itodo, 1984). As a result of this work, a subsequent preliminary investigation into preparation and testing of a pulpy kidney disease vaccine formulated with a local toxigenic strain of *Cl. perfringens* type D (Itodo et al., 1986) was done. The results obtained from this investigation form the basis for the present study.

With the obvious potential significance in pathogenesis and the economic impact *Cl. perfringens* enterotoxaemia would have in our livestock industry (Itodo, 1987), This study is being carried out with the following objectives:

(1) To produce 5 enterotoxaemia vaccines each with 2 antigenic components using local strains of *Cl. perfringens.*

(2) To assess the shelf life, potency, safety and immunogenicity of such vaccines in laboratory animals (mice, guinea pigs) and host animals (sheep).

(3) To study the effect of the adjuvant systems: sodium alginate and potash alum used in the preparation of the vaccines.

(4) The vaccine that elicited the highest titre of protective antibodies in vaccinated sheep (and rabbits) and found to be also cheaper to produce, easy to administer and has a good keeping quality (long shelf life) would be subjected to further tests including field test with the aim of developing a potent enterotoxaemia (Pulpy Kidney Disease) vaccine for routine vaccination in areas where the disease is enzootic or expected to occur in Nigeria.

CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 The Organism

Clostridium perfringens, is a member of the family *Bacillaceae* and of the genus *Clostridium*. It is also called *Cl. welchii* by some workers, a name derived from one of two different names *Bacterium welchii* and *Bacterium emphysematosum* that Migula suggested for it in 1900 (Simonds, 1915). *Cl. perfringens* is a gram-positive, anaerobic rod and non-motile spore-former. Strains of this species are divided into five types (A,B,C,D and E) based on their ability to produce four major lethal toxins: alpha beta, epsilon and iota (Brooks et al., 1957; Hauschild, 1970; Smith, 1975; McDonel, 1980). Eight other toxins or antigenic substances, (minor toxins) some of which have pathogenic significance or act as virulence factors may be produced. The most important of these is enterotoxin; followed by Kappa (collagenase); mu (hyaluronidase); and two haemolysins, delta and theta. The four major toxins produced by *Cl. perfringens* are responsible for several enterotoxaemic conditions of animals and humans (Itodo, 1991; Okewole, et al., 1991).

Several authors (Yamagishi, 1964; Smith, 1955; Princewill and Agba, 1982; Kholer, 1973; Itodo, 1984; Niilo, 1986) have demonstrated the ease with which this ubiquitous organism can be isolated and identified from soil, sewage, faeces, water and animal and human intestinal tracts.

Type A strains of low toxigenicity are the most commonly found in normal intestinal microflora and soil while types B,C,D and E appear to be restricted to the intestinal tracts of animals (Smith, 1975). However, types B, C and D have been isolated from soil in areas where

enterotoxaemias are enzootic or around animal stalls or runs (Sterne, 1981: Itodo, 1984; Niilo, 1986). Types B,C,D and E persist for only a short time in the soil (Smith, 1975). Animal faeces are responsible for deposition of types B,C,D and E in the soil, water, feeds and sewage. The frequency of isolation of this organism varied from one animal farm to the other. For sheep it was between 34.6±8% to 38.7±8%, while for cattle it ranged from 22.9±2% to 27.7±9% (Itodo, 1992). Kholer (1973) observed that varying the diet may change the numbers of *Cl. perfringens* in the faeces of same individual. Itodo (1984) also observed significantly larger number of the organism in faeces of sheep and cattle during the rains than during the dry season.

2.2. Antigenic **Characteristics - Toxins**

At least 12 different soluble antigens, referred to as toxins, that may be involved in the pathogenesis of *Cl. perfringens* in man and animal are produced. All the toxins appear to be proteins, are readily inactivated by heat and formaldehyde, are antigenic and combine with their antibodies in definite proportions (Willis, 1964; Smith, 1975; McDonel, 1980).

2.2.1 Alpha Toxin

While strains of all five types of *Cl. perfringens* produce this toxin, type A strains usually produce the greatest quantities. Alpha toxin has been demonstrated to have phospholipase C (lecithinase), lethal, necrotizing, haemolytic and cytolytic activities (Willis, 1977). The phospholipase C enzyme catalyzes the hydrolysis of the phosphodiester bond in position 3 of the lecithin molecule to produce phosphorylcholine and water-soluble 1, 2-diacylglyceride. Alpha toxins enzymatic nature

was first observed by Nagler (1939) and Seiffert (1939). The attack on lecithin is inhibited by alpha-antitoxin (Hayward, 1943) and is made use of in half-antitoxin egg yolk agar plates for the identification of *Cl. perfringens*. Alpha toxin has a molecular weight of about 30,000 (Mollby, 1978). Phospholipase C is a comparatively thermostable enzyme which can retain 45 percent of its activity when heated in a sealed ampule for 10 to 15 minutes at 100°C (Smith and Gardner, 1950).

An important biological activity of alpha toxin is its haemolytic nature. Haemolysis due to alpha toxin occurs in most erythrocytes except those of horses and goats. Erythrocytes of cattle and mice seem most susceptible while those of rabbits, sheep and human are moderately susceptible (Smith, 1975). Erythrocytes of many species do not lyse when exposed to toxin at 37°C. However, when cooled to 4°C rapid lysis occurs (hot-cold phenomenon). Alpha toxin is a well known cause of gas gangrene in humans (Willis, 1969).

Alpha toxin production also varies considerably with growth medium and amount of growth is not necessarily correlated with toxin production (Smith, 1975). Chopped meat medium (Nishida et al., 1962) with either fructose, dextrin or starch as the carbohydrate component and pH of 6.5-7.0 is the most convenient medium for the production of alpha toxin (Cruickshank et al., 1975; Smith, 1975).

2.2.2 Beta Toxin

This toxin (molecular weight, 30,000) is produced exclusively by *Cl. perfringens* types B and C. It is heat labile. While numerous other toxins are produced by types B and C, it is generally believed that beta toxin is the one primarily responsible for the symptoms noted in necrotic

enteritis of animals (Willis, 1969; Hauschild, 1971). Some slight differences in symptoms between enteritis caused by type B versus type C may be attributed to the production by type B of epsilon toxin (Bullen and Batty, 1956). The characteristic purplish necrotic area produced when beta toxin is injected by intradermal route is wider and irregular due to the presence of mu toxin while those of type C (most of which are without mu toxin) are smaller and more nearly circular. Heat stable inhibitors of trypsin, proteases as naturally present in sweet potatoes and soyabean have been shown to readily destroy beta toxin activity (McDonel,1980).

Beta toxin is necrotizing and haemolytic. Its maximum concentration occurs in young cultures grown in freshly prepared chopped meat broth containing 2% peptone, 0.5% sodium phosphate and 1% glucose, adjusted to pH 7.2. The culture should be harvested early, usually four to five hours after inoculation, to avoid inactivation of toxin by bacterial proteolytic enzymes (Thompson, 1963; Smith, 1975).

2.2.3. Epsilon Toxin

Cl. perfringens types B and D produce an essentially inactive proto-toxin that when digested with trypsin becomes highly potent toxin called epsilon toxin. This toxin is of particular interest in veterinary medicine because it causes a rapidly fatal enterotoxaemia (also called pulpy kidney disease and overeating disease) of sheep and other animals (Hauschild, 1971). It has a molecular weight values ranging from 24,000 - 34,250 (Habeeb, 1972) while the molecular weight of the proto-toxin is estimated at 25,000 - 32,700 (Habeeb, 1975; Worthington and Mulders; 1977).

Epsilon toxin is not haemolytic but is necrotizing on intradermal injection and is highly lethal. It affects the central nervous system by causing vascular permeability in the brain followed by liquefaction necrosis (Griner, 1961). The effect of epsilon toxin on the body, and one that is most important in the pathogenesis of the disease caused by type D strains, is its ability to increase the permeability of the intestines (Smith, 1955, 1975; Bullen and Batty, 1956). The proto-toxin is produced in greatest amount during the logarithmic phase of growth, in chopped meat broth adjusted to pH 6.7 - 7.4 (Pivnick et al., 1965).

2.2.4 Iota Toxin

Iota toxin is produced only by strains of *Cl. perfringens* type E. It is produced as a relatively non-toxic proto-toxin formed during the period of active growth of the organisms and is usually activated by the proteolytic enzymes produced by them. Iota toxin is lethal on intravenous injection and necrotizing but is not appreciably haemolytic. It increases permeability of the capillaries on injection into the skin (Craig and Miles, 1961).

Iota toxin is inactivated by temperature as low as 55^0. It's lethal, necrotizing and permeability inducing properties are inhibited by appropriate antitoxin (McDonel, 1980).

2.3. Biological Assay of *Clostridium perfringens* Toxin (Toxicity)

The toxic activity of *Cl. perfringens* toxin can be demonstrated by injecting 0.2 ml of test toxin intravenously, or 0.5 ml of toxin by intraperitoneal route in mouse or 0.2 ml of toxin intracutaneously in depilated albino guinea pig or rabbit as previously described (Oakley, 1943; Smith, 1975; Brit. Vet. Codex, 1970; Willis, 1964). The death of the

mice and the presence of characteristic necrotic lesions on the skin of guinea pigs or rabbits observed 48 to 72 hours later indicate the presence of a potent toxin (Mihut and Danes, 1989).

2.4. Enterotoxaemia Disease

Enterotoxaemia of sheep is mainly associated with *Cl. perfringens*, type D epsilon toxin (Bullen and Batty, 1957; Popoff, 1984). However, *Cl. perfringens* types A,B,C and *Cl. septicum* have also been implicated in some cases (Sterne, 1981). MacAdam and Zwart (1967) first diagnosed cases of ovine enterotoxaemia in Nigeria by demonstrating the causative agent, *Cl. perfringens* type D and its epsilon toxin in association with sudden death or profuse bloody diarrhoea. Since then very few workers have reported this very important disease in the country. A survey of clostridial organism causing diseases among livestock in Nigeria showed that 64% of all the clostridial isolates were *Cl. perfringens* (Princewill and Agba, 1982). Ugochukwu and Nwaneri (1984) reported incidence of 97.3% of ovine enterotoxaemia in Ibadan, Nigeria. Cases of sudden death associated with type D epsilon toxin was also reported in Vom, Nigeria, (Itodo et al., 1983). In a 2 year case study, it was observed that out of 180 samples (intestinal contents) from sheep dying suddenly, 153 were positive for *Cl. perfringens* type D epsilon toxin (Itodo, 1991). The disease affected more lambs (3 - 20 week old) during the period December to May than adult sheep at the rate of 25% and 13% respectively, while in the months of June to November, older sheep, (6 - 18 months old) were more affected than lambs at the rate of 68% and 47% respectively. There were no significant sex differences in prevalence (Itodo, 1991).

In Nigeria, the cold, wet, rainy season and the presence of abundant lush pasture for grazing appears to be a predisposing factor to the disease (Itodo, 1991). Itodo et al., (1986a) identified toxin-types of *Cl. perfringens* isolated from sheep, cattle and pasture soils revealing the occurrence of the organism as type A, 53.1%; type B, 7.1%; Type C, 5.9%; type D, 18.4% and 7.9% were type E while 7.5% the isolates were untypable and 2.4% were lost. The rate of detection of epsilon toxin varies according to month and season of the year, being highest in the wet months of June to September. For the same period an average rate (51.1%) of detection of epsilon toxin was recorded (Itodo, 1991).

Cl. perfringens produces various diseases in a variety of hosts worldwide (Table 2.4.1). The terminology used to describe these diseases can be variable and confusing because of the number of toxins and pathological manifestations that are recognized (McDonnel, 1980; Sterne, 1981). The most important enterotoxaemia, the classical, rapidly fatal type D enterotoxaemia, also known as sudden death, pulpy kidney and overeating disease is distributed throughout the world. It affects sheep of all ages except newborns (Griner et al., 1956, Smith, 1975; Sterne, 1981; Itodo and Ike, 1990; Itodo, 1991).

Next in economic importance are the haemorrhagic or necrotic enterotoxaemias caused by *Cl. perfringens* type C. Different varieties of this type have been found in different geographic locations throughout the world (Brooks et al., 1957; Niilo, 1980). It causes high mortality in 1 to 10 day old piglets, lambs, calves, foals, pigeons and chickens (Szent-Ivanyi and Szabo, 1956; Moon and Bergeland, 1962; Grieg, 1975; Sterne, 1981, Stubbings, 1990). The disease sometimes

occurs in older animals but it is primarily a problem of the newborn. Hogh (1967) reported a fatality rate of 12.5% to 83.3% among infected swine litters. "Struck", a disease of adult sheep caused by type C is usually associated with liver fluke infestation (Sterne, 1981). As with other enterotoxaemias, animals in good condition (the best feeders) appear to suffer most. The disease course is so rapid that the first signs are the finding of dead sheep often on its sternum (Jensen, 1974; Sterne, 1981).

Cl. perfringens type B causes lamb dysentery, a haemorrhagic and rapidly fatal enterotoxaemia of 1 to 2 week-old lambs and occasionally in neonatal calves and goats (English, 1966; Hart and Hooper, 1967; Sterne, 1981; Simos, 1982). Young animals probably get infected by contact from immune adult animals which act as carriers (Smith, 1975).

Type A strains of *Cl. perfringens* have been reported to cause enterotoxaemic conditions of nursing lambs (McGowan et al., 1958; Sterne, 1981) and of captive wild goats, *Capra ibex sibericus* (Russel, 1970) similar conditions have been described in reindeer (Kummeneje and Bakken, 1973) and piglets (Olubumi and Taylor, 1985; Okewole et al., 1991). Subcutaneous infections with gas bubbles in chickens (Saunders and Bickford, 1965) and in pigs (Okewole, et al., 1992) have been reported.

Cl. perfringens type E strains do not cause any significant disease condition in domestic animals except for the single case in calves reported in Australia by Hart and Hooper (1967). Type E strains may be occasionally pathogenic for rabbits (Baskerville et al., 1980; Sterne, 1981).

2.4.1 Pathogenesis of Disease

The disease conditions caused by *Clostridium perfringens* species is usually due to exotoxins produced by them (Mcdonel,1980). In all clostridial enterotoxaemias the mode of entry of toxigenic *Cl. perfringens* is by ingestion (Sterne, 1981). In some cases the causative strain may be present in the intestinal tract long before predisposing conditions (like abrupt changes in feeding habits, voracity or over- feeding) trigger the onset of the disease (Mumford, 1961; Griner, 1963; Urguter, 1965; Macadam and Zwart, 1967; Mansson et al., 1971; Kholer, 1973; Sterne, 1981; Princewill, 1983, Itodo, 1991). It is the microbe-toxin-host interaction that determines the initiation and outcome of the various enterotoxaemias (Table 2.4.2).

2.4.2. Type D

Where the disease is enzootic, normal sheep harbour *Cl. perfringens* type D in various parts of the alimentary tract. These strains provide the source for a fatal intoxication if conditions occur that are favourable for rapid and profuse growth of organisms in the intestine. Such conditions are associated with a sudden change from relatively poor diets to rich diets or gorging on palatable feed; hence the term over-eating disease. When sudden change occurs, the rumen flora which normally consists mostly of cellulose digesting microbes, becomes unbalanced and much undigested food passes into the small intestine. The presence of undigested starch in the intestine favours profuse growth of the organism and the consequent production of toxin (Bullen, 1970; Smith, 1975). Epsilon proto-toxin of relatively low toxicity is released by the multiplying bacteria in the lumen of the small

intestine and then rapidly activated by digestive enzymes such as trypsin.

When high concentration of epsilon toxin persist in the intestine for several hours, an increase in permeability of the intestine occurs leading to the absorption of large amounts of the toxin into the systemic circulation (Bullen, 1970). The active toxin selectively affects the brain, and to a certain degree, kidney and other tissues. The effect in the brain is manifested in the alteration of brain capillaries making them permeable to plasma proteins and water. This results in rapid extravasation of fluid into the brain and increased intracerebral pressure. This accounts for clinical signs of central nervous system derangement such as incoordination and convulsions (Griner, 1961). Oedema and effusions also occur to varying degree in the lungs, pericardium and other serous cavities (Blackwell, et al., 1991). Ultrastructural studies on the brain have shown that tight junctions in the vascular endothelium break down causing perivascular astrocyte processes to swell and rupture. This leads to the development of perivascular and intracellular oedema followed by focal to diffuse areas of degeneration and necrosis (Buxton and Morgan, 1976). Macroscopic foci of bilaterally symmetrical encephalomalacia have been observed in the campus striatum, thalamic area, midbrain, hippocampus and cerebellar peduncles, the extent of which vary with acuteness of the disease (Griner, 1961, Buxton, et al. 1978).

In type D enterotoxaemia, there are pathognomonic signs of hyperglycaemia and glucosuria due to glycogenolysis of liver glycogen reserves (Gardner, 1973). Hyperglycaemia coupled with tubular damage

in the kidneys leads to glucosuria. The pulpy kidney of sheep is a postmortem autolytic change occurring rapidly in damaged tissue.

2.4.3. Type C

Studies on pathogenesis of *Cl. perfringens* type C enterotoxaemia have been concerned mainly with susceptibility, clinical disease, and histopathology, but not much is known about its toxic mode of action. The initial activity of beta toxin in the intestine differs from that of epsilon toxin. Beta toxin is sensitive to proteolytic enzymes, its full effect on the mucosa mainly the jejunum, can take place only under conditions of diminished proteolytic enzyme activity such as lack of pancreatic secretion or presence of protease inhibitors in the ingesta (Niilo, 1986). Unlike epsilon toxin, it appears that beta toxin is not the sole attribute of virulence by which this type produces disease. Direct pathogenic action on the jejunal mucosa is preceded by attachment of type C organisms to the villous epithelium (Arbuckle, 1972). This is followed by necrosis and sloughing of the epithelium first on the villi and then extending to crypt epithelium as well as mesenchymal elements of lamina propria and muscularis, accompanied by haemorrhage (Bergeland, 1972). Masses of clostridia usually can be seen in necrotic tissue.

The production of other toxins such as alpha (phospholipase), kappa (collagenase), mu (hyaluronidase), and even the haemolysins may aid in pathogenesis due to their intimate contact with cellular elements of the mucosa (Niilo, 1986).

Beta toxin causes an increase in capillary permeability, and some of it is absorbed from the intestine as evidenced by finding the toxin, in

some cases, in pleural, peritoneal or pericardial effusions (Bergeland, 1972). However, it is not known how much the circulating toxin contributes to the pathogenesis of the disease. Experimental results on laboratory animals have shown that beta toxin, when administered intravenously raises blood pressure, possibly by acting on preganglionic fibres or central components of the autonomic nervous system and by release of catecholamines, may lead to cardiac failure (Sakarai et al., 1981). No other major pathophysiological effects as seen in epsilon intoxications have been observed with beta toxin.

Neonatal type C enterotoxaemia may result when pancreatic enzymes are not available in adequate amounts in the newborn to inactivate beta toxin, or when trypsin inhibitors are present in the colostrum. Low protein diets containing sweet potato act as trypsin inhibitors. Reduced pancreatic activity and sudden changes to rich meals are essentially contributory to human necrotic enteritis in Papua New Guinea (Lawrence and Cook, 1980). The pathogenesis of type C enterotoxaemia in older lambs (Struck), however, has not been satisfactorily explained and requires further elucidation.

2.4.4. Type B

Studies on *Cl. perfringens* type B enterotoxaemia (lamb dysentery) have been limited to epizootiology, immunization, and clinical observation in areas where the disease occurs (Sterne, 1981). Gross lesions are confined to the small intestine, and the initiation of the pathogenic process is like that of type C enterotoxaemia since the organism also produces beta toxin (Bergeland, 1972). In addition small amounts of epsilon toxin are produced and may add to the systemic effects (McDonel, 1980).

2.4.5. Type A

The enterotoxin of *Cl. perfringens* type A, the cause of human food poisoning has been studied extensively in animal models (Niilo, 1980, McDonel, 1980). It has only a peripheral importance in animal pathology, as such the pathogenesis will not be discussed here.

