ANTIGENS OF *BRUCELLA MELITENSIS* IN IMMUNODIFFUSION AND SEROLOGICAL DIAGNOSIS OF CAPRINE BRUCELLOSIS.

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DECLARATION:

This thesis is my original work and has not been presented for a degree in any other University.

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This thesis has been submitted for examination with our approval as University supervisors.

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SUMMARY

The sonic extracts of Brucella melitensis Strain 16M were studied in agar gel immunodiffusion test after they had undergone differential centrifugation and concentration. The number of precipitin lines obtained varied with the antigenic fractions used and the method of hyperimmune serum preparation. The maximum number of precipitin lines demonstrated was six when a sonic extract of phenol treated B. melitensis cell suspension, which had been centrifuged at 100,000 g for 60 minutes and concentrated (denoted $P_{100}C$), was reacted against ahyperimmune serum prepared by intravenously inoculating a live B. melitensis suspension in saline into a rabbit. Line 4 was only observed when the above antigen was reacted with the standard B. melitensis hyperimmune serum (denoted MHS). Line 5 was found to be due to a group specific antigenic component. Precipitin line number 6 was elicited by a lipopolysacchride protein complex of the cell wall which apparently carries the agglutinogens M and A. The rest of the lines were considered due to subsurface or cytoplasmic antigens.

Phenolised antigenic fractions,were found to give better reactions than acetone fractions in the indirect hemagglutination test with tanned sheep red blood cells. The above antigen ($P_{100}C$) gave the

best reaction of all the phenol antigenic fractions. Gluteraldehyde fixed tanned sheep erythrocytes gave even better reaction than tanned cells in the IHA reaction. All antigenic fractions, both phenolised and acetonised, were capable of eliciting complement fixation reactions. However, these fractions offered little advantage over the whole cell antigen in the CFT.

Micro-AGIT was found to be more efficient than either the macro- or minimicro-methods. Antigen $P_{100}C$ was found to give the most consistent results in the AGIT and hence was used to detect infection in naturally and experimentally infected goats. These results were compared to the results of SAT, CFT and RBPT. The AGIT was useful for confirming animals with equivocal titres in the SAT and CFT. The RBPT was oversensitive and also gave a number of false negative reactions. The CFT was the most specific test giving the maximum number of reactors.

The SAT would be of little value if used in combination with other tests. The combination CFT - RBPT or CFT - AGIT would provide most of the necessary information. However, for the detection of maximum number of reactors in an infected herd of goats, all four tests would be useful. In places where CFT facilities are not available, the combination of RBPT - AGIT would help in detection of a good number of reactors. The use of IHAT for

the diagnosis of caprine brucellosis should be assessed further.

The serological response of infected goats tended to be similar. Agglutinins appeared first, followed by antibodies for the AGIT and RBPT, and finally CFT. The CFT, RBPT and AGIT remained reactive for a long period whereas the SAT became suspicious or negative early in infected goats. The initiation and magnitude of the response depended on the dose and the route of infection with *B. melitensis*.

Abortions were the prominent sign with a suggestion of presence of infertility in the infected goats. *Brucella* organism was isolated only in the early stages of infection.

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SECTION I

INTRODUCTION

Brucellosis is a zoonotic disease primarily affecting cattle, sheep, pigs, dogs and rodents. These species are also the main source of infection for humans. The Genus Brucella is a coherent assembly of closely similar organisms separated from one another by differences in metabolic characteristics or different sensitivities to dyes (Jones (44)). Table 1 gives the general differential characteristics of Brucella species with their host preferences (Anon (10)).

Brucellosis presents a major public health problem and is of great economic importance, all over the world. In Kenya, Brucella melitensis has been isolated more frequently than Brucella abortus as the causal organism of human brucellosis (Wright, Cooke and D'Souza (113), Manson-Bahr (55), Cox (25) and Oomen (74)). It was not until 1970 when Philpott and Auko (79) reported abortion in goats in East Africa associated with the presence of B. melitensis. A limited survey and other field evidence suggested that the disease is common in certain areas of Kenya. Since then, the incidence of B. melitensis infection in goats has been increasingly reported by the Veterinary Research

TABLE 1: GENERAL CHARACTERISTICS OF SPECIES AND BIOTYPES IN THE GENUS BRUCELLA.*

Species	Biotype	Lysis by phage		CO_2 required	H_2S produced	Growth on dyes [a]					Agglutination by			Most common host reservoir
		RTD	$10^4 \times$ RTD			Basic fuchsin		Thionin			mono-specific sera [b]		Anti-rough serum	
						II	III	I	II	III	A	M		
Br. melitensis	1	−	−	−	−	+	+	−	+	+	−	+	−	Sheep, goats
	2	−	−	−	−	+	+	−	+	+	+	−	−	,,
	3	−	−	−	−	+	+	−	+	+	+	+	−	,,
Br. abortus	1	+	+	±	+	+	+	−	−	−	+	−	−	Cattle
	2	+	+	+	+	−	−	−	−	−	+	−	−	,,
	3	+	+	±	+	+	+	+	+	+	+	−	−	,,
	4	+	+	±	+	+	+	−	−	−	−	+	−	,,
	5	+	+	−	−	+	+	−	+	+	−	+	−	,,
	6	+	+	−	±	+	+	−	+	+	+	−	−	,,
	7	+	+	−	±	+	+	−	+	+	+	+	−	,,
	8	+	+	+	−	+	+	−	+	+	−	+	−	,,
	9	+	+	±	+	+	+	−	+	+	−	+	−	,,
Br. suis	1	−	+	−	+	−	−	+	+	+	+	−	−	Pigs
	2	−	+	−	−	−	−	−	+	+	+	−	−	Pigs, hares
	3	−	+	−	−	+	+	+	+	+	+	−	−	Pigs
	4	−	+	−	−	+	+	+	+	+	+	+	−	Reindeer
Br. neotomae		−	+	−	+	−	−	−	−	+	+	−	−	Wood rat
Br. ovis		−	−	+	−	+	+	+	+	+	−	−	+	Sheep (rams)
Br. canis		−	−	−	−	−	−	+	+	+	−	−	+	Dogs

[a] Species differentiation is obtained on Albimi or tryptose agar with the following graded concentrations of dyes : 1 : 25 000 (I), 1 : 50 000 (II), 1 : 100 000 (III). Other concentrations may be preferable with other growth media. Interpretation of results should be controlled with the reference strains of each species.

[b] A = *abortus* ; M = *melitensis*.

* Source:- from Anon, 1971. (10).

Laboratory, Kabete (Unplublished Data, Annual Reports (1971-1974) of Serology Section).

The control of brucellosis in animals aids prevention of human infections. In any control programme diagnosis forms an essential part. Various diagnostic methods for brucellosis have been developed. Among the tests that are widely accepted at present are :-

1) the Milk Ring Test (MRT);

2) the Serum (Tube) Agglutination Test (SAT);

3) the Complement Fixation Test (CFT);

and more recently, especially for bovine and porcine brucellosis,

4) the Buffered Brucella Antigen (BBA) either as the Card Test or the Rose Bengal Plate Test(RBPT).

All these tests employ whole cell Brucella antigens.

Although the SAT is widely used for the diagnosis of caprine brucellosis, evidence suggests that the CFT is more specific (Anon (10)). Alton (3), Unel, Williams and Stableforth (107)). The RBPT would be a more convenient field test (Philopott and Auko (79)) but more evidence of its efficiency is required. Since serological tests can be negative in infected animals that are excreting Brucella, there is still a concentrated effort to identify

reliable serological tests which are simple, sensitive, accurate and efficient for the diagnosis and determination of the immune status of animals following either infection or vaccination.

The main objective of this study was to characterize the antigens of Brucella melitensis Strain 16M in the Agar Gel Immunodiffusion Test (AGIT) and to determine whether these antigen preparations were applicable for the CFT and the Indirect (Passive) Hemagglutination Test (IHAT). A second objective was to compare the AGIT, SAT, CFT and RBPT for the diagnosis of caprine brucellosis.

SECTION II

LITERATURE REVIEW

A) BRUCELLA MELITENSIS INFECTION IN GOATS AND SHEEP

The description of Malta Fever by Marston 1861, the isolation of the causal organism by Bruce in 1886, and the discovery by Zammit in 1905 that goats served as the reservoir of infection, were all landmarks in the understanding of brucellosis (Alton (4)). The disease is widely distributed throughout the world but predominantly occurs in the countries surrounding the Mediterranean Sea, in Southern and Eastern U.S.S.R, Mongolia and Iran. Other areas where the disease is prevelant are Pakistan, India, the Southerly areas of the Near East, Mexico, Southern U.S.A, Latin America and most parts of Africa.

Brucellosis is commonly introduced into a susceptible herd by an infected goat. Initially, the spread of the disease may be slow, however a number of animals may become infected at about the same time and an outbreak develops. Clinical signs may range from abortion and mastitis to no symptoms whatsoever (Stableforth (102)). Abortion produces a massive contamination of the environment as the excretion of the organism in the foetus, placentas,

and vaginal discharges of infected animals is enormous. The organism is also excreted in the milk, urine, and feces. The principal portal of entry of Brucella is through ingestion. Other routes of infection may be inhalation into the nasal passages, through the ocular membranes and through intact skin (Alton (4)).

In an infected animal, the organisms reach the regional lymph nodes via the lymphatics. The infection becomes generalised as a bacteremia if the resistance of the regional lymph nodes is overcome. If the animal is pregnant, the organisms are likely to proliferate in the uterus causing abortion, and to a lesser extent in the Udder where mastitis may occur. Later the disease becomes chronic and localises in the Udder. Some infected goats abort only once, others repeatedly, some goats giving birth at the normal time excrete brucellae in large numbers. Most goats are capable of selfcure, but the process may take years (Alton (4)).

The pattern of immunoglobulin production after infection has been studied in both animals and man (Anon (10)). Following natural infection, both IgM and IgG appear. The IgM values decline earlier than the IgG and later, especially in chronic stages, the predominant and often the only immunoglobulin present is IgG. The various globulins in

different animal species appear to differ in their ability to fix complement and to cause agglutination. In cattle both IgM and IgG are capable of fixing complement and to cause agglutination whereas in man IgM is the predominant agglutinating antibody and IgG fixes complement. In some amimals, especially man and sheep and occasionally in other species, the antibody in chronic infection is non-agglutinating or 'incomplete' and can only be detected by using the Anti-Globulin test (Coomb's reaction). Corbel (22) has shown that the RBPT and the CFT activity is associated with IgG.

The control of the disease is based on testing and slaughter of infected animals, hygienic measures and vaccination. Surveillance is very important once control or eradication procedures have been initiated in a country.

B) ANTIGENS OF BRUCELLAE.

1) Whole Cell Antigens:

Brucella occurs in three main colony forms; smooth, rough and mucoid. All Brucella cultures have a tendency to undergo variation during growth (Alton and Jones (5)). Variants arise due to spontaneous appearance of mutants which are particularly apt to appear in liquid media and old cultures (Stableforth (102)). The change occurs more readily with

some strains than others; is more common with B. melitensis than B. abortus and is almost always irreversible (Wilson and Miles (111)).

Loss of specific smooth antigen occurs when brucellae undergo smooth (s) to rough (R) variation. This latter variation is accompanied by a reduction in virulence, colony morphology alteration and antigenic difference. Cell suspensions of smooth cultures are not agglutinated by heat and salt although, occasionally, may be slightly agglutinated by acids. Cells from rough cultures are agglutinated by heat, acids and frequently by salts. Apparently there is at least one antigen common to all rough strains of Brucella species (Wilson and Miles (110)).

To avoid non-specific antigenic differences smooth cultures are employed for the production of Brucella antigens. Depending on the extraction methods and extraction procedures used, different concentrations of multiple antigens are obtained. Agglutination and absorption tests were one of the first methods employed for revealing the antigens of Brucella.

Antigen M in smooth B. melitensis and Antigen A in smooth B. abortus are the specific antigens differentiating the two species. A non-specific group (G) antigen is present in all

Brucella species (Olitzki and Gurevitch (71)). Wilson and Miles (110) concluded that antigens A and M are present in different quantitative ratio in smooth B. abortus, B. melitensis and B. suis. By optimal proportion in agglutination tests, Miles (57) found B. abortus to have an A:M ratio of about 20:1 whereas in B. melitensis it was 1:20.

A more detailed antigenic analysis of the genus Brucella was presented by Renoux and Mahaffey (89). The smooth cultures of the three species B. abortus, B. suis and B. melitensis have the antigens A, M, Z and R in different quantitative distributions. B. ovis contains only R and Z and the rough cultures of all species contain only R with or without the Z antigens. Alton (1) assumed that B. abortus had the antigens Am and B. melitensis, antigens Ma, where the capital letters represent the major antigen and the small letters the minor antigen. Similarly, the rough cultures had the antigenic structure Rm or Ra depending on the species of Brucella.

The surface antigens of B. ovis, B. canis and rough strains of B. abortus and B. melitensis are similar but not identical. Little antigenic relationship is seen between the surface antigens of B. ovis, B. canis and smooth B. abortus or

B. melitensis (Diaz et al (32)). Cross-absorption of B. neotomae, B. abortus and B. melitensis antisera with the three antigens revealed B. neotomae and B. abortus to be identical.(Stonner and Lackman (103)).

The above results, together with the findings of Miles (57) suggest that the minor antibody component of an antiserum can be absorbed out leaving most of the major antibody component, thereby providing monospecific reagents for the identification of Brucella cells. It can be presumed that the failure of monospecific serum to agglutinate the heterologous Brucella species is due to insufficient antigen to allow firm agglutination. Therefore the use of quantitative agglutination and absorption tests allow differentiation between B. melitensis on one hand and B. abortus and B. suis on the other, if they are in smooth phase. Also, these procedures help in identifying the rough strains from the smooth ones (Anon (10)).