2.4.6. Differential Diagnosis of Disease

Unless there is overwhelming evidence that the flock is immune to type D, enterotoxaemia should be suspected whenever a number of sudden deaths occur (Sterne, 1981), accompanied by the symptoms and post-mortem findings described above. Detection of sugar in the urine of such cases is sufficient confirmation of disease. In making the diagnosis in adult sheep, which do not show pulpy kidney, is easily confused with hypomagnesaemia which also manifests in nervous signs and sudden deaths coupled with epicardial extravasation (Sterne, 1981, Simos, 1982).

Type C enterotoxaemia can be confirmed by the demonstration of *Cl. perfringens* beta toxin in intestinal contents or in the peritoneal fluid. At the same time black disease can be excluded by testing the peritoneal fluid for *Cl. novyi* beta lecithinase (Itodo et al., 1987). Clinically, 'Struck' can be distinguished from pulpy kidney by the absence of neurological symptoms, occasional presence of lameness, resistance of the animal to being moved and sternal position at death (Sterne, 1981). At postmortem examination struck is accompanied by pericardial and thoracic effusions but does not show epicardial and endocardial echymosis referred to as 'splashing' as may be seen in pulpy kidney.

In cases of struck, muscle lesions, if present, resemble putrefactive change and differ greatly from the dark red, spongy rancid-smelling necrosis characteristic of *Cl. chauvoei* or from the pale muscle embedded in an extensive oedema characteristic of black disease (McDonel, 1980).

Cl. perfringens type B infection can be differentiated from acute neonatal *Escherichia coli* enteritis by the presence of characteristic ulceration on the reddened, inflamed mucosa. Also, as in the case of lamb dysentery, large Gram-positive rods are plentiful in smears made from intestinal scrapings, which does not show the large Gram-positive rods that are plentiful on smear preparations from intestinal scrapings of animals dying of lamb dysentery. Also ulcerations on the reddened, inflamed mucosa as can be seen in lamb dysentery is absent in *E. coli* enteritis (Sterne, 1981).

2.4.7. Control and Prevention of *Cl. perfringens* Enterotoxaemias

Due to the acuteness of the clinical disease, no treatment is possible more so that the use of antibiotics or chemotherapeutics is not effective (Sterne, 1981). Effective protection of susceptible animals can be achieved by vaccination with appropriate toxoid (or bacterin) or by administration of hyper-immune antiserum. Field experience have shown that hyper-immune antiserum is the only treatment likely to be of value (English, 1966). Doses of antiserum varying from 2 to 2.5 ml have been used successfully in the various species, but in general death occurs too quickly for treatment to be effective. For lambs, 5-10 ml prophylactic doses of antiserum, types C and D are injected subcutaneously. The therapeutic dose injected subcutaneously or

intravenously is double the prophylactic dose. The use of antitoxin particularly against lamb dysentery shortly after birth can be helpful but it is costly. It is, therefore, suggested that a proper diagnosis of the specific clostridial infection be carried out during outbreaks and this should be followed up by prescription of the correct vaccine for active immunization (Sterne, 1981; Niilo, 1986). Active immunization of pregnant animals provide passive protection to their neonates by transmission of colostral antitoxin (Bergeland, 1972; Smith, 1975; Sterne, 1981).

2.5. Vaccines

Clostridium perfringens vaccines are cultures in fluid medium or filtrates of such cultures or materials derived therefrom, and consist of one or more types of *Cl. perfringens*, inactivated in such a manner that immunogenic activity is retained (British Veterinary Codex, 1970; Hydler, 1973).

The vaccines may be prepared from toxigenic strains of appropriate type of *Cl perfringens* under conditions which favour adequate toxin production (British Veterinary Codex, 1970; Smith, 1975). The lethal activity and the mouse lethal dose of the bacteria-free toxin is then exposed to the action of formaldehyde at 7.4–7.6 pH and at 37^{0}C temperature until they are sterile and non-toxic (Thompson and Batty, 1953). This method of preparation is applicable to *Cl. perfringens* types B, C and D. The actions of clostridial vaccines may be enhanced by incorporating adjuvants such as potash alum, oil or sodium alginate (Thompson and Batty, 1953; Thompson et al., 1969; Bhatty, 1983).

Vaccines in form of toxoids or bacterins with either one or more

antigenic components have been used to protect susceptible animals against enterotoxaemias (Kerry and Craig, 1979). Toxoids with adjuvants were found equally good if not better than bacterins (Bullen and Batty, 1953).

2.5.1. Immune Response of Host Animals

Humoral immunity plays an important role in the defence of the body against enterotoxaemias caused by *Cl. perfringens* (Thompson and Batty, 1953; Bullen and Batty, 1957; Hepple and Chodnik, 1959; Helwig et al., 1967; Bergeland, 1972; Itodo et al., 1986b). In immune sheep (exposed to *Cl. perfringens* type D), epsilon toxin is absorbed from the intestine in amounts sufficient to stimulate a rapid increase in the level of circulating epsilon antitoxin. Circulating antibodies against both epsilon and beta toxins may be measured by the toxin neutralization tests in mice (Carter, 1967; British Veterinary Codex, 1970) or quantified by the rocket immunoelectrophoresis (Weeke, 1973). The response of cattle to vaccination with *Cl. perfringens* type D toxoid was reported as satisfactory by Helwig, et al., (1967). Although the lowest level of circulating antibody which will give protection for 3 to 4 weeks in sheep was estimated to be 0.1 unit per ml of serum, double vaccination was considered suitable to protect against enterotoxemia in yearlings and adult cattle as well as in sheep preferably at 4 to 6 weeks interval between inoculations (Thompson and Batty, 1953; British Veterinary Codex, 1970).

Table 2.4.1: Enterotoxaemias, major toxins, and related disease conditions caused by *Cl. perfringens*

Cl. perfringens Type	Toxins	Disease
A Classical	Alpha (phospholipase C)	Gas gangrene in humans and animals, possibly colitis in horses and necrotic enteritis in fowl.
	Food poisoning enterotoxin	Human food poisoning
B_1 varieties	Beta (+ epsilon)	Lamb dysentery; neonatal haemorrhagic enteritis in calves, foals; haemorrhagic enterotoxemia in adult sheep and goats.
C_1 varieties	Beta	Neonatal haemorrhagic/necrotic enterotoxaemia (enteritis) in lambs, calves foals, piglets; acute enterotoxaemia (struck) in sheep, necrotic enteritis in fowl.
D	Epsilon	Classical enterotoxemia in sheep, goats and cattle.
E	Iota	Rabbit

Source: L. Nilo (1986)

Table 2.4.2 Pathogenesis pathways of enterotoxaemias caused by *Cl. perfringens* types C and D

Aspect of infection	C	D
Intestine, diet factors	Growth	Growth
Toxinogenesis	Beta toxin	Epsilon proto-toxin
Effect by digestive enzymes on toxin	inactivation	Activation to epsilon toxin
Effect by Immunity	Protection (toxin *neutralization*)	Protection (toxin *neutralization*).
Action on intestinal mucosa	Necrosis, haemorrhage, absorption	Capillary permeability, absorption
Target organ	Intestine	Vascular endothelium of brain/other organs
Clinical signs	Necrotic	Brain edema, CNS *disturbance*, hyperglucosuria, sudden *death*
Species affected	Newborn lambs, calves, foals, fowl, sheep, pigs.	Sheep, goats, cattle.
Commonly known disease	Haemorrhagic or necrotic enteritis	Enterotoxaemia
Prognosis	Fatal	Fatal

Source: L. Nilo (1986)

CHAPTER THREE

EXPERIMENT 3.1. PREPARATION AND TESTING OF *CLOSTRIDIUM PERFRINGENS* TYPES C AND D BACTERINS AND TOXOIDS.

3.1.1. ABSTRACT

Local strains of toxigenic *Clostridium perfringens* types C (KNC 831) and D (PD881) were used to produce five different vaccines each having two antigenic properties (beta and epsilon toxins). One batch was a plain bacterin (FC), while the remaining four batches were adsorbed in two different adjuvant systems, potash alum and sodium alginate. Thus there were bacterin in potash alum (PaA), bacterin in sodium alginate (SaA), toxoid in potash alum (PaT) and toxoid in sodium alginate (SaT). All the vaccines were subjected to standard quality control tests and were found safe for use in sheep and rabbits.

Antibody production as a result of vaccination with these vaccines was at its peak at 4 weeks post vaccination. Potash alum toxoid (PaT) produced the highest level of beta-antitoxin, 8.33+1.45 i.u.per ml and this was significantly higher ($P<0.05$) than that of any other vaccine. Epsilon-antitoxin level of 16.58+2.99 i.u. per ml produced by sodium alginate toxoid (SaT) was significantly higher ($P<0.05$) than that produced by any other vaccine.

Compared to sheep, a significantly higher($P< 0.05$) serum antibody titre was induced in rabbits. The pattern of antibody production in rabbit was similar to that in sheep. Potash alum toxoid (PaT) produced the highest beta-antitoxin titre of 42.0 i.u. per ml while sodium alginate toxoid (SaT) produced the highest epsilon-antitoxin titre of 44.7 i.u. per ml. The lowest levels of both beta and epsilon antibodies, 10.0 and 12.0 i.u. per

ml respectively, were produced by formol culture or the plain bacterin (FC). All the vaccines can be said to be highly immunogenic. Sodium alginate toxoid (SaT) was, however, the easiest to administer and it produced significantly higher ($P<0.05$) antibody titre than any other vaccine.

3.1.2. INTRODUCTION

Work reported by Thompson and Batty (1953) and Thompson et al (1969) highlighted the importance of the use of toxigenic *Cl. perfringens* strains and other clostridia in the production of vaccines to control enterotoxemia and other sheep diseases of clostridial origin. For active immunization of sheep, it is necessary to produce a vaccine which can impart a long lasting immunity such as that obtained with vaccines containing adjuvants. Such adjuvants include potash alum, oil-emulsion or sodium alginate.

Oil adjuvant vaccine has a marked superiority over ordinary bacterin and alum precipitated vaccine (Thompson and Batty, 1953). Despite the fact that oil adjuvant vaccines have been acclaimed by workers to be very potent against clostridial diseases of livestock, their use is still restricted due to problems associated with its production and administration. The production is cumbersome and requires special equipment. The emulsion prepared with oil is thick and creamy, and therefore, cannot pass easily through hypodermic needles. Hence its use in large scale vaccination in enzootic areas is traumatic.

D'Salvin (1950) employed sodium alginate successfully as an adjuvant in the preparation of bacterial hyperimmune serum in rabbits. Amies (1959) used 1% sodium alginate in normal saline or distilled water for production

of diphtheria toxoid replacing potash alum. Experiments carried out with the two vaccines showed that the use of alginate increased the immune response tenfold. In a recent study, a formalin inactivated and sodium alginate adsorbed *Salmonella gallinarum* (strain 9S) vaccine was reported to have a longer shelf life than the live vaccine and also had a protective potency which was as effective as that of the live one (Itodo et al., 1989). According to Bhatty (1983), Palit and Nilakantan used the oil emulsion and sodium alginate in the preparation of anthrax spore vaccine which when tested in cattle, sheep and goats, the efficacy achieved with the sodium alginate vaccine was better than that of the oil adjuvant vaccine.

This experiment was designed to prepare *Clostridium perfringens* vaccine in adjuvants(sodium alginate and potash alum) and to compare the duration of immunity conferred by potash alum, sodium alginate and plain bacterin vaccines prepared using Nigerian strians of *Cl. perfringens* culture isolates.

3.1.3. MATERIALS AND METHODS

Five different vaccines each containing two antigenic components of *Cl. perfringens* types C (beta toxin) and D (epsilon toxin), were produced using two local strains of toxigenic *Cl. perfringens*. The characteristics of the vaccine strains, particularly their toxicity, and virulence, which may be lost during storage (Lozano *et al.*,1970), and therefore their eligibility for use in vaccine production were re-examined (Itodo *et al*,1984).

Seed Cultures:

Highly toxigenic local strains of *Cl. perfringens* types C (KNC 831) and D (PD 881), which were maintained by once a year passage into pigeons, to enhance and maintain their virulence. A seed culture about 1-2

ml was inoculated into a young pigeon in the sternal muscle (the bird died in less than six hours of inoculation). Soon after, the muscular exudate would be asceptically collected and grown in reinforced clostridial medium for 24 hours at 37°C, lyophilized and stored until required.

Borate Buffered Saline (BBS):

Sodium pyroborate, 57 g; boric acid, 84 g and sodium chloride, 99 g were mixed dry in an electric lender and 74 g of this stock buffer salts (mixture) is added to 15 g sodium chloride (0.3%) and dissolved in 5000 ml distilled water. The pH was adjusted to 8.4 using 10 Normal sterile sodium hydroxide. This solution was used to make toxin antitoxin mixtures up to required volume.

Standardard antisera and toxins:

International standard antisera (10 i.u per ml) and toxins (L+ dose each per 0.1 ml of their respective solutions) kindly supplied by the Wellcome Research Laboratoreis, U.K., were used to set up control tests.

Vaccine Preparation:

A seed culture, 1 ml, pure 5-hour culture in cooked meat medium, was inoculated into a young pigeon in the thigh or sternal muscle which killed bird within 5 - 6 hours. Soon after death, 2 ml of the muscular exudate was aseptically collected in a syringe and inoculated into 10 ml of reinforced clostridial medium (RCM) in test tubes. The 5 - 6 hour growths of this culture were then inoculated into production flasks, each containing RCM broth (1000 ml) and incubated at 37°C. After 12 hours of incubation for type C or 20 hours for type D, the growth was stopped and tested for purity and aerobic sterility by cultural and microscopic examination. The total viable count per ml was determined (Miles and

Misra,1938) on blood agar plates incubated anaerobically. The pH was adjusted for optimum growth and toxin production during incubaton.The pH was adjusted to 7.2 for type C and 7.4 for type D with 10 N sterile sodium hydroxide using the Lovibond comparator.

About 5 ml of the product from each flask was removed and tested for presence of toxin in mice. At least 2 mice were inoculated intravenously via the tail vein with 0.2 ml of the clean centrifuged product and the mice usually died within 8 - 24 hours.

Trypsin (Merck) was added to the production flask containing type D, to a final concentration of 0.25% and mixed thoroughly with the culture and the flasks were again incubated at 37^{0}C for one and half hours. A test of the supernatant was again carried out in mice in a dose of 0.05 ml which killed them within a very short time, indicating that the epsilon proto-toxin had been converted to very active epsilon toxin after treatment with trypsin. Two mice (controls) which were each given 0.05 ml of the untrypsinized culture (proto-toxin) survived. Dilutions of supernatants of types C and D were each made, using sterile normal saline solution with the pooled bacteria-free toxin and the lethal dose (toxin content) per ml of each product was determined. This varies from 1000 to 8000 mouse minimum lethal dose per ml (British Veterinary Codex, 1970).

Plain bacterin:

Formalin was added to the whole cultures of types C and D (after trypsinization), separately, to give a final concentration of 0.6%. The formalinized cultures were pooled together in equal volumes, thoroughly shaken and left to stand at 37^{0}C for 14 days till the product becomes non-toxic to mice in a dose of 0.2 ml intravenously. The two dilution effects

would have reduced the fixed toxin content in the product to approximately 850 and 1000 mouse minimum lethal dose for type C and D respectively.

Adjuvant vaccines:

The formalized combined whole culture of types C and D was divided into two. Potash alum (Kodak Limited,London)was added to one part to a final concentration of 1%. The other part was mixed with 2% sodium alginate (BDH Chemicals Limited,London)in distilled water in equal volumes. The formalinized mixed culture of types C and D was centrifuged at 15,000 g to obtain the supernatant (toxoid). This toxoid was treated similarly as above with the two different adjuvant system. All vaccines were dispensed aseptically into amber-coloured sterile bottles, covered, labelled and stored at 4^0C until untill tested for sterility, safety and potency.

In all, five types of *Cl. perfringens* vaccines were prepared:

I.FC = Anaculture (formalinized whole culture or bacterin)

II.PaA = Anaculture + Potash alum

III.SaA = Anaculture + Sodium alginate

IV.PaT = Toxoid + Potash alum

V.SaT = Toxoid + Sodium alginate

Standardization of Vaccines

Toxin Assessment: Adapting the method described in the British Veterinary Codex (1970), dilutions of the bacteria-free toxin were made and their minimum lethal dose (toxin content) per ml of type C or type D cultures determined as illustrated below for type D:

(A)	1 ml of toxin + 9 ml of saline	1:10
(B)	1 ml of (A) + 9 ml of saline	1:100
(C)	5 ml of (B) + 5 ml of saline	1:200
(D)	1 ml of (C) + 1 ml of saline	1:400
(E)	1 ml of (C) + 2 ml of saline	1:600

(F) 1 ml of (C) + 3 ml of saline 1:800
(G) 1 ml of (C) + 4 ml of saline 1:1000

Three mice were each injected intravenouslv (caudal vein) with 0.2 ml of each of the above dilutions (per toxin type) and results read as shown below:

Dilutio	Dose	Units	No.of mice injected	No.of mice dead	Mortality ratio
C. 1:200	0.2ml	1000	3	3	3/3
B. 1:400	0.2ml	2000	3	0	0/3
C. 1:600	0.2ml	3000	3	0	0/3
D. 1:800	0.2ml	4000	3	0	0/3

The above supernatant contains 1000 units per ml of (epsilon) toxin

Sterility test: Blood agar plates and reinforced clostridium media were inoculated with a sample (1 ml) of each product,incubated aerobically and anaerobicallv at 37^{0}C and observed for a period of 7 days (British Veterinary Codex, 1970).

Safetv test:

1. Two sheep were each given 10 ml of vaccine subcutaneously;
2. Two rabbits were each given 3 ml of vaccine subcutaneously;
3. Two guinea pigs were each given 1 ml of vaccine subcutaneously;
4. Two mice were each given 0.5 ml of vaccine subcutaneously; and observed for 7 days.

Potency testing of Vaccine

The potency of the vaccines was tested in sheep and rabbits. Five groups of ten, six to ten month-old, unvaccinated Yankasa sheep, housed in clean accommodation for the duration of the study to reduce any effect of pasture derived organisms on the antibody response to the clostridial vaccines (Bullen and Batty, 1957), were used. Five groups of five four-

month-old New Zealand White X rabbits each were also used to assess the efficacy of the vaccine batches.

Pre-vaccination sera were collected from both sheep and rabbits and stored at -20^{0}C until used. A field dose of vaccine, 2.5 ml per rabbit and 5 ml per sheep was used (Thompson and Batty,1953,Itodo *et al*,1984). Sheep were each given 5 ml of vaccine by subcutaneous route. A second dose (5 ml) of vaccine was repeated two weeks later. Rabbits were each given 2.5 ml of vaccine by subcutaneous route. A second dose of vaccine of 2.5 ml was repeated two weeks later. After a further interval of 14 days rabbits were bled via the ear vein. The antitoxin content of rabbit sera, mixed in two pools, was estimated as recommended for potency tests (British Veterinary Codex,1970).

After vaccination of sheep with each of the five preparations the concentration (i.u per ml) of beta and epsilon antitoxin in serum samples taken via the jugular vein at the following times during the course of the experiment was measured and compared:

1. Two weeks after the first and at the time of second injection showing response to the sensitizing dose.
2. Four weeks after the first injection (or 2 weeks after booster dose) showing the values obtained at or about the peak of the secondary response.
3. At 12 weeks, and ten months thereafter in order to follow the duration of the immunity in the sheep.

Assay of the Vaccines:

The titres of anti-beta and anti-epsilon toxin from vaccinated sheep and rabbits were determined by toxin-antitoxin neutralization tests in mice

and rabbits were determined by toxin-antitoxin neutralization tests in mice (employing standard toxins and sera kindly supplied by Wellcome Research Laboratories, U.K.) as described in the British Veterinary Codex (1970). Prevaccination and postvaccination sera collected from the above sheep and rabbits were mixed (neutralized) by inversion, with equal volumes of their respective dilutions of toxins in tubes, and left to stand at room temperature for 30 minutes. Each mixture was injected into each of 2 white mice with 0.5 ml intravenously. Control mice, 2 per each level, were injected with only 0.5 ml saline containing the appropriate dose of toxin. They were observed for 5 days and results recorded.

Assay of *Cl. perfringens* beta antitoxin component

Standard serum The interntional Standard *Cl. perfringens* type B antitoxin was diluted in BBs to 10 i.u. per ml.

Test toxin Batch S/WB1 has an L+ dose of 0.22 mgm.