2) Antigenic Extracts:

Miles and Pirie (58) extracted antigenic material from B. melitensis using 7 percent phenol, and ammonium sulphate fractionation, and designated it PLAPS. The constituents of the antigen were phospholipids (PL), amino-polyhydroxy compound (AP) and arginine (S). On disaggregation of AP the antigenic

determinants A and M of Wilson and Miles (110) and Miles (57) were revealed. They found that AP was three times as effective as PLAPS in inhibiting B. melitensis agglutination using the monospecific sera. The ratio of homologous and heterologous inhibiting titre was of the same order, that is, 80 for B. melitensis AP and 20 to 25 for B. abortus. This indicates that the A : M ratio in B. melitensis is substantially greater than 1:20 found by agglutination tests. A similar complex was extracted by Paterson, Pirie and Stableforth (77) but they failed to isolate a monospecific antigen and postulated that AP was associated with the monospecificity of the antigen.

Sulitzeanu (105) suggested that the agglutinogen was present in a soluble as well as insoluble form. Both the agglutinating and protective properties of Brucella antisera could be removed by absorption with the insoluble antigens. The agglutinogen (A and M) carrying lipopolysacchride antigen of B. abortus and B. melitensis has been found to be the main antigen reacting in the SAT, RBPT and Coomb's reaction (Diaz and Levieux (35)).

Recently, the agar gel immunodiffusion and immunoelectrophoresis have revealed more information on the structure of Brucella. Water soluble extracts

of disintegrated cells of Brucella have yielded gel diffusable antigens which are nearly identical in all Brucella species, either rough or smooth forms. These are probably associated with internal or cytoplasmic protein and nucleoprotein antigens that are distinctive for the genus Brucella (Anon (10)). Determinants specific for both A and M are carried by the lipopolysacchride complex extracted from smooth cells.

Olitzki (70) examined the antigen-antibody reaction between Brucella antigens and their corresponding antibodies by the gel diffusion test and noted the appearance of at least six precipitin lines. The number of lines produced depended on the immunization procedure (Olitzki and Sulitzeanu (72)). Using the intravenous route, the immune sera produced six precipitin lines and an additional diffuse line with Brucella extracts. Three or four additional lines were seen with immune sera produced after antigens were injected with Freund's adjuvant either intramuscularly or subcutaneously.

Soluble Brucella antigens extracted by various methods (sonication, chemical, differential centrifugation and column chromatography) have yielded different numbers of precipitin lines on immunodiffusion and immunoelectrophoresis with homologous and heterologous antisera (Parnas, Cegielka and Burdzy (76), Silverman and Elberg (99),

Bruce and Jones (16), Chen and Elberg (20), Baughn and Freeman (13), Hinsdill and Berman (41), Diaz, Jones and Wilson (31), Olitzki (70) and Kulshreshtha, Atal and Wahi (48)). The number of lines usually decreased when the sera were either absorbed with homologous antigens or diluted.

Several workers (Parnas et al (76), Olitzki (70) Silverman and Elberg (99), Carrere, Roux and Serre (19), Roux and Serre (96), Gajos (37) and Diaz et al (31)) have found a close antigenic relationship between B.abortus, B. suis and B. melitensis but were unable to demonstrate a species specific antigen in either immunodiffusion or immunoelectrophoretic tests.

Specific antigen factors (Fraction 5 from B. melitensis of Redfearn (83) and cell-wall membranes of Roux and Serre (96)) exist within the Brucella species since it is possible to obtain monospecific sera absorption. References to the identification of an antigen from extracts of smooth Brucella species similar to that of AP substance described by Miles and Pirie (58) have been made but with different nomenclature. Hinsdill and Berman (41) called it Component IX; Serre, Asselineau, Lacave and Bascoul (98) referred to it as LPS sol Ph and PS, McGhee and Freeman (56) as antigen F and Diaz, Jones, Leong and Wilson (33) as Component X. This antigenic fraction is a

lipopolysacchride protein complex on the surface of the Brucella cell-wall since antibody against it is absorbed by whole cells. In immunodiffusion or immunoelectrophoresis, this antigen invariably forms a precipitin line or band close to the antigen well when smooth Brucella antigenic extracts are reacted against smooth Brucella antisera (Diaz et al (31, 32, 33, and 34), Kulshreshtha et al (48) and Corbel (23)). In addition, this antigen is specific for hemagglutination with normal red blood cells and agglutination, and is affected by S and R variation (Diaz et al (33)). Diaz, Jones, Leong and Wilson (34) have suggested that Component M from B. melitensis and A from B. abortus correlate with M and A agglutinogens of Wilson and Miles (110). They concluded that the A and M antigens are present as a single complex, in different proportions depending on the species and biotype, and that this component is lipopolysacchride-protein in nature and diffuses poorly through agar gel.

There are other antigens which do not appear to be species specific and probably are subsurface or intracellular components (Hinsdill and Berman (41), Carrere et al (19), Roux and Serre (96), Serre et al (98), Diaz et al (31, 33 and 34), Baughn and Freeman (13), Kulshreshtha et al (48), Myers, Jones and Varela-Diaz (64), Corbel (23) and Freeman,

McGhee and Baughn (36)). These antigens sensitize tanned red blood cells for IHA reactions, diffuse through agar gel freely and are present in both smooth and rough forms of Brucella (Diaz et al (33)).

Antigenic formulae for Brucella species was proposed by Freeman et al (36) from the studies done on the immunoelectrophoretic separation of the soluble antigens. Preliminary characterization of the individual antigenic components of three major species was attempted by chemical, physical and enzymatic treatment on sonic extracts. Antigens 6 and 7 were found to be of considerable intrest since these were only demonstrated in B. melitensis antigenic fractions. These antigens are known to be present both in B. abortus and B. suis but are believed to be present in small quantities as they were not revealed by immunoelectrophoresis. These antigens are probably identical to those described by Diaz et al (33).

C. SEROLOGICAL TESTS.

There are two main reasons why immunological diagnostic methods for brucellosis are needed; firstly, to determine the epizootiological factors influencing the prevention, eradication and surveillance of brucellosis, and secondly, for the diagnosis of the clinical disease, latent infection or vaccinal status of an individual human or animal.

Evidence as to the efficiency of the various diagnostic tests for B. melitensis infection is somewhat limited (Anon (10)). Positive serological reactions are common in goats from which no Brucella can be isolated at autopsy, and conversely, animals known to be infected may have no detectable antibodies. Therefore the results of serological tests are applied on a herd basis. One or several tests repeated at frequent intervals will give more information, especially on individual animals.

1) The Complement Fixation Test (CFT):

The CFT has been used along with the agglutination test in earlier investigations on specific diagnosis of bovine brucellosis in Denmark (Holth (43)) and England (MacFeyden and Stockman (54)).

Much has been reported about the sensitivity, reliability and specificity of the CFT and its advantages compared to other serological tests for the diagnosis of brucellosis in cattle (Larsen (53); Boerner and Stubbs (15), Rice, Boulanger, Mackie and Moore (92), Jones, Hendricks and Berman (46), and Mylrea (66)), and sheep and goats (Gaumont (38), Alton (3) and Unel, Williams and Stableforth (107)).

Renoux, Plommet and Philippon (90) found the results obtained in microplate CFT identical to those obtained by the tube CFT for brucellosis. Philpott and Auko (79) who used the microplate CFT

for diagnosis of B. melitensis infection in sheep and goats, reported that more reactors were detected by the CFT than either by the SAT or RBPT. Alton (2) found the CFT particularly useful in differentiating the vaccinal titres fron the infection titres in adult animals.

2) The Serum Agglutination Test (SAT):

The SAT was originally developed by Wright and Smith (112) and was first applied in conjunction with the CFT for diagnosis of bovine brucellosis in 1909 (Grinsted (40) and Holth (43)). Both tests were found to be reliable in detecting infected animals. Brucella infection in goats was first recognised by the agglutination test (Zammit (114)). The SAT proved to be consistent and reliable (Polding (80 and 81)). Currently, the SAT is perhaps the most widely used serological test for the diagnosis of brucellosis and for assessing eradication campaigns (Morgan (59)).

The SAT can not be used for individual animal diagnosis of brucellosis because a negative reaction does not indicate freedom from Brucella infection (Burnet and Lagoanere (17), Renoux (84), Renoux and Alton (87 and 88), Morgan, Mackinnon, and Lawson and Cullen (61) and Nicoletti and Muraschi (69)). Furthermore in the incubative and chronic stages of the disease there is a difficulty in differentiating antibodies induced during

infection or by vaccination (Morgan (59)). For instance, goats which are vaccinated with live B. melitensis Rev 1 vaccine or killed B. melitensis H 38 adjuvant vaccine, may have suspicious or positive titres several years after vaccination (Anon (10) and Alton (2)).

3) The Rose Bengal Plate Test (RBPT):

The RBPT is comparatively a new test which is simple, rapid and sensitive (Anon (10)).

Originally, Rose and Ropeke (95) modified the plate agglutination test by using antigen buffered to pH 4 immediately before use. This modification was found to alleviate non-specific agglutination while the activity of specific Brucella antibody was unaffected. Both the Card test and RBPT are slight modifications of the Acid Plate Antigen Test (APAT). The usefulness of the APAT as a supplementary test in screening field samples was noted by Lambert and Amerault (51 and 52).

Reports on the use of the RBPT for the diagnosis of goat and sheep brucellosis are few although the test has been commonly used for the diagnosis of cattle, pig and human brucellosis.

A close correlation has been reported between the SAT, CFT and RBPT when bovine, ovine caprine and human sera are tested for brucellosis (Morgan et al (61), Nicoletti and Fadai-Ghotbi (68),

Oomen and Waghela (75) and Anon (11)). In infected cattle herds, the RBPT apparently can identify infected animals at an earlier stage than the SAT and is often positive, along with the CFT, when the SAT is negative or inconclusive. However, some workers have found the RBPT to be oversensitive compared with the SAT or CFT (Anon (9), Davies (28)).

4) The Agar Gel Immunodiffusion Test (AGIT):

Literature on the use of immunodiffusion as a diagnostic test for brucellosis is meagre. Bruce and Jones (16) compared the AGIT with the SAT and CFT. They found 90 percent agreement between agglutination and precipitin reaction. It was suggested that the appearance of precipitins was closely related to the stage of infection in the animal; when the infection is overcome, the precipitins disappear more quickly than agglutinins.

Myers and Sinuik (65) described a simple gel diffusion techniques for the diagnosis of ram epididymitis. The test, using saline extracts of B. ovis was as sensitive as the CFT and more practical (Myers et al (64)). However, the test did not appear to be as sensitive as the established methods (SAT, CFT and RBPT) for the diagnosis of B. melitensis and B. abortus infections.

Corbel (23) suggested that the AGIT may assist

in the identification of antibodies specific to smooth Brucella infection in sera that give equivocal results in the SAT and CFT.

5) The Indirect Hemagglutination Test (IHAT):

Several investigators have applied the IHA reaction to the studies of brucellosis and found the test to be sensitve and Reliable (Ris and Te Punga (94), Ris (93), Versilova (109), Diaz, Chordi, Toromo and Rodriguez-Burgos (29), Pathak (78), Chen and Elberg (20), Kulshreshtha and Ramanchandran (50), Polyakov, Rassudov, Soshiev, Lozovoi and Sagatovskii (82), Renoux, Plomet and Philippon (91), Chernysheva, Vashkevich, Stepushin and Ivanov (21), Belchenko and Ivanov (14), and Skarshevskaya and Dakhno (100)). Most of the authors have used normal or tanned sheep red blood cells for the IHA reaction. Renoux (85) described a method using chromium chloride as a coupling agent.

The sensitivity and specificity of the test would appear to depend on the nature of the antigen used for the sensitization of the cells (Anon (10) and Hirschberg and Yarbrough (42)). Polyakov et al (82) found that antigen extracted by deoxycholate offered the possibility of obtaining a stable

system, with a high activity in the IHA reaction for detecting Brucella antibodies. Both sonicated and chemical extracts were found suitable for the sensitization of red blood cells.

Corbel and Day (24) assessed the IHAT for bovine brucellosis using erythrocytes sensitised with either lipopolysacchride (LPS) or intracellular (IC) antigens of B. abortus. The IHAT when compared with the CFT, SAT and RBPT gave a higher titre and was generally more sensitive. However, the IHAT offered little advantage over the other tests.

SECTION III

MATERIALS AND METHODS

A) SERUM SAMPLES FROM GOATS.

Serum samples for evaluation of the various serological tests were obtained from two sources.

1) Naturally infected goats.

2) Experimentally infected goats.

These sera were tested in the SAT, CFT, RBPT and AGIT. The IHAT was not used to assay the sera.

1) Naturally Infected Goats:

a) Farm 1.

Approximately 1000 goats originally from Wajir and Mandera districts of North Eastern Province were brought to a ranch in Athi River for breeding purposes. An outbreak of abortion was first reported in July 1972. Brucellosis was confirmed both serologically and bacteriologically. In August 1972, another outbreak of abortion was reported in the same flock. Blood samples for serum were taken fron 136 goats. Milk and vaginal swabs from aborted females, taken on the same day, yielded two isolates of B. melitensis biotype 1 on culture. The isolate was confirmed by the Central Veterinary Laboratory, Weybridge, U.K.

b) Farm 2.

A group of indigenous and crossbred female goats which were being upgraded by Sannen billies had an abortion outbreak. B. melitensis biotype 1 was isolated from vaginal swabs and milk samples (Philpott and Auko (79)). Seventy sera from this herd were tested using the four serological tests.

2. Experimentally Infected Goats:

Twenty four goats, mainly adults, were bought from Limuru and Gatundu areas of Central Province. On arrival at Kabete, they were ear-tagged, sprayed with toxaphene for ectoparasites and drenched with thiabendazole for endoparasites. The goats were tested serologically and bacteriologically for brucellosis five times over a period of about six months prior to infection. All were negative during this period. Some of the female goats were pregnant at the time of infection.

All goats were kept in an isolation block. Prior to moving the animals to the isolation unit, they were sprayed and drenched as before. Goats were divided into three groups of eight animals each. In Group1, five female goats were sub-cutaneously infected with a dose of 1.4×10^6 live B. melitensis Strain H 38 cells. Two females and

one male were left incontact. In Group 2, five female goats were infected with a dose of 7.0×10^4 live organisms by conjunctival instillation into one eye. Two female goats and one male goat were left incontact. Group 3 consisting of six females and 2 males was kept as a control group.