0.022 gram dry toxin dissolved in 10 ml. in peptone broth gives 2.2 mgm per ml.

Toxin control:Tube	1	2	3	4	5
Toxin solution	0.26	0.32	0.40	0.48	0.60
Antitoxin(10 i.u./ml)	0.40	0.40	0.40	0.40	0.40
BBs	1.34	1.28	1.20	1.12	1.00
Dry toxin(mg/mouse)	0.141	0.176	0.22	0.264	0.317
Test Serum					
Serum (neat)	1.60	0.80	0.40	0.20	0.10
Toxin solution	0.40	0.40	0.40	0.40	0.40
BBS	0.00	0.80	1.20	1.40	1.50
Units/ml tested for	2.50	5.00	10.00	20.00	40.00

Assay of Cl. perfringens epsilon antitoxin

Standard serum

The international Standard *Cl. perfingens* type D antitoxin was diluted n BBS to 10 i.u. per ml.

Test toxin

Batch S/WD2 has an L+ dose of 0.0119 mgm.

0.0119 gram of toxin was dissolved in 100ml peptone broth to give 0.119 mgm per ml.

Toxin control:Tube	1	2	3	4	5
Toxin solution (ml)	0.26	0.32	0.40	0.48	0.60
Antitoxin (10 i.u/ml)	0.40	0.40	0.40	0.40	0.40
BBS (ml)	1.34	1.28	1.20	1.12	1.00
Serum under test					
Serum (neat) (ml)	1.60	0.80	0.40	0.20	0.10
Toxin solution (ml)	0.40	0.40	0.40	0.40	0.40
BBS (ml)	0.00	0.80	1.20	1.40	1.50
Units /ml tested for:	2.50	5.00	10.00	20.00	40.00

For a closer result, a dose range (Tables 3.1.1 and 3.1.2) was used as explained below:

The preliminary assay using two-fold dilutions indicates the probable unitage of an unknown serum. For example, if a *Cl. perfringens* beta antitoxin assay results in the death of mice given only 0.2 ml serum to protect them against one L+ dose of toxin, whereas the mice given 0.4 ml survived, the dose which afford 50% protection lies somewhere near 0.3 ml, which represents a value of 3.75 units per ml of serum. (The higher the level of L+ dose used the more the antitoxic value).

Looking at the largest rectangles of Tables 3.1.1 and 3.1.2 you can find the value nearest to 3.75. This figure is 3.81 which occurs in three places. The priority of choice is:

For accuracy, use the value nearest the top of the page.

For economy of serum, use the value nearest the bottom of the page.

For economy of toxin, use the value furthest to the right in its table provided the test toxin contains enough lehal doses in the dilution indicated at the head of the column.

If there is adequate supply of toxin and serum, the value of 3.81 in the second column of Table 3.1.2 is first choice. A range about this value can be constructed so that it overlaps the preliminary range (2.50- 5.00 units per ml).

In this example this will be the first eight doses of column 1, Table 3.1.1 which are compounded as follows:-

Tube	1	2	3	4	5	6	7	8
Serum vol.in ml (from column 1)	1.60	1.44	1.30	1.17	1.05	0.95	0.85	0.76
BBS (ml)	0.00	0.16	0.30	0.43	0.55	0.65	0.75	0.84
Toxin (1 L+ dose in 1 ml solution)	0.40	0.40	0.40	0.40	0.4	0.40	0.40	0.40

Each tube now contains 2 ml mixture. The mice, each injected with 0.5 ml from tube 1 would receive 0.40 ml serum plus one L+ dose of toxin. If only one of this pair died during the observation period (i. e. the 50% endpoint) the serum would have an anti-beta toxin vale of 2.50 units per ml. if 50% protection was afforded by the mixture in tube 2, the serum would have a value of 2.77 units per ml and so on.

Table 3.1.1 shows that the range would be 2.50, 2.77, 3.08, 3.42, 3.81, 4.21, 4.7 and 5.26 units per ml, which covers the range 2.50–5.00 units at log. intervals. The limitations of pipette accuracy dictate that the figures drift away from log. intervals towards the bottom of the page. The number of unitage of antisera (anti-beta or anti-epsilon) in sheep and rabbits was thus calculated from average of three tests. The amount of test serum which protects 50% of the mice when equated with the amount of International Standard serum protecting 50% of the mice is regarded as one international unit).

Two way analysis of variance was used to compare the mean values of antitoxic components produced by vaccines at various periods (See Appendixes 3.3.I. and 3.3.2).

3.1.4 RESULTS

The colonial morphology (Plate 3.1.1) and cultural characteristics (Plates 3.1.2 &3.1.3) were typical of *Clostridium perfringens* strains used for the vaccine production. The estimated beta and epsilon toxin content (average of 3 tests) in reinforced clostridial medium (RCM) culture of *Cl. perfringens* was 2500 and 4000 units (mouse minimum lethal dose) per ml respectively. The mean viable count for *Cl. perfringens* type C organisms was 9.5×10^7 while that for type D was 8.8×10^7 per ml.

There were no growths either on anaerobic or aerobic culture plates of any of the vaccine preparations. There were no deaths of any of the mice inoculated with the vaccines. The vaccines when used, did not induce adverse reactions on the experimental mice. There was a noticeable minor inflammatory reaction at the site of injection but this disappeared within three days. The temperature rises of 0.1 to 0.6^{0}C were recorded in the

vaccinates (sheep), 48 hours after first injection but normalized under 24 hours.

The sheep and rabbits used for the potency testing of vaccines had no demonstrable antitoxin titre of either beta or epsilon toxins prior to vaccination. The antitoxic titres obtained for sheep sera tested at various periods after vaccination are shown on Tables 3.1.3, 3.1.4, 3.1.5, 3.1.6 and 3.1.7. The summary of means of beta antitoxin (Table 3.1.8) and epsilon antitoxin (Table 3.1.9) values examined as per 2, 4, 12, and 40 weeks after vaccination with the vaccines are also indicated. Pairing the beta antibody mean values present in sheep sera post vaccination per duration, as shown in Appendix 3.3.I, the level of beta antitoxin at 4 weeks (5.57 i.u. per ml) was significantly higher ($P<0.05$) than that at 2 weeks (2.49 i.u. per ml). The least significant difference at 5% level treatment was 1.207 (Appendix 3.3.I). Similarly, the level of beta antitoxin at 4 weeks was higher ($P<0.05$) than the levels recorded at 12 (3.05 i.u per ml) and 40 weeks (0.82 i.u. per ml). At 12 weeks the level of beta antitoxin titre was also significantly higher ($P<0.05$) than that at 40 weeks. The amount of beta antitoxin measured at 12 weeks was not significantly different ($P>0.05$) from that at 2 weeks while that at 2 weeks was significantly higher ($P<0.05$) than that at 40 weeks. Pairing the mean beta antitoxic values produced by vaccines potassium aluminum sulphate toxoid (PaT) produced significantly higher ($P<0.05$) titre (3.29 i.u. per ml) than that (1.13 i.u. per ml) by the plain bacterin and that (2.55 i.u. per ml) by the sodium alginate toxoid. The mean beta antitoxin titre (2.65 i.u. per ml) produced by potassium aluminum sulphate anaculture (PaA) was significantly higher ($P<0.05$) than that (1.13 i.u. per ml) produced by the plain bacterin. The 2.55 i.u. per ml beta toxin

produced by sodium alginate toxoid was not significantly different (P>0.05) than that (1.13 i.u. per ml) produced by the plain bacterin. There was no significant difference (P>0.05) in the mean beta antibody titres produced when potassium aluminum sulphate toxoid (PaT) and PaA, PaT and SaA, PaT and SaT, PaA and SaA, PaA and SaT, SaT and SaA, and SaA and FC (See Appendix 3.3.1) were compared. Looking at the level of epsilon antitoxin, the titre (8.55 i.u. per ml) measured at 4 weeks was significantly higher (P<0.05) than that (3.21, and 3.20 i.u. per ml) at 2, and 40 weeks respectively. The least significant difference at 5% level was 3.32 (Appendix 3.3.2). The presence of epsilon antibody level (5.77 i.u. per ml) at 12 weeks was not significantly different (p>0.05) than that (3.21 i.u. per ml) measured at 2 weeks and that (3.20 i.u. per ml) at 40 weeks. The level of epsilon antitoxin recorded at 2 weeks was not significantly (P>0.05) different from that at 40 weeks. Comparing vaccines, the mean epsilon antibody titre (4.50 i.u. per ml) induced by PaT was not significantly higher (P>0.05) than that (2.18 i.u per ml) by FC, that (3.11 i.u. per ml) by PaA or that (4.13 i.u. per ml) by SaA. There was a significant difference (P<0.05) between the epsilon antibodies (4.50 i.u. per ml) produced by PaT and that (8.21 i.u per ml) by SaT. Potassium aluminum sulphate anaculture (PaA) mean epsilon antitoxin level was not significantly different from either that (2.18 i.u. per ml) of FC or that of SaA. There was, however, a significant difference between the mean epsilon antibodies produced by PaA and that (8.21 i.u. per ml) by SaT. Sodium alginate toxoid (SaT) produced a mean epsilon antitoxin titre which was significantly higher than that of either FC or SaA. Sodium alginate anaculture (SaA) produced a mean epsilon antitoxin which was not significantly higher than that of FC (See

Appendix 3.3.2).

Production of antibodies (against beta and epsilon toxins) was highest at 4 weeks for all vaccines with potash alum toxoid (PaT) producing beta antitoxin of 8.33±1.45 i.u. per ml (Table 3.1.8) while sodium alginate toxoid (SaT) induced the highest epsilon antitoxic component of 16.58±2.99 i.u. per ml (Table 3.1.9). Similar pattern of antibody production was noticed for potash alum-anaculture (PaA) and sodium alginate-anaculture (SaA) which induced 5.64±0.88 and 4.32±1.36 i.u per ml of beta antitoxin respectively, while epsilon antitoxin production was 6.46±1.26 and 7.66±1.22 i.u. per ml respectively, 4 weeks after vaccination. In both sheep and rabbits, potash alum vaccines induced the highest beta antitoxin titres (Tables 3.1.6, 3.1.10) while sodium alginate vaccines produced the highest epsilon antibodies (Tables 3.1.7, 3.1.10) at 4 weeks for sheep and 2 weeks for rabbits irrespective of the type of vaccines (toxoid or anaculture) used.

At 4 weeks post vaccination, the highest titre of beta antitoxin of 8.33±1.45 i.u. per ml was produced by potash alum toxoid (PaT) and was significantly higher ($P<0.05$) than that from any other vaccine preparation. At 40 weeks the level of beta antitoxin elicited by the vaccines diminished to below 2.0 i.u. per ml (Figure 3.1.1).

Epsilon antitoxin titre was highest (16.58±2.99 i.u. per ml) at 4 weeks post vaccination and was produced by sodium alginate toxoid (SaT). This was significantly higher ($P<0.05$) than that from any other vaccine. Figure 3.1.1 shows the overall picture of the level of the antitoxin components produced by the vaccines in vaccinated sheep sera up to 40 weeks. Table 3.1.10 shows the beta and epsilon antitoxic values produced in rabbits 14

days after second injection. Concentrations of antitoxin components elicited in rabbits varied from vaccine type to vaccine type. Potash alum toxoid (PaT) produced the highest level of beta antitoxin concentration (42.0 i.u. per ml), while the minimum amount (10.0 i.u. per ml) was produced by formol culture or anaculture (FC). Potash alum adsorbed anaculture (PaA) and sodium alginate adsorbed anaculture (SaA) produced 19.5 and 22.0 i.u. per ml respectively. Sodium alginate toxoid produced the highest epsilon antitoxin value of 44.7 i.u. per ml while PaA and SaA produced 22.3 and 15.5 i.u. per ml respectively. Formol culture (FC) produced the least (12.0 i.u. per ml) for the same period in rabbits (Table 3.1.10).

3.1.5 DISCUSSION

The fairly high humoral immune response displayed by sheep and rabbits to the five vaccine preparations is a demonstration of the high immunogenic properties of the two local isolates of *Cl. perfringens* types C and D used in the vaccine preparation. Each of the bivalent vaccines containing beta and epsilon components produced antibodies in vaccinated sheep. The antibodies persisted at a satisfactory level (1 to 5 i.u per ml), except for plain bacterin which induced <0.1 i. u per ml of beta antitoxin at ten months. The immunity in the majority of animals would have fallen below the protective level 10 months after vaccination. This agrees with earlier work done by Jensen (1967c) and Thompson and Batty (1953). This calls for annual booster dose with preferably ordinary plain bacterin since the use of adjuvant vaccine as a booster has little or no advantage over the plain bacterin (Thompson et al., 1969).

Generally, toxoids with adjuvants elicited higher antibody levels than anaculture plus adjuvant while the above two preparations produced higher

antibody titres than ordinary plain bacterin. This agrees with previous findings (Thompson and Batty, 1953, Thompson et al., 1969). Most countries use vaccines containing adjuvants such as aluminum hydroxide gel, potassium aluminum sulphate, aluminum phosphate and water in oil emulsions (Sterne,1981).

The absence of any adverse reaction in any one of the vaccinates makes the adjuvants used preferable to the various oil emulsions which may produce in an unacceptable proportion of animals, severe granulomatous lesions following administration by the subcutaneous or intramuscular routes (Thompson et al., 1969). The alginate alone is non immunogenic, forms a stable colloidal transparent solution in water and is devoid of disadvantages inherent in commonly used chemical oil adjuvants (Bhatty, 1983).

The levels of antibodies produced in rabbits further confirms the efficiency of the vaccines prepared from the local isolates. Generally, besides FC which produced just about the minimum requirements of 10.0 i.u. per ml for beta and 12.0 i.u. per ml of epsilon which is twice the minimum satisfactory level for *Cl. perfringens* types B, C or D vaccines (British Veterinary Codex, 1970), all the vaccines produced two to four times the minimum internationally acceptable antitoxic values (10.0 i.u. for beta and 5.0 i.u. for epsilon) in rabbits. Rabbits developed higher titres of antibodies to beta or epsilon toxins irrespective of the type of vaccine used than sheep. This agrees with reports by Frerichs and Gray (1975).

Although the antitoxic titres diminished during the course of the study, the mean values at 40 weeks, except for animals vaccinated with FC (plain bacterin), still remained within the protective levels of 0.1-0.2 i.u.

per ml of epsilon antitoxin and twice that value for beta antitoxin (Thompson and Batty, 1953; Frerichs and Gray, 1975).

The pattern of mean antitoxic values for any given component of the five vaccines assayed was similar in sheep and rabbits. Although sheep may respond, on the average, three and a half times less well than rabbits *in terms of epsilon antitoxin*, a vaccine eliciting a titre of 5 i.u. per ml serum in rabbits should be of satisfactory potency (British Veterinary Codex, 1970). It is, however, important to note here that a product which induces only a low but adequate antibody response may be just as effective in protecting animal against field challenge as a product giving a much greater antibody response. Thus, the production of high antitoxic values do not necessarily reflect comparative efficacy in the field. The method used permits the vaccines potency to be described only in terms of their capacity to produce a certain concentration of antitoxin in the sera of rabbits and sheep, this concentration can be expressed in international units of antitoxin (British Veterinary Codex,1970). This, therefore, does not express vaccine's potency in international units of vaccine.

From the results obtained, it is evident that a long term protection can be achieved by vaccinating sheep, twice 3 - 4 weeks apart, with SaT or PaT followed by a yearly booster dose.

Sodium alginate toxoid (SaT) is selected for further studies based on the acceptability of the adjuvant system used and the ability of the vaccine's antigenic properties to induce consistently antibodies much higher than the minimum protective level in sheep. Sodium alginate is compatible at the percentage used with phenol, formaldehyde, merthiolate and other preservatives and is not affected by repeated thawing and

freezing (Bhatty,1983). Sodium alginate is also soluble in water and easier to combine and administer to animals compared to other adjuvants. Even though, immunity depends on the quality of the antigen carried on the adjuvant surface, from this study it has been confirmed that sodium alginate adsorbed vaccine produces only mild reaction on tissue surface. It is important to note that the combination of *Cl. perfringens* cultures in equal volumes would have reduced the beta and epsilon contents to half due to the dilution effect. This did not however affect the high antibody titres displayed by the sodium alginate vaccines. For these reasons the sodium alginate adjuvant is preferred to the other preparations.

Table 3.1.1. Constructed dose scheme for the determination of anti-beta and anti-epsilon toxin levels in serum of vaccinated sheep and rabbits.

Volume of serum	Toxin dose contained in 0.1 ml. solution			
	L+	L+/5	L+/10	L+/20
1.60	2.50	0.50	0.25	0.125
1.44	2.77	0.55	0.28	0.14
1.30	3.08	0.61	0.31	0.15
1.17	3.42	0.68	0.34	0.17
1.05	3.81	0.76	0.38	0.19
0.95	4.21	0.84	0.42	0.21
0.85	4.70	0.94	0.47	0.23
0.76	5.26	1.05	0.53	0.26
0.69	5.80	1.16	0.58	0.29
0.62	6.45	1.29	0.64	0.32
0.56	7.14	1.43	0.71	0.36
0.50	8.00	1.60	0.80	0.40
0.45	8.89	1.78	0.89	0.44
0.41	9.76	1.95	0.98	0.49
0.36	11.11	2.22	1.11	0.55
0.33	12.12	2.42	1.21	0.61
0.29	13.79	2.76	1.38	0.69
0.27	14.81	2.96	1.84	0.74
0.24	16.67	3.33	1.67	0.82
0.21	19.05	3.81	1.90	0.95
0.19	21.05	4.21	2.10	1.05
0.17	23.53	4.70	2.35	1.17
0.16	25.00	5.00	2.50	1.25
0.14	28.57	5.71	2.86	1.42
0.13	30.77	6.15	3.08	1.53
0.11	36.36	7.27	3.64	1.82
0.10	40.00	8.00	4.00	2.00
0.09	44.44	8.89	4.44	2.20
0.08	50.00	10.00	5.00	2.50
0.07	57.14	11.43	5.71	2.85
0.06	66.67	3.33	6.67	3.33
0.05	80.00	16.00	8.00	4.00
0.04	100.00	20.00	10.00	5.00
0.03	133.33	26.67	13.33	6.67
0.02	200.22	40.00	20.00	10.00
0.01	400.00	80.00	40.00	20.00

Source: British Veterinary Codex Supplement, 1970.

Table 3.1.2. Constructed dose scheme for the determination of anti-beta and anti-epsilon toxin levels in serum of vaccinated sheep and rabbits.

Volume of serum	Toxin dose contained in 0.1 ml solution			
	L+	L+/5	L+/10	L+/20
1.46	2.64	0.55	0.27	0.14
1.33	3.01	0.60	0.30	0.15
1.21	3.31	0.66	0.33	0.16
1.00	4.00	0.80	0.40	0.20
0.90	4.44	0.89	0.44	0.22
0.81	4.94	0.99	0.49	0.25
0.73	5.48	1.10	0.55	0.27
0.66	6.06	1.21	0.60	0.30
0.59	6.78	1.36	0.68	0.34
0.53	7.55	1.51	0.75	0.38
0.48	8.33	1.67	0.83	0.41
0.43	9.30	1.86	0.93	0.46
0.39	10.26	2.05	1.01	0.50
0.35	11.43	2.29	1.14	0.57
0.31	12.90	2.58	1.29	0.64
0.28	14.29	2.86	1.43	0.71
0.25	16.00	3.20	1.60	0.80
0.23	17.39	3.48	1.74	0.87
0.21	19.05	3.81	1.90	0.95
0.18	22.22	4.44	2.22	1.11
0.17	23.53	4.71	2.35	1.18
0.15	26.67	5.33	2.67	1.33
0.13	30.77	6.15	3.03	1.45
0.12	33.33	6.67	3.33	1.67
0.11	36.36	7.27	3.64	1.82
0.10	40.00	8.00	4.00	2.00
0.09	44.44	8.90	4.44	2.22
0.08	50.00	10.00	5.00	2.50
0.07	57.14	11.43	5.71	2.85
0.06	66.67	13.33	6.67	3.33
0.05	80.00	16.00	8.00	4.00

Source: British Veterinary Codex Supplement,1970.

Table 3.1.3. Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks post vaccination in sheep vaccinated with *Clostridium perfringens* type C and D formalinized culture (FC).