All goats were bled regularly, initially at 2 day intervals for 3 weeks, then usually at weekly intervals for 44 weeks. Vaginal swabs were taken regularly. Milk samples were taken whenever the individual goats were lactating.

A summary of these infection procedures is given in Table 2.

B) INFECTED MATERIAL.

Infected materials such as the milk samples, aborted fetal tissues and tissues of carcases of either farm or experimental goats were cultured for Brucella on two types of media in duplicate plates:

1) Blood agar;

2) Serum Dextrose agar (SDA) containing Polymixin B (600 units/100ml), Bacitracin (2000 units/100ml) and Cycloheximide (10 mg/100 ml) according to Alton and Jones (5). One pair of the duplicate plates was incubated at 37°C in air for 3 days and the other pair incubated in an atmosphere of 10% carbon dioxide for 3 days at 37°C.

Table 2: Goats Experimentally Infected with *B. melitensis**; the Dose and Route of Infection:

GROUP ONE	INFECTION:	*B. melitensis* Strain H38
	DOSE:	1.4×10^6 organisms/ml
	ROUTE:	Subcutaneous over the shoulder
	ANIMAL NO.:	3120(F)**; 3132(F); 3138(F); 3140(F); 3143(F).
	INCONTACT:	
	ANIMAL NO.:	3129(F); 3135(F); 3123(M)**.
GROUP TWO	INFECTION:	*B. melitensis* Strain H38
	DOSE:	7.0×10^4 organisms/ml
	ROUTE:	Conjunctival Instillation
	ANIMAL NO.:	3119(F); 3128(F); 3130(F); 3134(F); 3139(F).
	INCONTACT:	
	ANIMAL NO.:	3126(F); 313(F); 3122(M).
GROUP THREE	CONTROL:	
	ANIMAL NO.:	3121(F); 3124(F); 3125(F); 3127(F); 3133(F); 3141(F); 3142(M); 3136(M)***.

*All animals bled initially at 2 day intervals and then at weekly intervals.

**Sex: F = Female; M = Male.

***Goat No. 3136 moved to Group 1 on day 155 to replace No. 3123 which died.

Brucella-like organisms were tested against monospecific serum for B. melitensis.

All milk samples were tested by the milk ring test (MRT) (Alton and Jones (5)). Briefly, the test was performed by adding a drop (0.03 ml) of stained Brucella antigen to 1 ml of the test milk in a test tube. The tube was incubated at 37°C for 60 minutes. The test was read. The test was kept at 4°C for 18 hours and the test re-read.

C. PRODUCTION OF THE HYPERIMMUNE SERA AND MONOSPECIFIC SERUM.

The hyperimmune sera were produced by inoculating adult male white rabbits with either live or killed B. melitensis Strain 16M. The concentration of Brucella cells was 3.5×10^7 cells per one ml. The protocal followed to produce hyperimmune sera is presented in Table 3. Serum from rabbit A was used as a standard hyperimmune serum (MHS). Monospecific serum for B. melitensis (MMS) was made according to the procedures described by Alton and Jones (5).

D. SEROLOGICAL TESTS.

1) The Serum (Tube) Agglutination Test (SAT):

(a) Antigen Preparation.

Antigen for the SAT was prepared on Serum Dextrose agar (SDA) from smooth B. abortus Strain 99

Table 3: Procedures for Production in Rabbits of Hyperimmune Sera against Smooth *Brucella melitensis* Strain 16M:

Rabbit No.	*B. melitensis* Cell Suspension. 3.5×10^7 org./ml	Dosage Regimen	Route of Inoculation*	Day of Exsanguination+
A	Live	1 ml	I.V.	6th day
B	Live	1 ml	I.V.	
		3 weeks later		
	Killed	2 ml	S.C.	28th day
C	Killed	1 ml	I.V.	
		3 weeks later		
	Killed	2 ml	S.C.	28th day
D	Killed in CFA**	0.5 ml	I.V.	
		2 ml	S.C.	
and		2 weeks later		
	Killed in CFA	2 ml	S.C.	
E		2 weeks later		
	Killed in CFA	2 ml	S.C.	42nd day

*I.V. = Intravenously; S.C. = Subcutaneously. +Day after the first inoculation.

**CFA = Complete Freund's Adjuvant.

according to Alton and JOnes (5), and stanardized against the International Standard for Anti-Brucella abortus Serum II (ISAbS). An antigen dilution was selected which gave 50 percent agglutination at 1:500 final dilution of ISAbS.

(b) Test Procedure.

The SAT was a five tube test, beginning with a serum dilution of 1:6.25, prior to the addition of the antigen. Doubling serum dilutions were made in 0.5 ml of 0.5 percent phenol in 5 percent saline and 0.5 ml of antigen at the working dilution was added to each tube. After shaking, the tubes were incubated at 37°C for 18 hours.

Controls included a known positive serum and a negative serum. The highest serum dilution showing 50 percent agglutination was taken as the 'end-point' of the serum.

(c) Interpretation of the Results.

The results were recorded in International Units (I.U.)/ml obtained by multiplying the reciprocal of the titres by 2. The results were interpreted as in Table 4.

2) The Complement Fixation Test (CFT):

(a) Antigen Preparation.

Antigen used in the CFT was kindly supplied by the Max V. Pettenkofer Institute (Berlin, West Germany). The dilution of antigen used was 1:100, as recommended by the Institute.

(b) Test Procedure.

The test was based on the microtitre system and was similar to the one described for use by the U.S. Dept. of Hlth., Edu. and Welfare (Anon (7)).

The test was a six well test with serum dilutions of 1:2.5 to 1:80. The test included one volume (0.025 ml) of serum dilution in veronal buffered diluent, pH 7.4 (VBD), one volume of antigen, two volumes of complement (five minimum hemolytic doses) and one volume of sensitized sheep red blood cells. Before use, all sera were diluted to 1:2.5 and inactivated at 62.5^{o}C for 30 minutes. The usual complement, antigen, anticomplementary, negative and positive serum controls were included.

(c) Interpretation of the Results.

The results were recorded according to the degree of fixation at a particular titre. One hundred percent fixation was recorded as 4, 75 percent as 3, 50 percent as 2 and 25 percent as 1. The results were interpreted as in Table 4.

3) The Rose Bengal Plate Test (RBPT):

(a) Antigen Preparation.

The antigen was prepared from B. abortus Strain 99, according to the method recommended by the U.S. Dept. of Agriculture (Anon (8)). The antigen contained an 8 percent concentration of stained Brucella cells buffered at pH 3.65 and

Table 4: Interpretation of the Titres in SAT and CFT:

	TESTS*	
INTERPRETATION	SAT**	CFT**
SUSPICIOUS	25 I.U.	2:2.5 to 1:5
POSITIVE	50 I.U. and over	2:5 and over

*SAT = Serum Agglutination Test.
CFT = Complement Fixation Test.

**SAT titres in International Units (I.U.).
CFT titres in serum dilutions. The titres are given in degrees of fixation at a particular titre: 1 = 25 percent; 2 = 50 percent; 3 = 75 percent; 4 = 100 percent.

standardized in relation to the antigen produced by the Central Veterinary Laboratory, Weybridge, U.K.