Wks	2		4		12		40	
Sheep No.	b	e	b	e	b	e	b	e
1	0.0	2.0	1.5	5.0	0.5	2.0	0.0	0.0
2	0.5	3.0	4.0	3.0	2.5	2.0	1.0	0.0
3	1.0	2.5	2.0	7.0	0.5	5.0	0.0	0.0
4	2.0	4.0	5.0	10.0	3.0	6.0	0.0	8.0
5	1.0	1.5	4.0	2.5	0.2	1.0	0.0	1.0
6	0.5	0.8	2.0	1.5	0.8	1.1	0.5	0.0
7	1.8	0.5	1.5	2.0	1.0	1.5	0.0	0.0
8	1.5	3.0	1.0	3.5	0.5	2.0	0.2	5.0
9	2.5	1.0	3.0	4.0	0.2	2.8	0.0	0.0
10	2.8	2.0	6.0	8.0	2.0	4.0	0.0	1.0
M+SE ab. i.u.	1.36±0.29	2.03±0.35	3.00±0.53	4.65±0.89	1.12±0.32	2.73±0.54	0.17±0.10	1.50±0.87

ab = antibody (antitoxin)
M+SE = Mean & Standard Error
I.U = *International units per ml.*

There was no measurable antitoxin titres pre-vaccination.

Table 3.1.4. Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks post vaccination in sheep vaccinated with *Clostridium perfringens* type C and D bacterin in potash alum adjuvant(PaA).

Wks	2		4		12		40	
Sheep No.	b	e	b	e	b	e	b	e
1	2.8	0.5	4.0	3.0	3.8	1.5	1.8	0.0
2	0.8	3.5	1.8	3.8	0.2	2.5	0.0	5.0
3	3.8	1.5	5.8	4.0	3.8	5.0	1.7	2.0
4	4.7	2.0	5.0	8.0	2.0	5.0	1.2	2.0
5	0.2	0.5	4.0	3.8	4.0	2.0	1.8	0.0
6	4.7	3.0	8.8	4.0	3.8	1.8	1.5	0.0
7	0.2	8.0	7.0	10.0	6.5	4.0	3.1	2.0
8	5.0	2.0	8.0	5.0	7.0	3.1	2.0	8.0
9	5.7	10.0	10.0	15.0	2.0	5.0	0.2	4.0
10	0.5	4.0	2.0	9.0	0.8	5.0	0.4	1.0
M+SE ab I.U.	2.84±0.70	3.20±1.04	5.64±0.88	6.46±1.26	3.39±0.70	3.49±0.47	1.37±0.30	2.40±0.82

ab = antibody (antitoxin)
M+SE = Mean & Standard Error
I.U = International units per ml.

There was no measurable antitoxin titres pre-vaccination.

Table 3.1.5. Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks post vaccination. in sheep vaccinated with *Clostridium perfringens* type C and D bacterin in sodium alginate adjuvant(SaA).

Wks	2		4		12		40	
Sheep No.	b	e	b	e	b	e	b	e
1	4.2	5.0	2.6	10.0	1.6	2.0	0.8	5.0
2	5.7	8.0	10.0	10.0	4.4	4.0	2.0	0.1
3	0.4	2.0	0.8	8.0	1.3	5.0	0.5	2.0
4	4.0	2.0	9.0	5.0	4.0	5.0	1.6	5.0
5	2.0	5.0	2.0	6.0	1.6	2.0	0.4	9.0
6	0.2	8.0	0.4	4.0	0.4	2.0	0.4	2.0
7	5.7	1.5	2.8	1.0	8.0	3.0	0.2	5.0
8	0.4	6.7	2.0	14.8	4.0	10.0	0.4	5.1
9	2.0	0.5	1.5	8.0	1.0	5.0	0.8	2.0
10	4.0	10.0	12.1	9.8	8.0	5.0	2.6	3.0
M+SE ab i.u.	2.86±0.68	4.87±1.04	4.32±1.36	7.66±1.22	3.43±0.88	4.30±0.76	0.97±0.26	3.82±0.80

ab = antibody (antitoxin)
M+SE = Mean & Standard Error
I.U = International units per ml.

There was no measurable antitoxin titres pre-vaccination.

Table 3.1.6. Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks pos vaccination in sheep vaccinated with *Clostridium perfringens* type C an D toxoid in potash alum adjuvant(PaT).

Wks	2		4		12		40	
Sheep No.	b	e	b	e	b	e	b	e
1	8.0	2.0	1.1	10.0	1.8	4.7	0.8	5.1
2	6.4	3.0	5.8	1.0	1.6	3.8	0.2	5.0
3	1.0	5.0	13.8	3.0	0.6	6.7	0.4	5.0
4	4.0	10.0	8.5	15.0	8.0	7.0	0.8	2.0
5	4.0	10.0	15.0	10.0	10.0	7.8	1.6	5.2
6	1.0	0.8	5.0	5.0	2.5	1.5	0.4	5.0
7	0.2	7.0	10.0	4.0	1.3	3.3	0.5	5.0
8	5.0	10.0	13.3	7.3	4.0	2.0	1.6	8.0
9	1.5	10.0	5.0	14.0	5.0	10.0	2.5	0.2
10	4.0	2.0	5.8	4.7	1.6	3.8	0.2	0.1
M+SE ab i.u.	3.56±0.79	5.96±1.22	8.33±1.45	7.40±1.49	3.64±1.00	5.09±0.87	0.90±0.24	4.66±0.79

ab = antibody (antitoxin)
M+SE = Mean & Standard Error
I.U = International units per ml.

There was no measurable antitoxin titres pre-vaccination.

Table 3.1.7. Distribution of beta(b) and epsilon(e) antitoxin titres* up to 40 weeks pos vaccination in sheep vaccinated with *Clostridium perfringens* type C an D toxoid in sodium alginate adjuvant(SaT).

Wks	2		4		12		40	
Sheep No.	b	e	b	e	b	e	b	e
1	2.5	5.0	8.8	13.0	6.5	9.8	1.0	5.2
2	1.5	8.0	8.0	4.0	5.0	9.0	1.0	8.0
3	0.5	9.0	15.0	22.0	6.0	17.0	2.0	2.1
4	2.0	10.0	10.5	13.8	2.0	10.0	0.5	2.0
5	3.0	7.0	9.0	25.0	5.0	20.0	0.4	5.2
6	0.5	12.0	6.8	36.0	2.5	30.0	0.8	8.2
7	5.0	8.0	8.0	15.0	4.0	10.0	0.2	5.1
8	0.8	2.0	2.8	10.0	2.0	8.0	0.5	0.4
9	1.0	0.5	3.0	7.0	1.5	4.8	0.2	4.0
10	1.5	10.0	2.8	20.0	2.0	14.0	0.4	0.2
M+SE ab i.u.	1.83±0.44	7.15±1.16	6.57±1.30	16.58±2.99	3.65±0.59	13.26±2.34	0.70±0.17	4.04±0.9

ab = antibody (antitoxin)
M+SE = Mean & Standard Error
I.U = International units per ml.
There was no measurable antitoxin titres pre-vaccination.

Table 3.1.8 **Summary of means of beta anti-toxin values*, for 2 weeks, 4 weeks, 12 weeks and 40 weeks after initial vaccination of sheep with the vaccines.**

	Vaccines				
Duration	FC	PaA	SaA	PaT	SaT
(Pre-Vac) Time 0	0	0	0	0	0
2 weeks	1.36±0.29	2.84±0.70	2.86±0.68	3.56±0.79	1.83±0.44
4 weeks	3.00±0.53	5.64±0.88	4.32±1.36	8.33±1.45	6.57±1.30
12 weeks	1.12±0.32	3.39±0.70	3.43±0.88	3.64±1.00	3.65±0.59
40 weeks	0.17±0.10	1.37±0.30	0.97±0.26	0.90±0.24	0.70±0.17
Totals	5.621	3.24	11.58	16.43	12.75

*International units per ml.

Table 3.1.9: Summary of means of epsilon anti-toxin values*, for 2 weeks, 4 weeks, 12 weeks and 40 weeks after initial vaccination with the vaccines.

	Vaccines				
Duration	FC	PaA	SaA	PaT	SaT
(Pre-Vac) Time 0	0	0	0	0	0
2 weeks	2.03+3.5	3.20±1.04	4.87±1.84	5.96±1.22	7.15±1.16
4 weeks	4.65+0.89	6.46±1.26	7.66±1.22	7.40±1.49	16.58±2.99
12 weeks	2.73+0.54	3.49+0.47	4.30+0.76	5.09±0.87	13.26+2.34
40 weeks	1.50+0.87	2.40±0.82	3.82±0.80	4.06±0.79	4.04±0.90
Totals	10.91	15.55	20.65	22.51	40.95

*International units per ml.

Table 3.1.10: **Antitoxin (Beta and Epsilon) values (i.u per ml) of rabbit sera 14 days after second injection with vaccines.**

	Vaccine Type									
	SaT		PaA		SaA		PaT		Fc	
No. of pool	Beta	Eps	Beta	Eps	Beta	Eps	Beta	Eps	Beta	Eps
1	18.0	44.4	25.0	21.0	24.0	17.0	44.0	16.7	8.0	10.0
2	16.0	45.0	14.0	23.5	20.0	14.0	40.0	14.0	12.0	14.0
Average	17.0	44.7	19.5	22.3	22.0	15.5	42.0	5.4	10.0	12.0

*Pre-vaccination sera contained no detectable antibody titre.

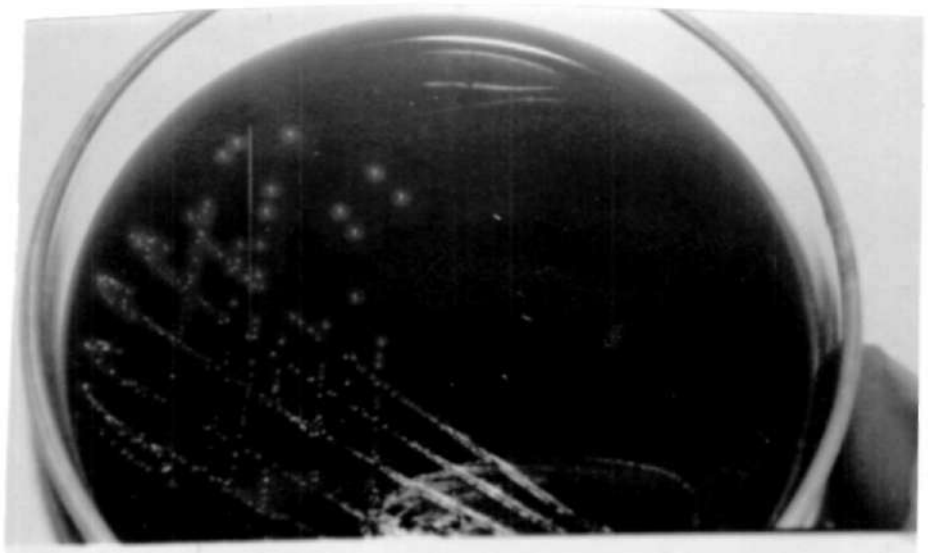


Plate 3.1.1 Colonies of CI. Perfringens surrounded by zones of haemolysis. (x2).

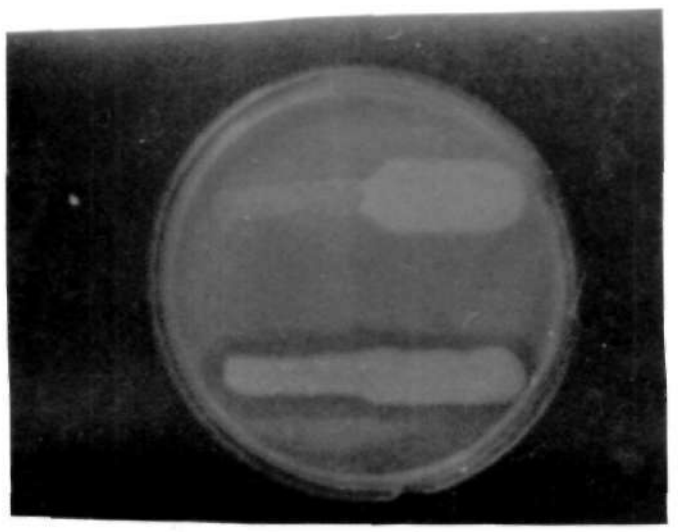


Plate 3.1.2 Culture of Cl. Welchii and Cl. bifermentans at 48 h on half-antitoxin egg yolk agar (x2).

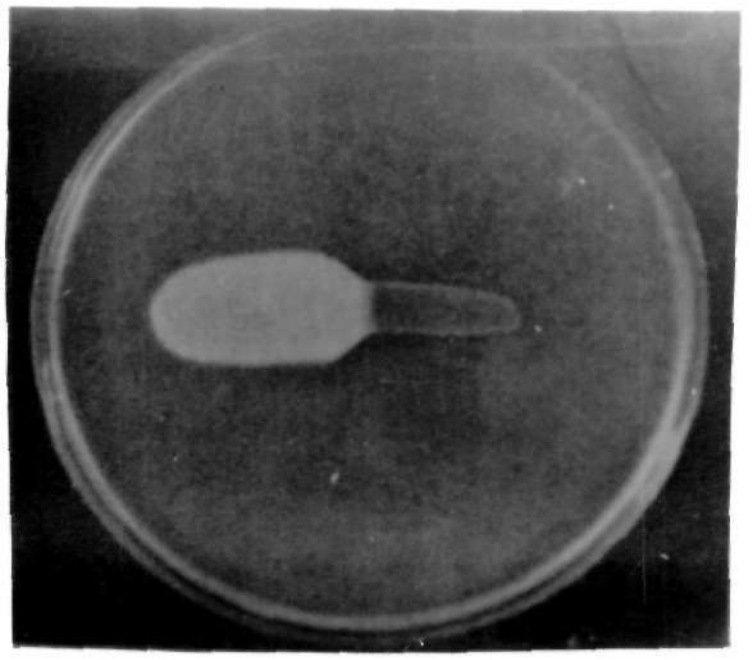


Plate 3.1.3: Culture of CI. welchii type A at 24 h on half-antitoxin on right-half of the plate. The diffuse lecithinase C opacity is inhibited by alpha-antitoxin (x2).

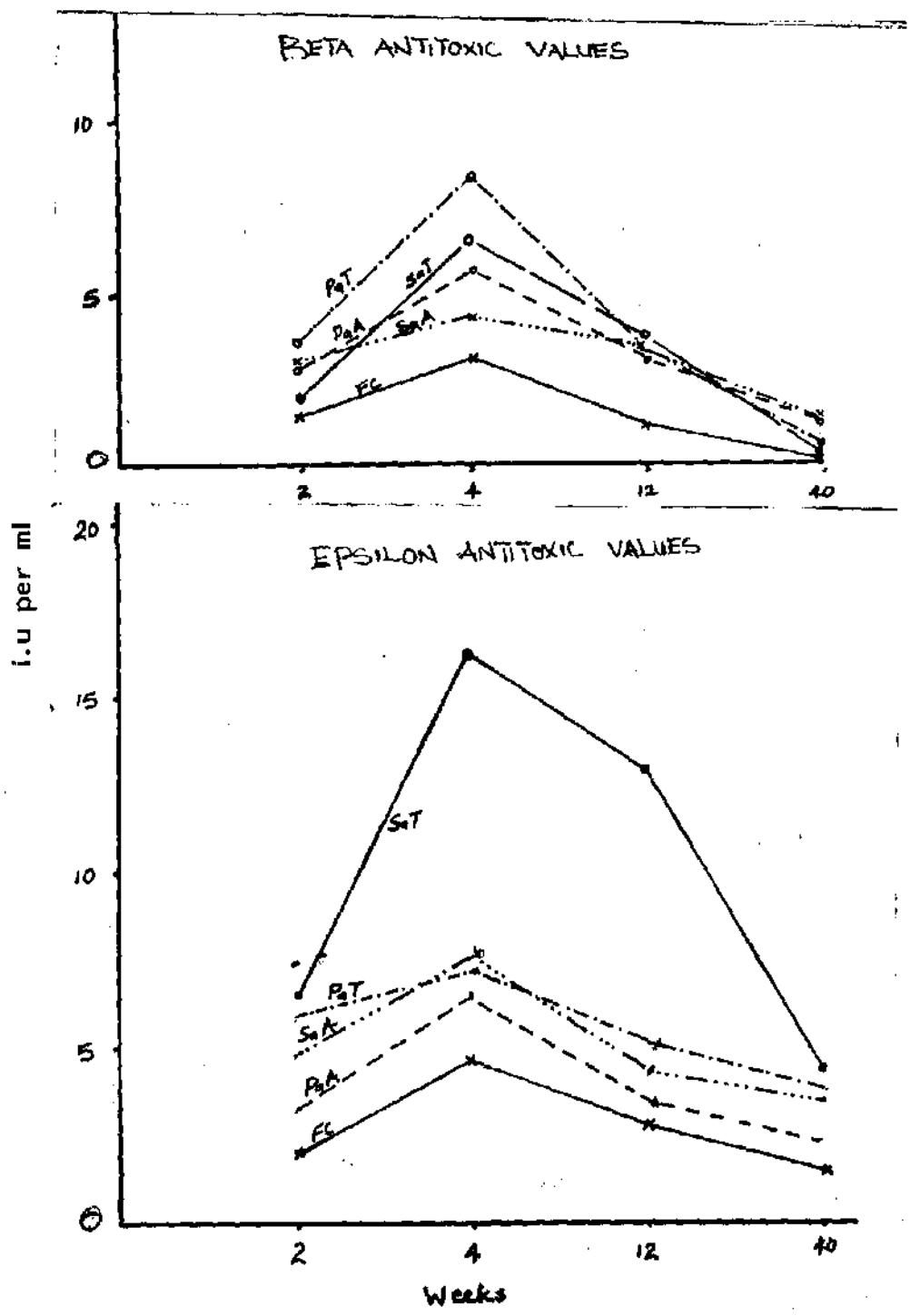


Figure 3.1.1: Overall coparison of mean serum beta and epsilon antitoxin titres produced by vaccines (FC, PaA, SaA, PaT and SaT) in sheep 2,4,12 and 40 weeks post-vaccination.

Appendix 3.1.1. Randomized Block Analysis (Anova Double Classification) of the mean values of beta antitoxin produced using vaccines after various periods.

Durations Treatment	Prevac	2wks	4wks	12wks	40wks	Teatment Total	Means
FC	0.00	1.36	3.00	1.12	0.17	5.65	1.13
PaA	0.00	2.84	5.64	3.39	1.37	13.24	2.65
SaA	0.00	2.86	4.32	3.43	0.97	11.58	2.32
PaT	0.00	3.56	8.33	3.64	0.90	16.43	3.29
SaT	0.00	1.83	6.57	3.65	0.70	12.75	2.55
Block Total	0.00	12.45	27.86	15.23	4.11	59.65	
Means	0.00	2.49	5.57	3.05	0.82		

No. of observations Bt = 25

Grand Total (G) = Σx = 59.65

Correction factor (C) = $\frac{G^2}{bt} = \frac{59.65^2}{25} = 142.33$

Total Sum of squares about the mean (TSS) = $\Sigma x^2 - C$ = 261 - 38 - 142.33 = 119.05

Sum of squares Treatment (SST=$(\Sigma B^2/t)-C$=154.77-142.33 =12.44

Sum of squares Blocks (SSB))=$(\Sigma T^2/b)-C$=236.01-142.33 =93.68

Sum of squares Error (SSE)=TSS-(SST x SSB)=119.05-(93.68+12.44) =12.93

Anova Table

Source of Variation	Sum of Squares	df	Ms	F
Treatment	12.44	4	3.11	3.84
Blocks	93.68	4	23.42	28.91
Error	12.93	16	0.81	

Calculated F (Treatment) = 3.84
Calculated F (Blocks) = 28.91
Tabulated F at (4,16 df) at P<0.05 = 3.01

There is significant difference in both the treatments and the blocks (P<0.05)

Testing least significant difference (LSD) at 5% level:

i) within treatment groups (vaccines) = t(0.05 16df) x $\sqrt{25^2/n}$

= 2.12 x 2 x $\sqrt{0.81/5}$ = 2.12 x 0.57 = 1.207

ii) within block groups (duration) LSD is also 1.207 since n=5 in both cases.