(b) Test Procedure.

The different test sera were placed in a row on a white enamel plate in volumes of 0.03 ml, then 0.03 ml of the antigen was added to each serum drop. The reactants were mixed with an applicator stick and shaken on a mechanical shaker for 4 minutes. The test was then read.

(c) Interpretation of the Results.

The test was recorded as positive when there was slight to complete agglutination.

4) The Agar Gel Immunodiffusion Test (AGIT):

(a) Antigen Preparation.

Freeze dried B. melitensis Strain 16M was obtained from the Central Veterinary Laboratory, Weybridge, U.K. The reconstituted strain was grown on SDA and harvested according to the method described by Alton and Jones (5). After harvesting, the organism was spun at 3000 g for 45 minutes. The supernatant was stored at -20°C. The sedimented cells were divided into two batches. Batch 1 was suspended in 0.5 percent phenol in 0.85 percent saline and Batch 2 in cold acetone. These batches were stored at 4°C.

The phenolised cells (Batch 1) were dialysed at 4°C against distilled water for two weeks. The suspension was sonicated for 10 minutes at full

power (MSE ULTRASONICATOR). The sonicated material was centrifuged at 3000 g for 60 minutes, and the supernatant tested by the AGIT against B. melitensis hyperimmune serum (MHS). If no reaction was noted a further 10 minutes of sonication was applied after mixing of the supernatant and sediment.

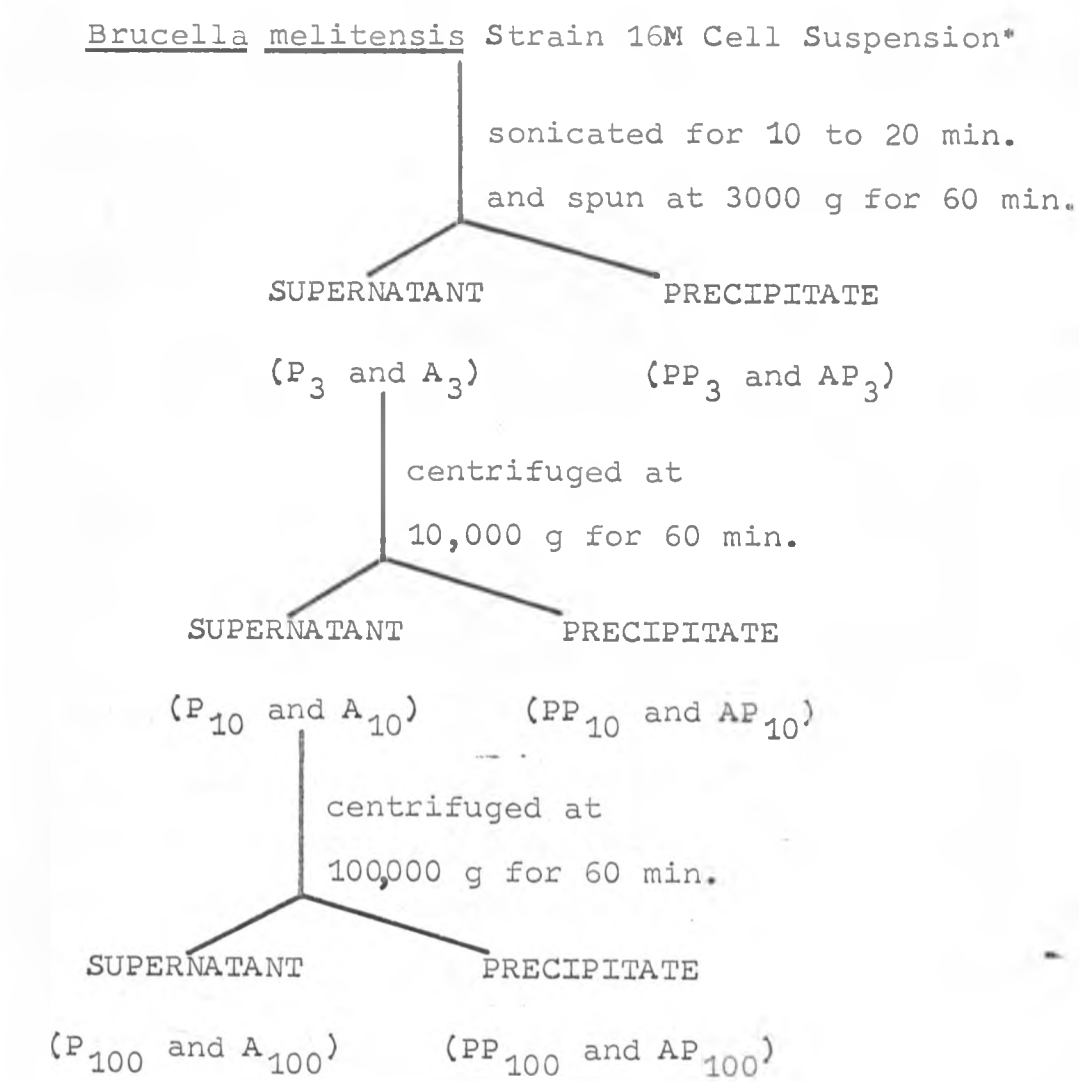
The bacterial suspension in acetone (Batch 2) was dried over $CaCl_2$ in a vacuum dessicator at room temperature. The dried material was ground to a powder and suspended in 20 ml of distilled water. Then the suspension was treated as for phenolised cell suspension (above).

After the final sonication, both batches were centrifuged at 3000 g for 60 minutes. The supernatant underwent differential centrifugation at 10,000 g and 100,000 g for 60 minutes each. The resulting fractions were lyophilized and stored at $-20^{o}C$.

All the resulting fractions were reconstituted to one-fifth of their original volume. All supernatant fractions were designated with a suffix 'C' to denote 'concentrated', for example, A_3C, $A_{100}C$, $P_{100}C$ and so on. All these fractions were tested against B. melitensis hyperimmune and monospecific sera.

The following flowchart shows the preparations of various fractions from the cell suspensions:

Flowchart showing the Fractionation of the Sonicated *Brucella melitensis* Strain 16M Cell Suspension:



* P = Batch 1 - cells in o.5 percent phenol saline; dialysed against distilled water. Suspension in 0.85 percent saline.

A = Batch 2 - cells in cold acetone dried under vacuum. Suspension in distilled in water.

Antigen fraction $P_{100}C$ was used to test the field and experimental sera from goats in the AGIT.

(b) Test Procedure.

Approximately 6 ml of 1percent Noble Agar (Difco) in VBD was carefully poured on glass slides, measuring 7.6 cm by 2.5 cm, previously coated with 3 percent Noble Agar. The agar was allowed to solidify for about thirty minutes.

To check whether the pH of 1 percent Noble Agar or the addition of a preservative affected the test, a pH range of 6.5 to 8.0 was selected and preservatives 0.01 percent merthiolate or 0.02 percent sodium azide was used.

Wells were cut using templates with the following designs:

Six wells with a diameter of either 2.0 mm (minimicromethod), 3.5 mm (micromethod) or 6.0 mm (macromethod), arranged around a central well of the same diameter, and at an edge to edge distance of 3.0 mm, 3.0 mm and 5.0 mm respectively. A small petri dish (45 mm diameter) instead of a slide was used for the macromethod. The agar was removed from wells using suction. The bottom of the wells was sealed with a small volume of 1 percent Noble Agar. Antigen was added in volumes of 0.01 ml, 0.02 ml and 0.04 ml; and the antisera in volumes of 0.02 ml, 0.03 ml and 0.09 ml respectively for each method.

The slides or petri dishes were kept in a moist chamber, at room temperature. The tests were read at 4 hours, 8 hours and daily for the next 14 days. After the final reading, the slides were washed in normal saline for 48 hours, followed by distilled water for another 48 hours. Saline and distilled water were changed at least twice daily. The slides were allowed to dry overnight at room temperature. During the drying, the agar was covered by cellulose acetate membrane which was pierced over the wells. The dried slides were then washed in distilled water and stained.

Various stains were used to visualize the reaction and to determine the chemical nature of the reacting antigens. These were:

Azocarmine Red	(0.05 percent)
Thiazine Red	(0.1 percent)
Amido Black	(0.1 percent)
Ponceau S	(0.1 percent)
Sudan Black	(0.1 percent)

and Periodic Acid Schiff reagent (PAS).

All stains, except for sudan black and PAS were diluted in 50 percent methanol with 5 percent acetic acid. Sudan black was diluted in 60 percent ethanol. To every 100 ml of this dye was added 0.2 ml of 25 percent sodium hydroxide. Slides were stained for 2 to 5 minutes (one minute with amido black),

and washed in 5 percent acetic acid in 50 percent methanol for 10 to 20 minutes. Sudan black stained slides were differentiated with 60 percent ethanol. Slides were then air dried. Staining with PAS was performed according to Crowle (26).

(c) Interpretation of the Results.

Any degree of precipitation was regarded as a positive reaction. The number of precipitin lines was also recorded.

5) The Indirect Hemagglutination Test (IHAT):

(a) Antigen Preparation.

The same antigens prepared for the AGIT were used for the study of the IHA reaction. Sheep red blood cells were sensitized in the following way:

A 1 percent washed red cell suspension was tanned with an optimum dilution of tannic acid; washed once with warm PBS (pH 7.4) and suspended to 5 percent in the same buffer. An equal amount of this cell suspension was added to an optimal concentration (predetermined by titration) of the antigen. This mixture was incubated at 37^{o}C for 45 minutes. The red cells were then washed three times in PBS (pH 7.4). A 0.5 percent cell suspension was finally made in 1 percent fetal calf serum (FCS) diluted in PBS.

Gluteraldehyde fixed cells were prepared by the following procedure:

Packed and washed cells were diluted in PBS to a 4 percent suspension. While being stirred, 0.2 ml to 2.0 ml of an aqueous solution of 2.5 percent of gluteraldehyde was added to the red cell suspension. The stirring was continued for 60 minutes at room temperature. The gluteraldehyde fixed cells were then sesitized as above.

(b) Test Procedure.

The technique for conducting the IHAT was similar to the one used by Zimmerman, Mathews and Wilson (116). All the sera were inactivated at 56°C for 30 minutes and diluted in 1 percent fetal calf serum (FCS), before use in the test. Heterophile antibodies were absorbed by adding one volume of the packed sheep red blood cells to nine volumes of test serum and allowing it to stand for 30 minutes at 37°C.

Initially, 0.05 ml of 1 percent FCS was dispenced in each well of a microtitre plate, except the first well to which was added 0.1 ml of 1:10 test serum dilution. A two-fold dilution was made starting from the first well, using 0.05 ml microdiluters. To each well 0.025 ml of the sensitized sheep red blood cell suspension was added. The plate was gently shaken and allowed to stand overnight at room temperature. The 11th and 12th row of wells were used as cell and buffer control. Known positive and negative serum were used as controls.

The test was regarded as either positive or negative according to the presence or absence of agglutination within each well.

SECTION IV

RESULTS

A). A COMPARISON OF ANTIGENS IN THE AGAR GEL IMMUNOFIFFUSION TEST (AGIT)

All the antigenic fractions were tested against *B. melitensis* hyperimmune sera and monospecific serum. The maximum number of precipitin lines demonstrated was six. These were designated according to their distance towards the antigen well from the antibody well, that is, the line nearest the antibody well was number 1 while the line closest to the antigen well was number 6 (Figs, 1 and 2). A broad diffuse halo around the antibody well was considered as non-specific since it was present even with negative sera.

The AGIT was found to give best reaction with the micromethod. The difference in the results using the same reactants with the three methods is as shown in the Figs.3, 4, 5. The same figures show that there was no change in the reaction, after 18 to 24 hours incubation, in the minimicromethod and micromethod. The reaction in the macromethod was complete in 72 hours. It was also found that pH of agar closer to 7.2 and 7.4 always gave the maximum lines, with maximum definition. A pH either

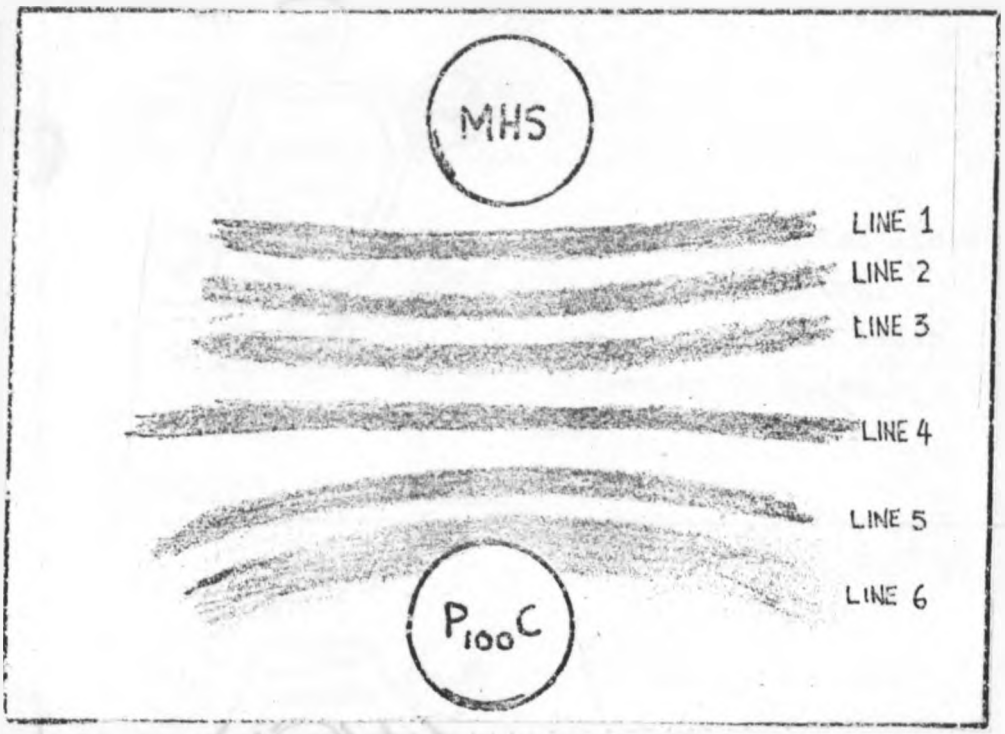


Figure 1: Diagrammatic Classification of the Six Precipitin Lines Observed in the AGIT.