Pairing the mean values:

Treatments (Vaccines)

PaT	Vs	Fc	3.29 -1.13	=	2.16P<0.05
PaT	Vs	PaA	3.29 -2.65	=	0.64 NS
PaT	Vs	SaA	3.29 -2.32	=	0.97 NS
PaT	Vs	SaT	3.29 -2.55	=	0.74 NS
PaA	Vs	Fc	2.65 -1.13	=	1.52P<0.05
PaA	Vs	SaA	2.65 -2.32	=	0.33 NS
PaA	Vs	SaT	2.65 -2.55	=	0.10 NS
SaT	Vs	FC	2.55 -1.13	=	0.42P<0.05
SaT	Vs	SaA	2.55 -2.32	=	0.23 NS
SaA	Vs	Fc	2.32 -1.13	=	1.19 NS

Blocks (Duration)

4wks	Vs	2 wks	5.57 -2.49	= 3.08P<0.05
4wks	Vs	12wks	5.57 -3.05	= 2.52P<0.05
4wks	Vs	40wks	5.57 -0.82	= 4.75P<0.05
12wks	Vs	40wks	3.05 -0.82	= 2.23P<0.05
12wks	Vs	2wks	3.05 -2.49	= 0.56 NS
2wks	Vs	40wks	2.49 -0.82	= 1.67P<0.05

Key: NS = Not Significant [when the difference between pairs of means is less than the calculated LSD at the appropriate level of significance (0.05)].

Appendix 3.1.2. **Randomixzed Block Analysis (Anova Double classification) of the mean values of Epsilon antitoxin produced using vaccines after various periods.**

Treatment Duration	Prevac	2wks	4wks	12wks	40wks	Treatment Total	Means
FC	0.00	2.03	4.65	2.73	1.50	10.91	2.18
PaA	0.00	3.20	6.46	3.49	2.40	15.55	3.11
SaA	0.00	4.87	7.66	4.30	3.82	20.65	4.13
PaT	0.00	5.96	7.40	5.09	4.06	22.51	4.50
SaT	0.00	7.15	16.58	13.26	4.04	41.63	8.21
Block total	0.00	23.21	42.75	28.87	15.82	110.65	
Means	0.00	3.21	8.55	5.77	3.20		

No. of observation (bt = 25
Grand Total (G) $=\Sigma x$ = 110.65
Correction factor (C) = G^2/bt = $(110.65)^2/25$ = 489.74

Total Sum of Squares about the mean (TSS)
= Σx^2-C
= 871.67-489.74 = 381.93

Sum of Squares Treatment(SST)
= $(\Sigma B^2/t)-C$
= 595.49 - 489.74
= 105.75

Sum of Squares Blocks (SSB)
= $(\Sigma T^2/b)-C$
= 863.85-489.74
= 374.11

Sum Of Squares Error (SSE)
= TSS - (SST+SSB)
= 381.93 - 479.86
= 97.93

Anova Table

Source of Variation	Sum of Square	df	Ms	F
Treatment	381.93	4	95.48	15.60
Block	374.11	4	93.537.63	15.28
Error	97.93	16	6.12	

Calculated F (Treatment) = 15.60
Calculated F (Blocks) = 15.28
Tabulated F (0.05) at (4,16 df) = 3.01

Testing least significant difference (LSD) at 5% level:

i) Within treatment groups (vaccines) LSD = t (0.05) x $\sqrt{25^2/n}$

= 2.12 x $\sqrt{2x1.42/5}$ = 2.12 x 1.56 = 3.31

ii) within block (duration) LSD is also 3.31 since n=5 in both cases.

Pairing the mean values:

Treatment (Vaccines)

PaT	Vs	Fc	4.50 -2.18	=	2.32 NS
PaT	Vs	PaA	4.50 -3.11	=	1.39 NS
PaT	Vs	SaA	4.50 -4.30	=	0.37 NS
PaT	Vs	SaT	4.50 -8.21	=	3.71P<0.05
PaA	Vs	Fc	3.11 -2.18	=	0.93 NS
PaA	Vs	SaA	3.11 -4.13	=	1.02 NS
PaA	Vs	SaT	3.11 -8.21	=	5.10P<0.05
SAT	Vs	Fc	8.21 -2.18	=	6.03P<0.05
SAT	Vs	SaA	8.21 -4.13	=	6.03P<0.05
SaA	Vs	Fc	4.13 -2.18	=	1.95 NS

Blocks(duration)

4wks	Vs	2wks	8.55 -3.21	=	5.34P<0.05
4wks	Vs	12wks	8.55 -5.77	=	2.78 NS
4wks	Vs	40wks	8.55 -3.20	=	5.35 P<0.05
12wks	Vs	40wks	5.77 -7.48	=	1.71 NS
12wks	Vs	2wks	5.77 -3.21	=	2.56 NS
2wks	Vs	40wks	3.21-3.20	=	0.01 NS

Key: NS = Not significant when the difference between pairs of means is less than the calculated LSD at the appropriate level of significance (0.05).

EXPERIMENT 3.2 ESTIMATION OF IgG AND IgM LEVELS IN POOLED SERUM SAMPLES OF SHEEP VACCINATED WITH SODIUM ALGINATE VACCINE (SaT) BY THE ROCKET IMMUNOELECTROPHORESIS METHODS.

3.2.1 ABSTRACT

Using specific antisera against sheep IgG and IgM, the mean plasma immunoglobulin concentrations pre-vaccination and post-vaccination in 10 Yankasa sheep were estimated by the rocket immunoelectrophoresis methods. The pre-vaccination concentration of IgG was 8.20±0.55 and post-vaccination IgG concentration at 7, 14, 28 and 42 days were, 16.70±2.59, 14.70±3.91, 23.20±1.22 and 21.70±4.30 mg per ml respectively. The pre-vaccination IgM concentration was 16.30±1.56 while post-vaccination levels at 7, 14, 28 and 42 days were 18.20±0.56, 21.50±1.25, 18.10±0.20 and 15.80+1.00 mg per ml respectively. The post-vaccination IgG concentrations were individually significantly higher ($P<0.05$) than the pre-vaccination values. IgM production was earlier and peaked at 14 days but declined more rapidly than IgG.

Post-vaccination beta antitoxin production at 7, 14, 28 and 42 days were 0.50±0.12, 4.80±1.55, 3.58±0.81, and 2.95±1.00 i.u. per ml while epsilon antitoxin levels for the same periods were 1.80±1.30, 7.00±3.30, 14.50±2.50 and 13.80±1.30 i.u. per ml respectively.

The effect of vaccination was clearly established by the rise in plasma immunoglobulins (IgG and IgM) and the corresponding post-vaccination rise of both beta and epsilon antitoxins.

3.2.2 INTRODUCTION

Protection by specific antibodies can be achieved either by vaccination to evoke a long lasting active immunity, or by the

administration of antiserum to give temporary passive immunity. Immunologic response to clostridial infections is by production of immunoglobulins, first by IgM class followed later by IgG class (Herbert, 1970, Williams et al., 1975). Antibodies in sheep are subdivided into IgG1, IgG2, IgM and IgA (Campbell et al 1977). The prevention of enterotoxaemia therefore requires adequate presence of specific immunoglobulins (IgG and IgM) in the plasma of susceptible animals and this can be achieved by vaccination with a good immunogenic vaccine. A rise in antibody level during a secondary response above that at primary response, due to vaccination, is a strong indication that the animal has been sensitized (Herbert, 1970).

The purpose of the present investigation was two-fold, (a) to investigate the effect of vaccination with sodium alginate toxoid (SaT) on immunoglobulin levels in vaccinated animals and (b) to establish the pattern of immunoglobulin (IgG and IgM) production during vaccination. The rocket immunoelectrophoresis methods as described by Weeke (1973) was used for this study. The antibody levels for the various periods were also measured by the toxin neutralization tests in mouse (Frerichs and Gray, 1975).

3.2.3 *MATERIALS AND METHODS*

Biologicals

Standard ovine IgG and IgM with their corresponding standard antisera were obtained from Miles Scientific Division,Miles Laboratories,Inc. Naperville,Ill.60566 USA.

Test Sera: Ten sheep were bled at various periods pre-vaccination and after first and second injections with *Cl. perfringens* types C and D

toxoid in sodium alginate adjuvant system (SaT). The pooled serum was tested for antibody levels using the passive mouse protection test (Frerichs and Gray, 1975) and the immunoglobulins IgG and IgM levels were assessed by the rocket-immunoelectrophoresis methods (Weeke, 1973). Five Yankasa sheep kept in the same environment with the vaccinates were used as controls. These were injected with 5 ml of sterile normal saline each time. The controls were bled along with the vaccinates and monitored for same serum parameters.

Buffer: Laurel (1965) barbital buffer containing barbital sodium, 131.4 g; barbital, 20.7 g; calcium lactate, 4.0 g and 10 litres of distilled water.

The barbital was dissolved in 1 litre of distilled water in boiling water bath under constant shaking. The barbital sodium was then added as well as the calcium lactate. Distilled water was added to make up to 10 litres. The pH was adjusted to 8.6 using 10 N sterile sodium hydroxide solution. It was stored at 4^0C until used. The stock buffer solution was always diluted 1:4 with distilled water (ionic strength- 0.02) before use.

Agarose

Ten gram of agarose was melted in 1000 ml of barbital buffer in a boiling water bath. It was dispensed in 20 ml quantities in universal bottles and stored at 4^0C.

Staining Solution

Commassie blue, 50 g was dissolved in 1000 ml glacial acetic and 4,500 ml 96% ethanol and 4,500 ml distilled water was added. It was left at room temperature until the next day. The solution was filtered via Whatman No. 1 filter paper. It was stored in a covered plastic box.

Destaining Solution

This was made up of 96% ethanol, 4,500 ml; glacial acetic acid, 4,500 ml and 4,500 ml distilled water. They were mixed and stored in closed containers until used.

Fixing Solution

Picric acid, 14 g was added to 1,000 ml distilled water and gently heated to 60^0C. The saturated picric acid was then filtered through Whatman No. 1 filter paper and 200 ml acetic acid was mixed with it. It was stored in dark glass container away from light.

Carbamylation of Protein

The anodic migration during electrophoresis increases for IgG, IgM proteins by carbamylation (Weeke, 1968): One tenth ml standard ovine IgG or IgM was mixed with 0.1 ml of 2 molar potassium thiocyanate. At the same time, 0.1 ml of the experimental serum samples were each mixed with 0.1 ml of 2 molar potassium thiocyanate and left at 50^0C in a water bath for 20 minutes. The reactions were stopped by transferring the samples into ice bath. The samples were then each diluted 1:15 with barbital buffer and used for electrophoresis.

Cooling system for electrophoresis

Tap water was passed through an ice bath in latex tubings wound round a 2 litre beaker, filled with ice blocks before it was fed into the electrophoresis chamber. The flow of water at the chamber's drainage tube was adjusted to 60 drops per minute. This brought the temperature in electrophoresis chamber to 12 - 15^0C throughout the electrophoresis run.

Rocket immunoelectrophoresis run for the estimation for IgG and IgM in serum samples

The following procedures as described by Weeke (1973) was used.

Thirty ml of 1% agarose was melted in a boiling water bath and placed in a 55°C water bath. Ovine IgG antiserum (0.36 ml.) was added to the cooled agarose (45-55°C) to give a 1.2% antibody concentration. The agarose was immediately poured into a 200 x 100 x 1.5 mm glass plate on a horizontal table. When the agar had set (10-15 minutes), wells were punched on the long side of the plate, 3 cm from each end with 2.5 mm diameter gel puncher using a template.

The glass plate was placed on the Vokam SAE27[61] electrophoresis chamber, and the gel was connected to the barbital buffer in buffer vessels by means of filter paper (No.1 Whatman, folded 4 times) wick. Water cooling was started and the current switched on to between 70 and 100 volts on the power supply corresponding to 2 volts per cm on the gel.

Standard ovine IgG was diluted to 10 mg per ml. in barbital buffer. The standard IgG and sheep serum samples were carbamylated. The sera which were pooled were from 10 sheep (vaccinated using the sodium alginate toxoid). Using a 5 microlitre Carlsberg pipette, 5 microlitre of 10 mg per ml carbamylated ovine IgG standard, diluted 1:15 with buffer, was added to the first and last wells to serve as controls. Five microlitre of carbamylated serum samples each diluted 1:15 with buffer, was dropped into each well as shown on Plates 3.2.1-4.

After the application of all the samples, electrophoresis was continued with high voltage (at 280 - 300 volts on the gel) for 4 hours

at constant voltage. During an electrophoresis run, antigens migrate in an electric field in agarose containing monospecific antibodies. The precipitin lines form rockets whose heights are directly proportional to the quantity of antigen present in serum (Weeke, 1973). After the run, the protein was fixed in picric acid by placing the gel in picric acid bath for 10 minutes. Excess picric acid was thereafter removed by washing in 96% ethanol for 5 - 10 minutes. The gel with fixed protein was then pressed for 10 - 15 minutes using a layer of Whatman No. 1 filter paper (avoiding air bubbles) and a layer of soft blotting paper with a slight pressure sustained by means of a book placed on top of the blotting papers. After 10-15 minutes, the pressure and blotting paper were removed.

The gel was then left in distilled water bath for 15 minutes in order to wash off all unprecipitated proteins and then pressed on immediately. It was further dried by placing it in 60^{0}C incubator with a fan until it was completely dry. The gel was placed in Commassie brilliant blue staining solution and stained for 10 minutes. After that, it was destained in acid-alcohol bath until only the protein precipitates were stained (30 minutes to 4 hours). The gel was finally dried at 60^{0}C incubator with fan for about 30 minutes. The height of rockets of samples were then measured and equated with that of the standard ovine IgG (control) and the mg per ml calculated from average of three tests (Table 3.2.1).

Using the same method, the IgM values of serum samples were recorded (Table 3.2.2). Beta and epsilon antitoxin titres were evaluated from pooled sera of the ten sheep vaccinated with SaT for the periods

7 to 42 days and compared with the IgG and IgM concentrations.

3.2.4. RESULTS

The mean IgG concentration in sheep serum pre-vaccination was 8.20 ±0.55 mg per ml while post-vaccination serum values at 7 days was 16.70 ±2.59 mg per ml, at 14 days was also 16.70 ± 3.91 mg per ml (Plate 3.2.1), at 28 days 23.20 ± 1.22 mg per ml and at 42 days 21.70 ± 4.30 mg per ml (Plate 3.2.2). Also the pre-vaccination mean IgM concentration was 16.30 ± 1.56 (Plate 3.2.3) while post vaccination IgM values at 7, 14, 28 and 42 days were 18.20 ± 0.56, 21.50 ± 1.25, 18.10 ± 2.20 and 15.80 ± 1.00 mg per ml respectively (Plate 3.2.4). There was a significant ($P<0.05$) rise in IgG level during the secondary response to vaccination, when compared to the pre-vaccination level (Figure 3.2.1).

The primary and secondary antibody responses are represented graphically on Figure 3.2.1. There was no measurable antitoxin in the pre-vaccination sera in the sheep. At 7, 14, 28 and 42 days, titres of antitoxin against beta toxin were 0.50 ± 0.12, 4.80 ± 1.55, 3.58 ± 0.81 and 2.95 ± 1.00 i.u. per ml respectively while the titres of antitoxin against epsilon toxin for the same periods were 1.80 ± 1.30, 7.00 ± 3.30, 14.50 ± 2.50 and 13.80 ± 1.30 i.u. per ml.

Control animals had no measurable antibody titres throughout the test period. Control animals also did not experience any rise in IgG and IgM mean levels which remained constant, 8.20 ± 0.45 and 16.50 ± 1.25 mg per ml respectively throughout the test period.

A rise in immunoglobulin production was noticed during the first week after vaccination. IgM was prominent with peak production at about the 14th day after which there was a gradual decline in serum

level. IgG class reached its peak production about the 28th day after the first and 14th day after the second (booster) inoculations (Tables 3.2.1-2, Figure 3.2.1). A high level of IgG persisted for a longer period (6 weeks after first inoculation) while IgM level declined to normal level by the 28th day .

3.2.4. DISCUSSION

The effect of vaccination was clearly demonstrated by the corresponding rise post-vaccination, in plasma immunoglobulins,beta and epsilon antitoxins.The control animals experienced no difference in mean antibody titres and mean immunoglobulin concentrations throughout the study period. The pattern of immunoglobulins produced whereby IgM class was prominent soon after vaccination while later IgG class were found and persisted long after, offers possible indication to appropriate response to vaccination (Herbert, 1970).

In carrying out this study it was necessary to identify and minimize variables involved in sampling. It is known that sex, breed and environment have a significant effect on immunoglobulin level (Williams *et al.*, 1975). These variables were therefore eliminated in the present study by using a single breed of sheep (Yankasa) of same age bracket, and bleeding sheep maintained on one farm on a single day. It is not always possible to take into account the immune status of any one animal which may have elevated immunoglobulin levels as a result of antigenic stimulation (Williams *et al.*, 1975). The screening of pooled sera samples by the rocket immunoelectrophoresis methods was therefore to estimate the immune status of the animals as a result of vaccination.

Generally it is desirable for a vaccine to possess the ability to stimulate the production of antibody for a long period of time even if at a relatively low titre. Vaccines should perhaps be judged on their ability to do this, rather than on the height of antibody titre which is reached immediately after booster.

Table 3.2.1: Results of Rocket immuno-electrophoresis quantitation of pre- and post vaccination serum IgG in a group of sheep 1,7,14,28 and 42 days after vaccination with *Cl. perfringens* sodium alginate toxoid.

		Average Rocket Height (mm)			
		Prevaccination	Post vaccination		
Well Number	Sample name	Day 1	7 Day	14 Day	Conc.(mg/ml)
26,25	Reference				
2,1	Standard	1.95			10.0
24-21	Unknown sample	1.60			8.2
19-12	"		3.25		16.7
11-4	"			3.25	16.7
B					
24.23	Reference	28 day	42 day		
2.1	standard	2.63			10.0
3-12	Unknown samples	6.10			23.2
13-22	"		5.70		21.7

Table 3.2.2: Results of Rocket immuno-electrophoresis quantitation of pre- and post vaccination serum IgM in a group of sheep 1,7,14,28 and 42 days after vaccination with *Cl. perfringens* sodium alginate toxoid.

		Average Rocket Height (mm)			
		Prevaccination	Post vaccination		
Well Number	Sample name	Day 1	Day 7	14 Day	Conc. (mg/ml)
A					
22,1	Reference Standard	2.25			10.0
2-7	Unknown Serum	3.68			16.30
8-13	"		4.1		18.2
14-21	"			4.83	21.5
B		28 day	42 day		
22,21 2,1	Reference Standard	2.43			10.0
13-20	Unknown samples	4.41			18.1
3-12	"		3.83		15.8

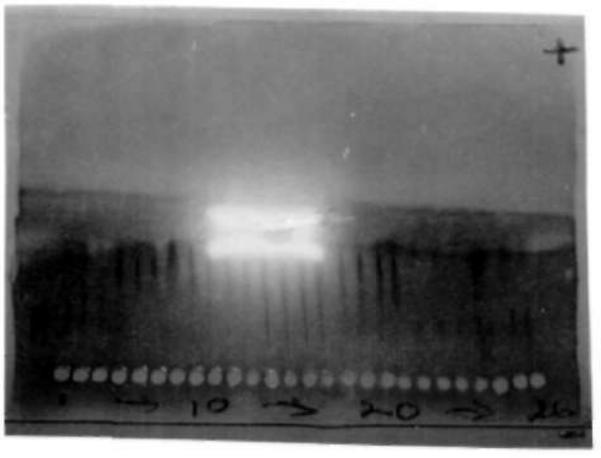


Plate 3.2.1: The rocket heights of IgG concentrations measured before vaccination with CI.perfringens toxoid (SaT).

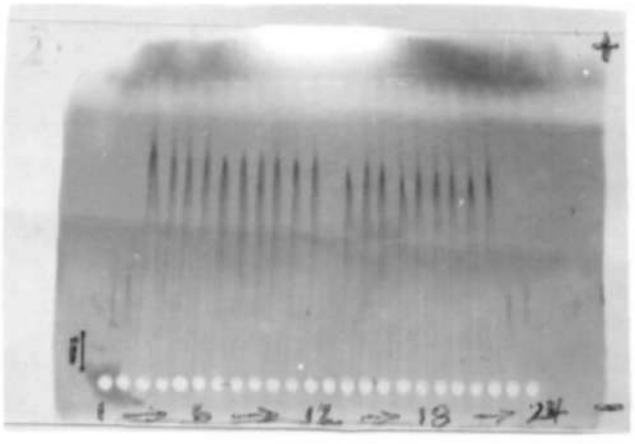


Plate 3.2.2: The rocket heights of IgG concentrations measures 7,14,28 and 42 days after vaccination with CI.perfringens toxoid (SaT).

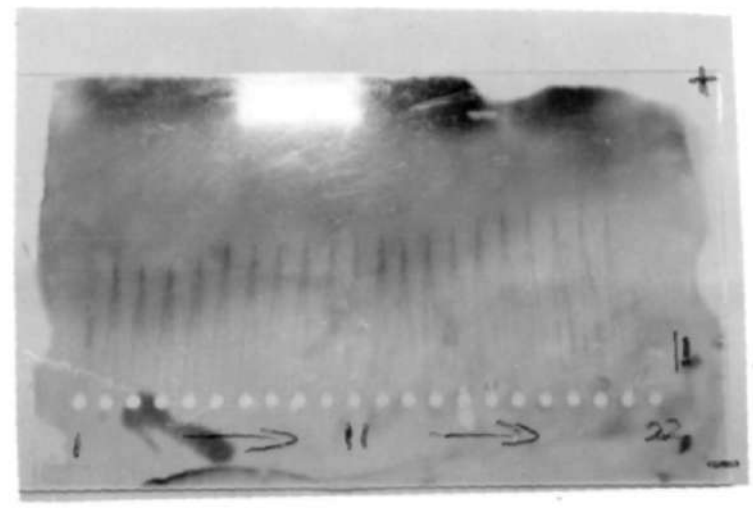


Plate 3.2.3: Rocket heights of IgM concentrations measured before vaccination with CI.perfringens toxoid (SaT).

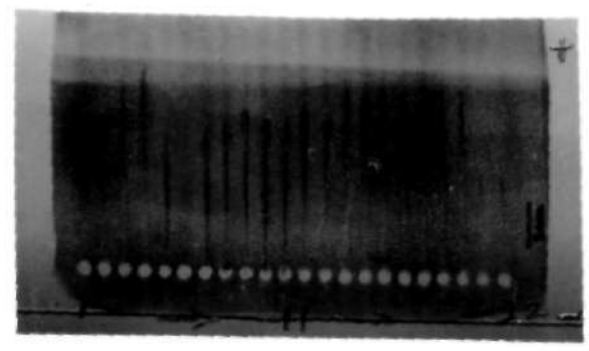
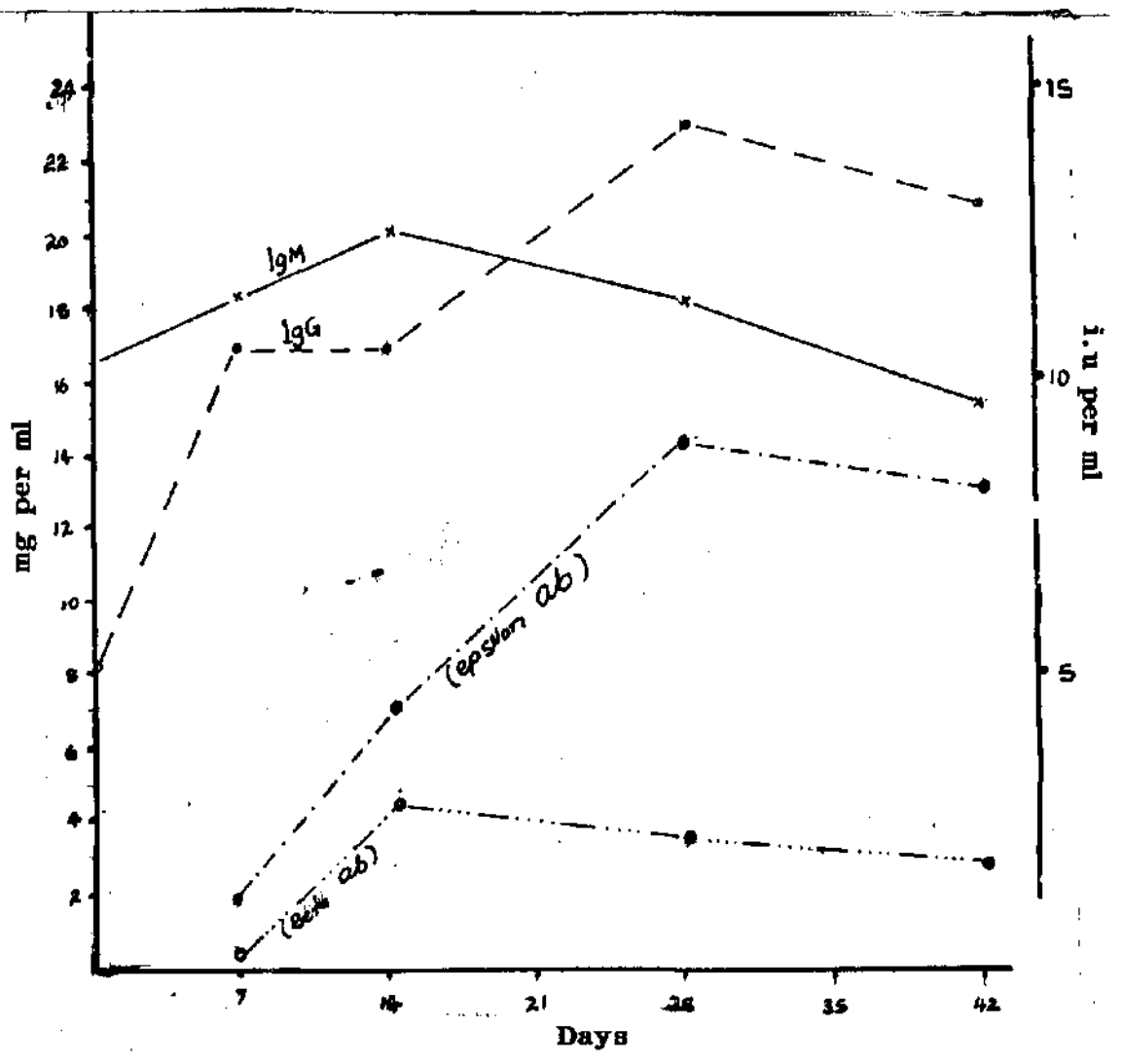


Plate 3.2.4: Rocket heights of IgM concentrations measured 7,14,28 and 42 days after vaccination with CI. perfringnes toxoid (SaT).



ab = antibody

Ig = immunoglobulin

Figure 3.2.1: IgG and Igm concentrations (mg/ml), beta and epsilon antitoxin titres measured 7 to 42 days after vaccination of sheep with sodium alginate toxoid (SAT).

EXPERIMENT 3.3 PRELIMINARY FIELD TRIAL WITH SODIUM ALGINATE CLOSTRIDIUM PERFRINGENS TOXOID AGAINST *ENTEROTOXAEMIA IN SHEEP*

3.3.1 ABSTRACT

A field - trial evaluation confirmed the efficacy of *Clostridium perfringens* types C and D vaccine as a suitable means for preventing sheep enterotoxaemia in Nigeria. In three private farms, known to experience the disease in Plateau State, a total of 2240 Yankasa sheep were identified to investigate whether immunization of feedlot sheep with sodium alginate *Clostridium perfringens* toxoid would reduce subsequent clinical symptoms and mortality due to natural enterotoxaemia. The vaccine comprised beta and epsilon toxins (products of local isolates of *Clostridium perfringens* types C and D) inactivated with 0.6% formalin. A total of 22 (1.9%) deaths were recorded among vaccinated animals while 271 (25.3%) animals died in unvaccinated flocks. The mean disease-specific mortality for vaccinates was 1.9% while that for controls (unvaccinated) was 25.3%. Vaccinal efficacy was defined in terms of smaller death rates as compared with the same factors in unvaccinated sheep. The vaccine efficacy was 98.6%. Unvaccinated sheep were 13.3 times more likely to be affected than vaccinated sheep. The association in the survival rate among sheep, from the 3 different farms, vaccinated with sodium alginate toxoid(SaT) and unvaccinated (controls) to enterotoxaemia was significant($P< 0.05$) by chi-square test [x^2 (cal.)=28.03, x^2 (tab.)=5.99, df=2].

3.3.2 *INTRODUCTION*

Enterotoxemia is a disease of economic importance among sheep in Nigeria, but immunization against the disease is not yet practiced (Njoku and Chineme, 1983, Itodo, 1987, Itodo, 1990). At present, the widely used immunizing agents against this disease are the alum precipitated and oil adjuvant vaccines. The duration of immunity conferred by the oil adjuvant vaccine, which is generally accepted as the better of the two, has been found to vary considerably and range between 8 and 14 months (Helwig et al., 1967, Thompson et al., 1969, Sterne, 1981). Although vaccines incorporating adjuvants such as sodium alginate have been used experimentally, they have not yet been established for regular use.

The husbandry patterns and management practices prevailing in most part of Nigeria where enterotoxaemia is enzootic do not permit frequent access to the animals for the purpose of vaccination. Furthermore, under the conditions prevailing in these areas the use of thick emulsion such as the oil adjuvant vaccine presents practical difficulties. Thus, there is a need for a vaccine which requires simple storage and transport facilities, which is easy to administer to animals and which will give adequate immunity of long duration.

Sodium alginate toxoid is a killed vaccine with two antigenic components (epsilon and beta toxoids) for vaccination against sheep enterotoxaemia. It is immunogenic, easier to prepare and administer and its colloidal nature tends to make it more stable when compared to the other preparations (Bhatty,1983).

The vaccine was tested in an enterotoxaemia enzootic areas of Plateau State (MacAdam and Zwart, 1967; Itodo and Ike, 1990). This disease which affects both sexes without bias, has a prevalence as high as 51.1% with a peak incidence during months of July to September in the area of study (Itodo, 1991). This field test is being done to confirm the efficacy of the sodium alginate adsorbed *Clostridium perfringens* types C and D toxoid as a means of preventing enterotoxaemia in sheep in Nigeria.

3.3.3 MATERIALS AND METHODS

The trials were carried out at three private farms located in enterotoxaemia enzootic areas (in Chaha, Barkin Ladi and Vom) of Plateau State. The three farms practiced the same management and husbandry methods. The farms are managed under extensive conditions with practically all sheep being maintained on grazing unimproved pasture with occasional supplementation with potato, groundnut and acha wastes, with periods of plenty (during the rains) alternating with times of scarcity during the dry season. The farms have ready access to veterinary services. The disease had neither been vaccinated against nor treated successfully in the area. Efforts to treat the condition under the general management methods had yielded no positive results because the disease cannot be treated with antibiotic or by chemotherapy except by immunization (Sterne, 1981).

Animals were of the Yankasa breed, 6 weeks to 10 months old and of both sexes. All animals were screened for protective antibodies by mouse protection tests before the commencement of the vaccination. Those found to have measurable antibody level (above 0.1 i.u per ml)

were excluded from the tests. In three trials a total of 1170 animals were vaccinated via the subcutaneous route with the Sodium Alginate Toxoid (SaT). The 1070 controls (unvaccinated sheep) were injected subcutaneously with sterile normal saline to compare the possible effect of injection. The vaccinates and controls were identified with indelible ink on the ears and neck region respectively. They were observed together in the feedlots under the same management condition, for ten months.

The vaccine (made up *of Clostridium perfringens* types C and D toxoids) dose was 5 ml and was administered subcutaneously twice 3 weeks apart in March 1992. The control animals were each injected similarly with 5 ml sterile normal saline.

Immunity in vaccinated or unvaccinated sheep was assessed by the mouse-antibody protection tests (Frerichs and Gray, 1975). Ten randomly selected vaccinates or controls were bled occasionally and the sera were used for the tests. A group of 3 mice was used for each serum sample. Each mouse was inoculated with 0.5 ml of undiluted serum by the subcutaneous route and challenged 30 minutes later with 1 mouse lethal dose of a fresh preparation of *Clostridium perfringens* types C or D toxins via the tail vein. Challenged mice were observed for 3 days. Mouse protective antibody was considered to be present if one or more survived challenge provided that all of an equal number of control mice died.

Mortality Data

All experimental animals were monitored throughout the ten month experimental period for signs of disease. Necropsy was performed on

dead animals within 24 hours of death. A gross diagnosis was recorded for each case. When the diagnosis was tentative, appropriate samples, gut content for toxin tests (mouse antibody-protection tests) and or urine sample for sugar and protein tests, were taken and processed by glucose oxidase and Biurett methods (Campbell and Kronfeld,1961) accordingly to confirm such diagnosis. Histopathological examinations were also done on kidney samples taken from confirmed cases and some vaccinated (normal) sheep. Tissues were immersion-fixed in 10% buffered formalin and processed to paraffin wax. sections were cut and stained with haematoxylin and eosin(HE). Disease-specific mortality was recorded for each group. The differences in mortality rate in the groups were compared and their association with vaccination was statistically analyzed by Chi square distribution. The vaccine efficiency was calculated by methodsiodds ratio) described earlier by Orenstein *et al.* (1988) (See Appendix 3.3.5).

3.3.4. *RESULTS*

There was no detectable immunity in the experimental animals prevaccination based on the mouse-antibody protection tests. When the result was doubtful such animal was excluded before embarking on the study. Tests carried out 3 weeks post-vaccination showed that all animals vaccinated had developed measurable antibody titres, 2.5-8.0 i.u. per ml. No adverse reactions were observed in any of the vaccinated animals.

Out of 2240 animals used for this test a total of 293 (13.0%) died of enterotoxaemia during the study period. A total of 22 (1.9%) deaths were recorded among vaccinated animals while 271 (25.3%) animals died

in unvaccinated flocks. The mean disease-specific mortality rate for vaccinates in the 3 farms was 1.9% while that for controls was 25.3%.

Two hundred and eighty four (96.9%) of the total deaths was due to *Cl. perfringens* type D while 9(3.1%) of the total deaths was due to *Cl. perfringens* type C. Only one (0.09%) death among vaccinated was due to *Cl. perfringens* type C.

Eight (36%) vaccinates and 81 (30%) controls died in the months of June and July. Most of the deaths, 14 (63%) vaccinates and 190 (70%) controls occurred in the months of August, September, and October. The number of animals affected and percentage mortality rates are shown in Table 3.3.1. The Chi-square analysis of the associations between vaccination and mortality rate, as observed on the 3 farms, is shown in Table 3.3.1. There was a significant difference [$P< 0.05$, x^2(cal.)=28.03, x^2(tab.)=5.99, df=2] between the vaccinates and the controls in the rate of survival as a result of vaccination. X^2test on association in the survival rate between vaccinated and unvaccinated sheep obtained from farms 1 and 2 showed a significant difference(Appendix 3.3.2) A similar result was obtained for farms 2 and 3 (Appendix 3.3.4). The difference in survival rate between vaccinated and unvaccinated animals from farms 1 and 3 (Appendix 3.3.3) was, however, not significant[X^2(cal.)=3.34,X^2(tab.)=3.84, df=1 $P>0.05$]. The vaccine efficiency was 98.6% (Appendix 3. 3.5).

Clincal Signs

The dominating symptoms demonstrated by most affected sheep were spastic movements, dullness, lassitude, and recumbency. Uncontrolled defeacation of semi-solidfeacal matter by some sheep in all

the farms were noticed. Retraction of the head and kicking of the hind limbs were signs which preceded death of some affected animals .

Necropsy Findings

The urinary bladders of affected animals were full with brown urine. There was inflammation of kidneys (pulpy) and livers of affected sheep (unvaccinated). The most consistent findings were congestion of the gastro-intestinal tract and excess fluid and fibrin in the pericardial sac.

Biochemistry

Urine from enterotoxaemia affected sheep was glucose positive (++) and positive for protein while same parameters were negative for vaccinated (normal) sheep.

Histopathology

Morbid examination of kidney samples from enterotoxaemia affected unvaccinated animals confirmed renal inflammation.

Kidney section from a dead unvaccinated sheep showed areas of coagulative necrosis with slight cellular infiltration surrounded by fibroblats affecting the cortex and medullary regions (Plate 3.3.3).

Kidney section from moribund unvaccinated sheep killed *in extrimis* showed haemorrhages, oedema and congestion with dilation of the blood vessels and fibrin deposits in the interstitial spaces (Plate 3.3.4).

Kidney section from vaccinated (normal animal) killed for comparism showed no significant pathological lesions (Plate 3.3.5).

3.3.5. DISCUSSION

The sodium alginate toxoid, a bivalent *Cl. perfringens* vaccine has demonstrated its efficiency by offering a significant protection to

vaccinated sheep in the 3 farms studied. The X^2 tests on the association of survival rates of animals protected with the vaccine in all 3 farms were significant, indicating a high pattern of protection to the animals. When the farms were paired,a similar significant association was observed except for that between farms 1 and 3 where there were 11 deaths (vaccinated), 56 deaths (unvaccinated) in the first farm and 7 deaths (vaccinated), 89 deaths (unvaccinated) in farm 3.

The findings that implicated *Cl. perfringens* as the major cause of death agrees with earlier reports (Itodo et al.,1986a; Itodo and Ike,1990). The presence of sugar in the urine of unvaccinated sheep which came down wih the disease coupled with the typical symptoms are indicative of the disease manifestation in sheep (Sterne, 1981).The protein in urine is associated with kidney inflammation (acute nephritis) which led to loss of plasma protein in urine as seen in this study.

Epizootiological analysis of vaccine efficacy in feedlot animals presents several problems. Epizootiological effect measures exist for determining vaccine efficacy.These include crude morbidity, crude and cause-specific mortality and incidence rates of fatal disease. They all have their advantages and disadvantages. Crude morbidity can be recorded and differences between vaccinated and unvaccinated groups determined. Given the acute nature of enterotoxaemia among sheep in the field and our present level of diagnostic technology the measure is very crude. Therefore, mortality may be a better measure of vaccine effect. For this study mortality due to enterotoxaemia was easy to diagnose without controversy and a specific cause of mortality can be determined with far greater accuracy than for morbidity. From the results the

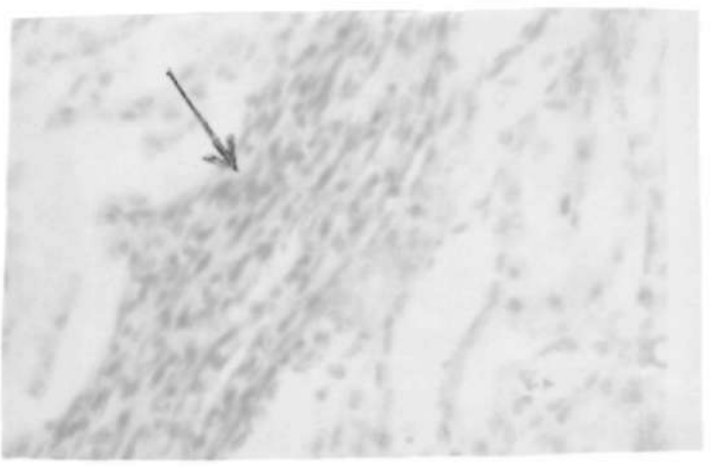
average mortality rate for the control was approximately 13.3 times higher than that of the vaccinates (Table 3.3.1). Over the entire study period the vaccinated group had a significantly low mortality rate. The vaccines overall protective effect was reduced by the death of few animals among vaccinates. The reason for this could be that these subjects developed low protective antibody due to some immunosuppressive phenomena. The other reason could be that of genetic variation in individual response to vaccination (Jansen, 1967c). Although sheep appear to respond satisfactorily to vaccination with *Clostridium perfringens* toxoids the lowest that will give protection appears to be of the order of 0.1 unit per ml for type D and twice that much for type C (Thompson and Batty, 1953).

Well designed field trials by others are necessary to determine if a beneficial effect of this prospective bivalent clostridial vaccine against enterotoxaemia occurs in other feedlot conditions.

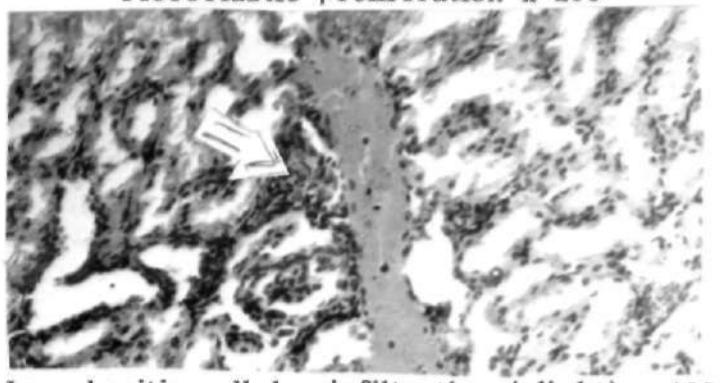
Table 3.3.1: Results of natural exposure of sheep vaccinated (A) with Sodium alginate toxoid (SaT) and unvaccinated controls (B) to enterotoxaemia.

Farms	No. of Animals		No. of animals Affected		Percentage (mortality rate)	
	A	B	A	B	A	B
1.	500	200	11	56	2.20	28.00
2.	320	470	4	126	1.25	26.81
3.	350	400	7	89	2.00	22.25
Total	1170	1070	22	271	1.88*	25.33*

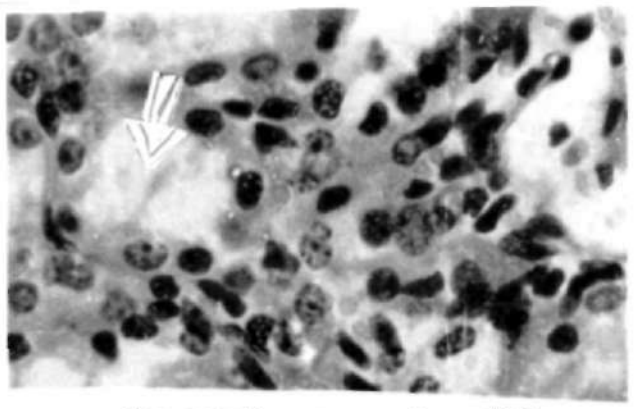
*Average percentage mortality



Fibroblastic proliferation x 200

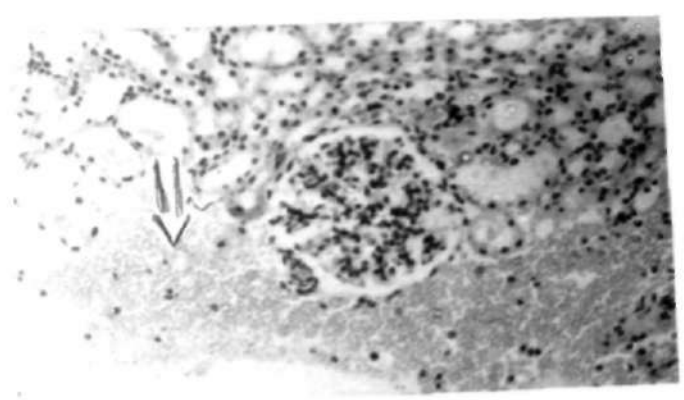


Lymphocitic cellular infiltration (slight) x 100

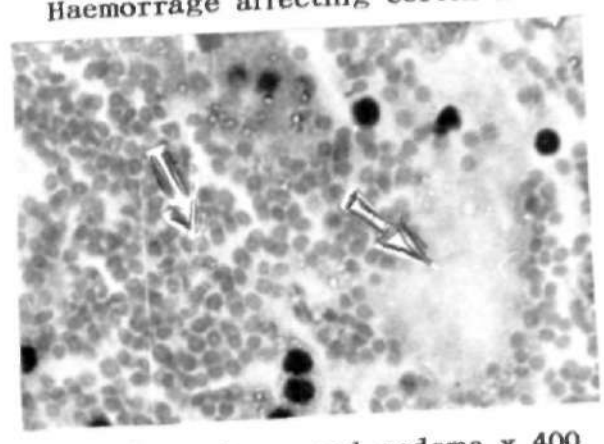


Coagulative necrosis x 200

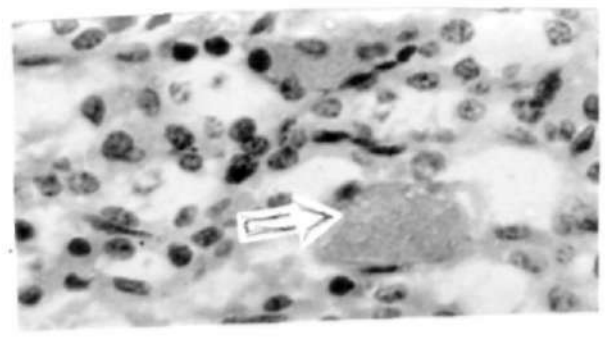
Plate 3.3.1: The cortex and medullary regions of kidney of unvaccinated sheed which died during natural exposure to clostridial enterotoxaemia, showing areas of coagulative necrosis with slight cellular infiltration (HE-Stain).



Haemorrage affecting cortex x 100

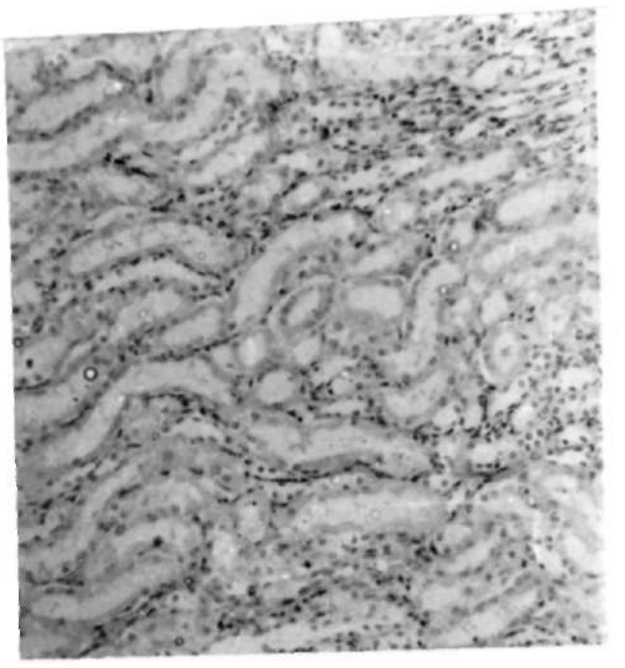


Haemorrhage and oedema x 400



Fibrin deposits x 100

Plate 3.3.2: Kidney section from a sick unvassinated sheep killed in extrimis, after exposure to clostridial enterotoxaemia, showing haemorrhages, oedema and congestion with dilation of the blood vessels and fibrin deposits in the interstitial space (HE-Stain).



Renal cortex (normal) x 100

Plate 3.3.3: Kidney section from vaccinated (normal) sheep showing no significant lesions, after exposure to clostridial enterotoxaemia (HE-Stain).

Appendix 3. 3.1. Calculation of Vaccine Efficiency(VE) (Orienstein et al,. 1988).

	ILL	NOT ILL
Vaccinated	22(a)	1148(b)
Unvaccinated	271(c)	199(d)

odds ratio = ad/bc

$$= \frac{22 \times 199}{271 \times 1148}$$

$$= 0.0141$$

VE(%) = (1-0.0141) x 100

= 98.59%

Appendix 3.3.2. x^1 Test on association in the survival rate of sheep, from 3 different farms, vaccinated with sodium alginate toxoid (SAT) and unvaccinated (controls) to enterotoxaemia

Farm	Vaccinated (a)			Unvaccinated (b)			Total
	O	E	$\frac{(O-E)^2}{E}$	O	E	$\frac{(O-E)^2}{E}$	
1	11	6.52	3.08	56	80.34	7.37	67
2	4	12.65	5.91	126	155.88	5.73	130
3	7	9.35	0.59	89	115.12	5.93	96
Total	22			271			226

O = Observed

E = Expected

x^2(Cal.) = 28.03, x^2(Tab.) = 5.99, df = 2, P<0.05.

Appendix 3.3.3. x^2 Test on association in the survival rate of sheep,obtained from farms 1 and 2 and vaccinated with sodium alginate toxoid (SAT) and unvaccinated (controls) to enterotoxaemia

Farm	Vaccinated (a)			Unvaccinated (b)			Total
	O	E	$\frac{(O-E)^2}{E}$	O	E	$\frac{(O-E)^2}{E}$	
1	11	5.10	6.83	56	61.90	0.56	67
2	4	9.90	3.52	126	120.10	0.29	130
Total	15			182			197

O = Observed

E = Expected

$x^2(Cal.) = 11.2$, $x^2(Tab.) = 3.84$, $df = 1$, $P<0.05$.

Appendix 3.3.4. x^2Test on association in the survival rate of sheep,obtained from farms 1 and 3 and vaccinated with sodium alginate toxoid (SAT) and unvaccinated (controls) to enterotoxaemia

Farm	Vaccinated (a)			Unvaccinated (b)			Total
	O	E	$\frac{(O-E)^2}{E}$	O	E	$\frac{(o-E)^2}{E}$	
1	11	7.40	1.75	56	59.60	0.22	67
3	7	10.60	1.22	89	85.40	0.15	960
Total	18			145			163

O = Observed

E = Expected

x^2(Cal.) = 3.34, x^2(Tab.) = 3.84, df = 1, P>0.05.

Appendix 3.3.5. x^2Test on association in the survival rate of sheep,obtained from farms 2 and 3 and vaccinated with sodium alginate toxoid (SAT) and unvaccinated (controls) to enterotoxaemia

Farm	Vaccinated (a)			Unvaccinated (b)			Total
	O	E	$\frac{(O-E)^2}{E}$	O	E	$\frac{(O-E)^2}{E}$	
2	4	11.35	4.76	126	221.83	41.40	130
3	7	8.38	0.23	89	163.91	34.16	960
Total	11			215			126

O = Observed

E = Expected

x^2(Cal.) = 80.55, x^2(Tab.) = 3.84, df = 1, $P<0.05$.

EXPERIMENT 3.4 DETERMINATION OF SHELF-LIFE OF THE SODIUM ALGINATE TOXOID(SaT) VACCINE

3.4.1 ABSTRACT

A batch of SaT was divided into two lots, one was stored at refrigeration temperature (4^0C) and another at 24^0C to 26^0C (room temperature).They were kept and tested at different periods of time for potency. The keeping quality of the vaccine was studied through a 12 month period. The consistency, safety and potency were the parameters used. Guinea pigs were actively immunized with SaT and subsequently challenged with 1 ml(1000 MLD_{50}) each of beta and epsilon toxins. It was shown that both lots of SaT were very potent beyond 10 months. The lot stored at 4^0C, however, protected all vaccinated guinea pigs throughout 12 months duration.

Chi-square analysis on associations in the survival rate of guinea pigs vaccinated with *Cl. perfringens* types C and D sodium alginate toxoid (SaT) vaccine stored separately through a 12 month period, at 4^0C and at 24-26^0C and challenged with epsilon toxin in one instance and beta toxin on the other were not significant ($P>0.05$). Although the storage temperature (24-26^0C) did not affect the vaccine efficacy significantly, there was a corresponding decrease in protection with increase in temperature. A 10 month period is recommended as the shelf-life.

3.4.2. INTRODUCTION

An important variable which affects the keeping quality of vaccines is temperature (Garba and Terry, 1986). Killed lyophilized vaccines relatively have a longer shelf-life than live wet vaccines. SaT

is a wet toxoid adsorbed in sodium alginate colloidal solution.

Sodium alginate toxoid (SaT) is a wet toxoid of *Cl. perfringens* types C and D grown in reinforced clostridial medium as described earlier in experiment 3.1. and adsorbed to sodium alginate adjuvant system. Formalin is an effective preservative and the toxoid prepared using formalin and sodium alginate as adjuvant is expected to be fairly heat stable (Bhatty, 1983). The shelf-life of a vaccine is the length of time for which the vaccine may be stored without losing its immunogenicity. This can be done by repeating the potency test at intervals whilst the product is kept at normal storage temperature, 5±3^{0}C (Davidson, 1975).

3.4.3 MATERIALS AND METHODS

A lot of sodium alginate toxoid Batch 2/92 was maintained at 4^{0}C and another was stored at room temperature, 24-26^{0}C for a 12 month period. Each lot was tested at the end of January, April, July, October and December. The toxin content of the clostridial culture before treatment with formalin was 1500 and 2000 units per ml for beta and epsilon respectively

Ten guinea pigs per group were inoculated subcutaneously with 1 ml dose of each lot of SaT vaccine. They were given a booster dose two weeks after. They were then challenged 14 days after booster dose. Five guinea pigs per group, as controls, were injected with sterile normal saline and challenged similarly along with the vaccinates. All animals were observed for one week after challenge.

Each guinea pig was challenged using 1 ml (1000 MLD_{50} of either epsilon or beta toxin via the ear vein. This dose kills guinea pig

within one hour of injection. MLD_{50} was also determined earlier by plotting % mortality against dosage (log form). The survival rate and therefore, the protective capability of the vaccine was then determined. The pre-formalinization toxin content of the vaccine was measured by method described earlier in experiment 3.1.

3.4.4. RESULTS

The survival rate, after challenge, of animals vaccinated with SaT vaccine stored at 4°C and 24-26°C room temperature is shown in Table 3.4.1. Animals immunised with vaccine stored at 4°C were all protected through the period of study. Ninety percent of the animals vaccinated with the lot stored at (24-26°C) and challenged with epsilon toxin were protected by the 12th month. By the 10th and 12th month the vaccine protected only 70% and 30% of animals challenged with beta toxin. All control guinea pigs died within 2 days of challenge.

3.4.5. *DISCUSSION*

A good vaccine should preferably retain its immunogenicity over a good period of time, otherwise it would be of limited value in the field. Initially,the vaccine was packed with an excess of effective antigens.Under constant storage conditions this gradually declines until a point is reached at which insufficient antigen remains to stimulate a good immune response. This experiment has shown that sodium alginate toxoid (SaT) can stay potent for up to 10 months at room temperature and 12 months provided a good pre-formalinization toxin content was obtained and the vaccine was stored at 4°C (British Veterinary Codex, 1970). The failure of the batch stored at 24 - 26°C to protect all animals by the 12th month may be related to the effect of heat. Despite the

acclaimed stable nature a formalinized toxin and the adjuvant used, the efficiency of the vaccine declined even though not significantly (P>0.05).There was a corresponding decrease in protection with a rise in temperature. For this reason, the expiry date should be well before the point of decline, that is at 10 months. The toxoid, a product of formalinized toxins produced by *Cl. perfringens* can be assessed either by vaccination and measuring how long the level of protection given to vaccinates lasts or by periodic vaccination and measurement of the antibody titres produced in vaccinates (Davidson,1975) as done in experiments 3.2 and 3.3. The only difference seen in this experiment was in the storage temperature. It is advised to store vaccines in refrigerators or appropriate coolers particularly during vaccination programme.

Table 3.4.1: Survival rate of guinea pigs challenged* with epsilon (E group) and beta (B group) toxins after vaccination wth sodium alginate toxoid (SaT) stored at 4^0 C and 24 -26^0 C room temperature.

Months	4^0C		24 - 26^0C	
	E	B	E	B
January	10/10	10/10	10/10	10/10
April	10/10	10/10	10/10	10/10
July	10/10	10/10	10/10	10/10
October	10/10	10/10	10/10	7/10
December	10/10	10/10	9/10	3/10

* 0.5 ml of epsilon (E) and 0.5 ml of beta (B) toxin.
Numerator = animals survived
Denominator = animals tested

CHAPTER FOUR

4.0 DISCUSSION AND CONCLUSION

In Nigeria, enterotoxaemia is sporadic in its incidence and mortality in herds and flocks making it the second most important clostridial disease of sheep after black quarter (Eze, 1975; Seifert, 1975; Itodo *et al.*, 1986a, Itodo, 1991).

Although treatment of enterotoxaemia is generally unsuccessful (Sterne, 1981) all conditions can be prevented by immunization. Bacterins, toxoids have been prepared fromtoxigenic strains of *Cl. perfringens* for the control and prevention of the disease in sheep in various countries (Bullen and Batty, 1957, Sterne 1981, Simos, 1982). Since earlier work by Thompson and Batty (1953), Jansen (1967a,1967b) and Thompson *et al.*, (1969), the importance of protecting livestock against the disease caused by clostridia has been realized. The use of prophylactic vaccines prepared with local toxigenic isolates for the immunization of animals should be encouraged since they are more likely to confer better immunity than imported strains. This would help to achieve the much desired locality-specific immune prophylaxis (Seifert, 1975). The production of a bivalent vaccine (SaT) comprising antigenic products of *Cl. perfringens* types C and D was done to facilitate effective routine vaccination of sheep in areas where the disease is endemic or expected to occur in Nigeria.

The sheep used in this study displayed a fairly high immune response to vaccinations with the vaccine preparations. This is an indication of the high immunogenic properties of the local isolates of *Cl. perfringens* types C and D used. There is however a need for a yearly

booster dose since the antibody level in the majority of animals would have fallen below the protective level 10-12 months after vaccination. The efficacy of the sodium alginate toxoid (SaT) was further demonstrated by the level of protection given to vaccinates in both challenge and field tests. The statistical significance in the association between the survival rate (of vaccinates) and vaccination, as per the field test, confirms the high efficiency record of the vaccine. Although the overall protective effect of the vaccine was reduced by the death of a few animals among vaccinates in the field tests, the level of protection given to vaccinates is commendable since it is rare to find a vaccine that can guarantee 100% protection to a flock. Within any group of animals the response of individuals to the administration of vaccine varies with their genetic make-up, state of health and management practices. The alginate vaccine is simple to prepare and easy to administer, in contrast to the oil vaccines (Bhatty, 1983). Sodium alginate forms a stable, colloidal transparent solution in water, and is devoid of disadvantages inherent in commonly used chemical oil adjuvants (Thompson *et al.*,1969). Based on its performance in this study it is found to be very suitable and recmmendable for vaccine preparation.

The pattern, quality and quantity of immunoglobulins produced in vaccinated animals as demonstrated by the rocket immunoelectrophoretic method is a further indication of the high immunogenic property of the vaccine (SaT). Although the height of antibody titre, after vaccination, may not be used to measure the level of protection, this could be a quantitative expression of response by a vaccinate. The ability to stimulate production of specific antibodies (IgG and IgM) by the vaccine

(SaT) is a good attribute of an efficient vaccine. Generally, it is desirable for a vaccine to possess the ability to stimulate the production of antibody for a long period of time even if at a relatively low titre (Davidson, 1975).

The level of protection given to vaccinates by the vaccine at the two different storage conditions suggests that the shelf-life extends well beyond 10 months. However, for a more dependent safety and potency of the vaccine, the expiry date should be fixed at 10 months after production and at a recommended storage temperature of 4^0C .

In conclusion, therefore, the objective of developing, a proved cheap and practicable method of producing a protective vaccine that can protect sheep against clostridial enterotoxaemia under extensive farming conditions has been attained. For a wider use of the vaccine, however, further field trials by others may be necessary to determine if a beneficial effect of the clostridial vaccine against enterotoxaemia occurs in other feedlot conditions.

REFERENCES

Amies, C.R. (1959). The use of topically formed calcium alginate as a depot substance in active immunization. Journal of Pathology and Bacteriology. 77: 435-442.

Arbuckle, J.B.R. (1972). The attachment of *Clostridium welchii* type C to the intestinal villi of pigs. Journal of Pathology 106: 65-72.

Baskerville, M., Wood, M., Seaner, J.H. (1980). *Clostridium perfringens* type E enterotoxaemia in rabbits. Veterinary Records. 107: 18-19.

Batty, I. and Walker, P.D. (1965). Colonial Morphology and Fluorescent labelled antibody staining in the identification of species of the Genus *Clostridium* . Journal of Applied Bacteriology 28:112-133.

Benjamin, M.M. (1961). Outline of Veterinary Clinical Pathology. Colorado State University, pp. 10, 69.

Bergeland, M.E. (1972). Pathogenesis of *Cl. perfringens* type C enteritis in swine. Journal of American Veterinary Association 160: 560-573

Bhatty, M.A. (1983). A preliminary comparison of Sodium Alginate and Oil Adjuvant Haemorrhagic Septicaemia Vaccines in cattle. Bulletin of Epizootic Diseases of Africa. 21: 171-174.

Blackwell, T. E., Butler, D.G.; Precott, J.F. and Wilcock, B.P. (1991). Differences and lesions in sheep and goats with enterotoxaemia induced by intraduodenal infusion of *Clostridium perfringens* type D. American Journal of Veterinary Research . 52, 1147-1152.

Blackwell, T.E. and Butler, D.G (1992). Clinical signs, treatment and post mortem lesions in dairy goats with enterotoxaemia. Journal of American Veterinary Medical Association 200: 214-217.

British Veterinary Codex (1970). Pharmaceutical Press. 1970 supplement. Second Edition. 124-295, 259-261.

Brooks, J.J.; Sterne, M. and Warrack, G.H. (1957). A reassessment of *Clostridium perfringens*. Journal of pathology and bacteriology 14: 185 - 195.

Bullen, J.J. and Batty, I. (1956). The effect of *Cl. perfringens* type D culture filtrates on permeability of the mouse intestine. Journal of pathology and Bacteriology 71: 311 - 323.

Bullen, J.J. and Batty I. (1957). Experimental enterotoxaemia of sheep: The effect of permeability of the intestine and the stimulation of antitoxin production in immune animals. Journal of Pathology and Bacteriology 68: 495 - 509.

Bullen J.J. (1970). Role of toxins in host parasite relationships. In: Microbial Toxins Vol. I. S.J. Aji. S. Kadis and T.C. Montie (Editors). Pp. 233 - 276. New York. Academic Press.

Buxton, D. and Morgan, K.T. (1976). Studies of lesions produced in the brain of colostrum deprived lambs by *Clostridium welchii* type D toxin. Journal of comparative Pathology 68: 435-447.

Buxton, D; Linklater, K.A. and Dyson, D.A. (1978). Pulpy Kidney Disease and its Diagnosis by Histological Examination. Veterinary Record. 102: 241.

Campbell, L.A. and Kronfeld, D.S. (1961). Estimation of low concentrations of plasma glucose using glucose oxidase. American Journal of Veterinary Research. 22: 587-590.

Campbell,G.;Siegel,M.J. and Brenda,J.K.(1977). Sheep immunoglobulins and their transmission to the neonatal lamb.New Zealand Veterinary Journal 25: 361-365.

Carl, S.R.; Jim, G.K. and Janzen, E.D.(1988). Efficacy of immunization of Feedlot calves with a commercial *Haemophilus somnus* Bacterin. Canadian Journal of Veterinary Research52:191-198.

Carter, G.R. (1967). Procedures in Diagnostic Bacteriology and Micology. Second Edition. Springfield, C.C. Thomas (Publishers).

Craig, J.P. and Miles, A.A. (1961). Some properties of the iota toxin of *Cl. welchii* including its action on capillary permeability. Journal of Pathology and Bacteriology. 81: 481 - 493.

Cruickshank R.; Duguid, J.P.; Marmion, B.P. and Swain, R.H.A. (1975). Textbook of Medical Microbiology, 12th Edition, 2: 473-476. Churchill Livingstone (Publishers). London, New York.

Cygan, Z. and Buczek, J. (1989). Immunogenicity of the autovaccine perfrinvac D. Medycyna 45: 614-616.

Davidson, I.(1975). Testing Veterinary Vaccines. Veterinary Record 97:389-392.

D'Salvin (1950) Production of antisera in rabbits using calcium alginate as an antigen depot. Nature . London, 165: 115.

English, Jr. J.E. (1966). Field Experience with Clostridium enterotoxaemia in young animals. Journal of American Veterinary Medical Association. 149: 1565-1570.

Eze, E.N. (1975). Survey of black-quarter outbreaks in Northern Provinces of Nigeria (covering the period April, 1, 1964 - March, 31, 1970). Bulletin of Epizootic Diseases for Africa. 21: 462.

Field, H.I. and Goodwin, R.F.W. (1959). The experimental reproduction of enterotoxaemia in piglets. Journal of Hygiene, 57: 87-93.

Frerichs, G.N. and Gray, A.K. (1975). The relation between the rabbit potency test and the response of sheep to sheep clostridial vaccines. Research in Veterinary Science 18: 70-75.

Garba, S.A. and Terry, R.J. (1986). Immunogenicity of oil-based Contagious bovine pleuropneumonia vaccine in cattle. Vaccine, 4: 266-270.

Gardner, D.E. (1973). Pathology of *Cl. welchii* type D enterotoxaemia II: Structural and ultrastructural alterations in tissues of lambs and mice. Journal of Comparative Pathology, 83: 509-524.

Grieg, A. (1975). An outbreak of *Cl. welchii* type C enterotoxaemia in young lambs in South West Scotland. Veterinary Record, 96: 179-184.

Griner, L.A. (1961). Enterotoxaemia of sheep. I: Effects of *Cl. perfringens* type D toxin on the brains of sheep and mice. American Journal of Veterinary Research, 22: 429-442.

Griner, L.A. (1963). Some factors influencing the incidence of enterotoxaemia in domestic animals. Bulletin of International Epizootics, 59: 1143-11451.

Griner, M.S.; Aichelman, W.W. and G.D. Brown (1956). *Cl. perfringens* type D (epsilon) enterotoxaemia in brown Swiss dairy calves. Journal of American Veterinary Medical Association; 122: 375-376.

Habeeb, A.F.S.A. (1972). Chemical and physicochemical properties of epsilon proto-toxin of *Clostridium perfringens* type D. Federal Proceedings 34: 932.

Habeeb, A.F.S.A. (1975). Studies on epsilon proto-toxin of *Clostridium perfringens* type D physicochemical and chemical properties of epsilon proto-toxin. Biochemical Biophysical Acta, 412: 62-69.

Hart, B. and Hooper, P.T. (1967). Enterotoxaemia of calves due to *Clostridium perfringens* type E. Australian Veterinary Journal, 43: 360-363.

Hauschild, A.H.W. (1970). *Clostridium perfringens* toxins, types B,C,D and E. In: Microbial Toxins, Vol. I. pp. 159-188. Academic press, New York.

Hauschild, A.H.W. (1971). *Clostridium perfringens* toxins B,C,D and E. In: Microbial Toxins, Vol. IIA Bacterial protein Toxins. pp. 159-188. Kadis S., Montie, T.C. and Aji S.J. (Editors). Academic press. New York.

Hayward, N.J. (1943). The rapid identification of *Cl. welchii* by tests in plate cultures. Journal of Pathology and Bacteriology, 55: 285-286.

Helwig, D.M.; Thomas, J.H. and Williams, L.G. (1967). The Bovine Enterotoxaemia complex: Its problems in diagnosis. Australian Veterinary Journal, 43: 364-367.

Hepple, S.R. and Chodnik, K.S. (1959). Immunization of lambs against *Cl. welchii* type D (pulpy) kidney disease) with purified toxoid, aluminum treated. Veterinary Record, 71: 201-206.

Herbert, W.J. (1970). Veterinary Immunology. First Edition. pp 187-200. Blackwell Scientific Publications Ltd., Oxford and Edinburgh.

Hirsch, A. and Grinsted, E. (1954). Methods for the growth and enumeration of anaerobic spore formers from cheese with observations on effect of nisin. Journal of Dairy Research 21: 101-110.

Hogh, P. (1967). Necrotizing infectious enteritis in piglets, caused by *Cl. perfringens* type C. I: Biochemical and toxigenic properties of *Clostridium* II: Incidence and clinical features. Acta Veterinaria Scandinavia, 8: 26-28.

Hydler, G. (1973). Enterotoxaemia or pulpy kidney disease vaccine. Indian Veterinary Journal 50: 972-978.

Itodo, A.E. (1984). Isolation and Serological Identification of *Cl. perfringens* in cattle, sheep and paddock soil. A Thesis presented for M.Sc. degree, Ahmadu Bello University, Zaria, Nigeria.

Itodo, A.E. (1987). Enterotoxemia and commercial livestock production in Nigeria. Nigerian Livestock Farmer, 7: 30.

Itodo, A.E. (1991). Association of *Clostridium perfringens* type D epsilon toxin with sudden death of sheep and in around Vom, Nigeria. Israel Journal of Veterinary Medicine. 46: 51-53.

Itodo, A.E. and R.O. Ike (1990). Enterotoxaemia in sheep and cattle in the Jos Plateau, Nigeria. Tropical Veterinarian 8: 120-122.

Itodo, A.E., Agba, M.I. and Okewole, P.A. (1983). *Clostridium perfringens* type D enterotoxaemia in cattle. Bulletin of Animal Health and Production for Africa, 31: 193-195.

Itodo, A.E., Adesiyun, A.A., Adekeye, J.O. and J.U. Umoh, (1986a). Toxin types of *Clostridium perfringens* strains isolated from sheep, cattle and paddock soils in Nigeria. Veterinary Microbiology 12: 93-96.

Itodo, A.E., Adesiyun, A.A., Umoh, J.U. and Adekeye, J.O. (1986b). Experimental Immunization and challenge of sheep with *Clostridium perfringens* type D. Zariya Veterinarian: 1: 1-5.

Itodo, A.E., Oyetunde, I.L.; Odeyemi, P.S. and Ibu, I.J., (1987). Bacteriology Examination of enterotoxaemia-like conditions in sheep. Nigerian Veterinary Journal, 15: 6-7.

Itodo, A.E., Molokwu, J.U., Nwobu, G.O. and Eze, E.N. (1989). Production and evaluation of the efficacy of a *Salmonella gallinarum* bacterin. Zariya Veterinarian, 4 :70-73.

Jansen, R. (1974). Disease of sheep. Lea and Febiger (publisher), Philadelphia.

Jansen, B.C. (1967a). The prevention of enterotoxaemia by vaccination. Bulletin of International Epizootics.67:1539-1567.

Jansen, B.C. (1967b). The production of basic immunity against pulpy kidney disease. Journal of Veterinary Research. 34: 65-80.

Jansen, B.C. (1967c). The duration of immunity to pulpy kidney disease of sheep. Journal of Veterinary Research. 34: 333-344.

Kerry, J.B. and Craig, G.R. (1979). Field studies in sheep with multi-component clostridial vaccines. Veterinary Record. 105: 551-554.

Kohler, B. (1973). Human and animal diseases caused by *Cl. perfringens* and their interactions. Gezamte Hygiene Gernzgen. 19: 228-234.

Kummeneie, K. and Bakken, G. (1973). *Cl. perfringens* enterotoxaemia in reindeer. Nordic Veterinary Medical Journal, 25: 195-202.

Labbe, R.G. (1983). Enumeration and confirmation of *Cl. perfringens*. Journal of Food Protection. 46: 60-73.

Laurel, C.B. (1965). Antibody-Antigen crossed electrophoresis. Journal of Analytical Biochemistry. 10: 358-359.

Lawrence, G. and Cooke, R. (1980). Experimental pigbel: The production and pathology of necrotizing enteritis due to *Clostridium welchii* type C in the guinea pig. British Journal of Experimental Pathology. 61: 261-271.

Lozano, E.A., Catlin, J.E. and Hawkins, W.W. (1970). Incidence of *Cl. perfringens* in neonatal enteritis of Montana calves. The Cornell Veterinarian 60: 347-358.

MacAdam, I. and Zwart, D. (1967). Enterotoxaemia in sheep in Nigeria. Bulletin of Epizootic Diseases for Africa, 15: 243-244.

Manson, I., Norberg, R., Olhagen, B. and Bierklund, N.E. (1971). Arthritis in pigs induced by dietary factors. Microbiologic, Clinical and histologic studies. Journal of Clinical and Experimental Immunology. 9: 677-693.

McDonel, J.L. (1980). *Cl. perfringens* toxins (types A, B, C, D and E). Pharmacology and Therapeutics. 10: 627-655.

McGowan, G., Moulton, J., and Rood, S.E., (1958). Lamb losses associated with *Cl. perfringens* type A. Journal of American Veterinary Medical Association. 133: 219-221.

Mihut, E. and Danes, M. (1989). Rapid toxicity test of *Clostridium perfringens* cultures. Lucrarile Institutui Cercetarisi Bioprepurate 'Pasteur' 18: 75-80.

Miles, A.A. and Misra, S.S. (1938). The estimation of the bactericidal power of the blood. Journal of Hygiene, Cambridge, 38: 732-748.

Mollby, R. (1978). Bacterial phospholipases. In: Bacterial Toxins and Cell Membranes, pp 367-424. Jeljalszewicz and Wadstron, T. (Editors). Academic Press, New York.

Moon, H.W. and Bergeland, ME. (1962). *Cl. perfringens* type C enterotoxaemia of newborn pigs. Canadian Veterinary Journal. 6: 159-161.

Mumford, D.H. (1961). Enterotoxaemia in cattle. Australian Veterinary Journal. 37: 122-126.

Nagler, F.P.O. (1939). Observation on a reaction between the lethal toxin of Cl. welchii type A and human serum. British Journal of Experimental Pathology. 20: 473.

Niilo, L. (1980). *Clostridium perfringens* in animal diseases. A review of current knowledge. Canadian Veterinary Journal. 21: 141-148.

Niilo, L. (1986). Enterotoxaemia due to *Clostridium perfringens*. In: Pathogenesis of Bacterial Infections in Animals. First edition. pp 80-86 Carlton, L. G. and Charles, O. T. (Editors). Iowa State University Press/Ames.

Nishida, S., Murakami, M. and T. Yamagishi (1962). Chopped meat broth as a medium for the production of toxins of Clostridia. I: Production of toxins by Cl. welchii. Japanese Journal of Microbiology, 6: 33-40.

Nioku, C.O. and C.N. Chineme (1983). Diarrhoeal Diseases of Nigerian Ruminants. Nigerian Veterinary Journal, 13: 57-63.

Oakley, C.L. (1943). The toxins of *Cl. welchii*. Bulletin of Hygiene, 18: 781-806.

Okewole, P.A., Itodo, A.E., Oyetunde, I.L., Chima, J.C., Irokanulo, E.A. and R.A. Ocholi (1991). *Clostridium perfringens* type A enterotoxaemia in pigs. A report of five cases. British Veterinary Journal, 147: 484.

Okewole, P.A., Chima, J.C. Oyetunde, I.L., Itodo, A.E., Irokanulo, E.A. and O.O. Chukwu (1992). Gas gangrene associated with *Clostridium perfringens* type A in an obstipated adult Yorkshire Boar. Bulletin of Animal Production for Africa, 40: 129-130.

Olubunmi, P.A. and Taylor, D.J. (1985). *Clostridium perfringens* type A in Enteric Diseases of pig. Tropical Veterinarian, 3: 28-33.

Orienstein, W.A., Bernier, R.H. and Hinman A.R. (1988). Assessing vaccine efficacy in the field. Further observations. Epidemiologic Review 10: 212-241.

Pivnick, H., Hauschild, A.H., Governstein, B. and A.F.S.A. Habeeb (1965). Effects of controlled pH on toxinogenesis by *Cl. perfringens* type D. Canadian Journal of Microbiology, 11:45-55.

Popoff, M.R., (1984) Bacteriological examination in enterotoxeamia of sandplamb. Veterinary Record 114:324

Princewill, T.J.T. and Agba, M.I. (1982). Examination of bovine faeces for isolation and identification of *Clostridium* species. Journal of Applied Bacteriology. 52: 97-102.

Princewill, T.J.T.(1983). Clostridial Infections of Nigerian Livestock. In: Proceedings pp. 47-51, National Workshop on Diseases of Livestock and Poultry. National Veterinary Research Institute, Vom, Nigeria.

Russel, W.C. (1970). Type A (*Cl. welchii*) enterotoxaemia in captive goats. Journal of American Veterinary Medical Association. 157: 643-646.

Sakarai, J., Fujii, Y., Matsura, M. and Endo, K. (1981). Pharmacological effect of beta-toxin of *Clostridium perfringens* type C on rats. Microbiology and Immunology, 24: 423-432.

Saunders, J.R. and Bickford, A.A. (1965). Clostridial infections of growing chickens. Journal of Avian Diseases, 9: 319-326.

Schillhorn Van Veen, T.J. (1973). Small Ruminant Health Problems in Northern Nigeria with emphasis on helminthiasis. Nigerian Veterinary Journal, 2: 26-31.

Seifert, G. (1939). A reaction in human serum with *Cl. perfringens* toxin. Zeitschrift fur Immunitatsforschung und experimentalle Therapie. 56: 516.

Seifert, H.S.H. (1975). Combating Anaerobic Epidemics among tropical cattle and sheep through locality specific immune prophylaxis. Animal Research and Development. 7: 72.

Simonds, J. P. (1915). Studies in *Bacillus welchii* with special reference to classification and its relation to diarrhoea. Monograph Number 5.1-6. Rockefeller Institute for Medical Research.

Simos, E. C. (1982). The present status of clostridial diseases of farm animals on small ruminants. IV. Symposium of the commission for the Study of Animal Diseases caused by Anaerobes. Proceedings. Pp 103 - 112. Paris.

Smith, L.DS. (1955). Introduction to the Pathogenic Bacteria. First edition. The University of Chicago Press. Chicago.

Smith, L.DS. (1975). *Clostridium perfringens*. In: The Pathogenic Anaerobic Bacteria. Second Edition pp 115-176. Springfield, Ill Charles C. Thomas (Publisher).

Smith, L.DS. and Gardner, V.M. (1960). The anomalous heat inactivation of *Clostridium perfringens* lecithinase. Achievements in Biochemistry. 25: 54-60.

Smith, W.D., Dawson, A. McI, Wells, P.W. and Burells, C. (1975). Immunoglobulin concentration in ovine body fluids. Research in Veterinary Science. 19: 189-194.

Sterne, M. (1981). Clostridial Infections. British Veterinary Journal. 137: 443-454.

Sterne, M. and Warrack, G.H. (1964). The types of *Clostridium perfringens*. Journal of Pathology and Bacteriology, 88: 278-293.

Stubbings, D.P. (1990). *Clostridium perfringens* enterotoxaemia in two young horses. Veterinary Record. 127: 431.

Szent-Ivanyi, T. and Szabo, S. (1956). Infectious Necrotic Enteritis in suckling pigs. I: Aetiology and Pathology. Hungarian Veterinary Journal. 6: 271-229

Thompson, A. and Batty, I. (1953). The Antigenic Efficiency of Pulpy Kidney Disease Vaccine. Veterinary Record, 65: 659-663.

Thompson, R.O. (1963). The fractionation of *Cl. welchii* E-Antigen on cellulose ion-exchange. Journal of General Bacteriology. 31: 79-90.

Thompson, R.O., Batty, I., Thompson, A., Kerry, J.B. Epps, H.B.G. and Foster, W.H. (1969). The immunogenicity of a multi-component clostridial oil emulsion vaccine in sheep. Veterinary Record. 85: 81-85.

Ugochukwu, E.I. and Nwaneri, B.C . (1984). An analysis of common Diseases of livestock in Ibadan, Nigeria (1978-1982). Nigerian Veterinary Journal, 13: 43-45.

Urguta, K.R. (1965). Bovine enterotoxaemia. Veterinariya Moskva (In Russian). 42: 34-36.

Walker, P.D., Harvis, E. and Moore, W.B. (1971). The Isolation of Clostridia from Animal Tissues. In: Isolation of Anaerobes, pp 25-38. Edited by D.A. Shapton and R.G. Board Academic Press. London and New York.

Weeke, B. (1968). Quantitative estimation of human immunogbobulins following cabamylation by electrophoresis in antibody-containing agarose. Scandinavian Journal of Clinical and Laboratory Investigation. 22: 107.

Weeke, B. (1973). Rocket Immunoelectrophoresis. Scandinavian Journal of Immunology, Second edition. Supplement, 1: 37-46.

Williams, M.R., Maxwell, D.A.G., Spooner, R.L. (1975) Quantitative studies on bovine immunoglobulins. Research in Veterinary Science 18: 314-321.

Willis, A.T. (1954). Anaerobic Bacteriology in Clinical Medicine: Second Edition. Butterworths, London Press.

Willis, A.T. (1969). Clostridial Wound Infections First Edition. Butterworths, London.

Willis, A.T. (1977). Anaerobic Bacteriology in Clinical Medicine, Third Edition. Butterworths, London.