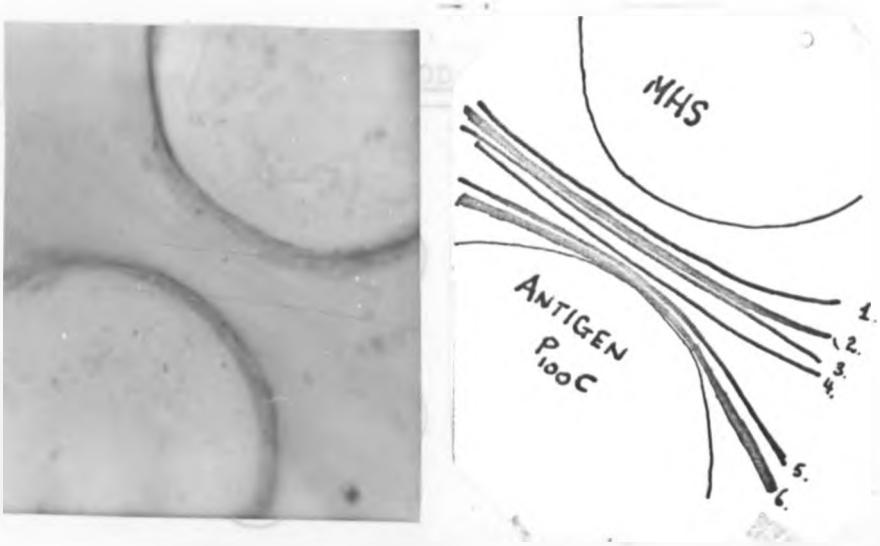
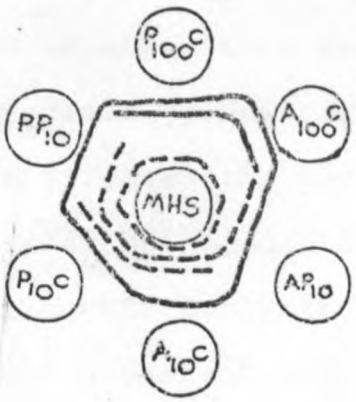
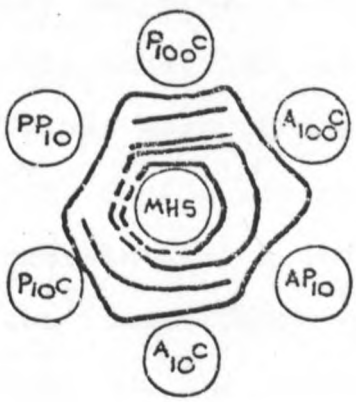


Figure 2: shows the Six Precipitin Lines Observed in the AGIT when Antigen $P_{100}C$ Reacted with B. melitensis Hyperimmune Serum A,(MHS).



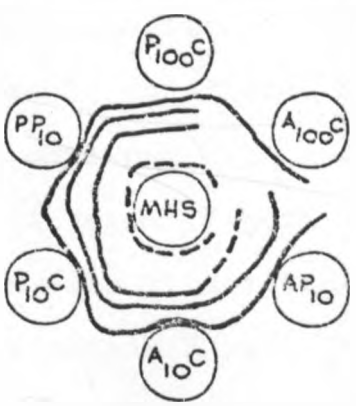
Reaction: Dotted lines seen by 24 hours.
Full lines seen by 72 hours.

Figure 3. MACROMETHOD



Reaction: Dotted lines seen by 7 to 8 hours.
Full lines seen by 18 hours.

Figure 4. MICROMETHOD



Reaction: Dotted lines seen by 7 hours.
Full lines seen by 18 hours.

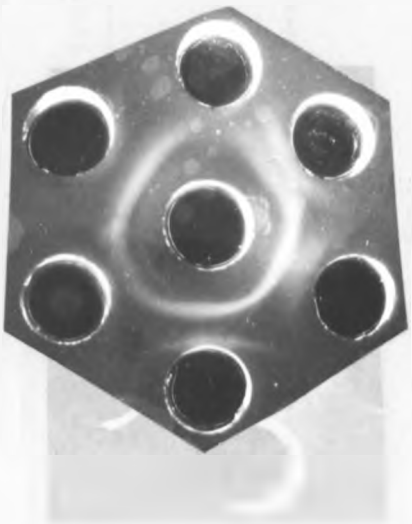
Figure 5. MINIMICROMETHOD

Figures 3 to 5: Differences in Reaction in the Three Designs of the Agar Gel Immunodiffusion Test. (Antigens in the outer wells; Antiserum in the central).

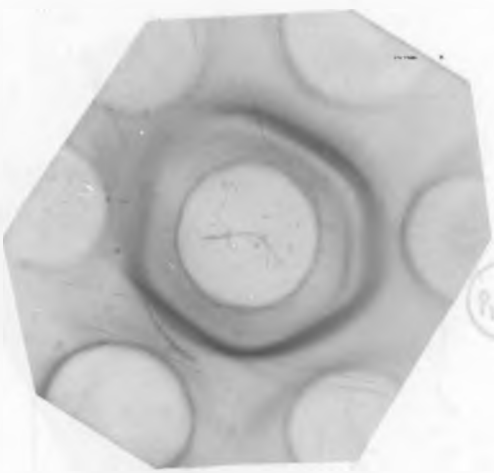
below or above this range did not bring out all lines clearly, Neither merthiolate nor sodium azide affected the test reaction significantly from that obtained using unpreserved agar. Both these agents inhibited contaminants which overcame the non-preserved agar within the first 18 hours of incubation.

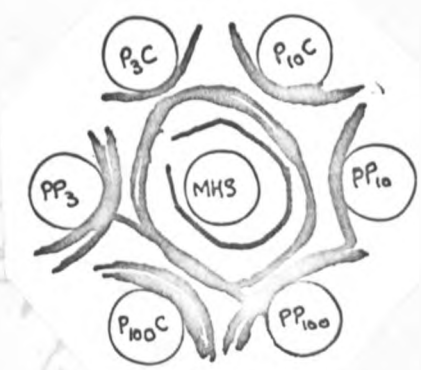
Staining of the reactions on the dried slides usually differentiated precipitin lines 2 and 3, and 5 and 6. Otherwise, staining did not reveal any more lines than it was possible to see on the slides prior to drying and staining (Figures 6 and 7). The protein stains and PAS reaction always stained all the lines. Sudan black stained lines 2 and 3 faintly, but the others were darkly stained.

Tables 5 and 6 show the results of the AGIT when various dilutions of the standard B. melitensis hyperimmune serum (MHS) were reacted against the antigenic fractions. Different numbers of lines were obtained with each entigen depending on the MHS dilution (Figures 8 and 9). These precipitin lines were classified by their identity to the lines obtained with fraction $P_{100}C$ which gave the highest numbers of lines, that is, numbers 1 to 6. Line number 4 was only seen with fraction $P_{100}C$ and not with others (Figures 10 and 11). Only the neat MHS showed maximum numbers of lines with each fraction.



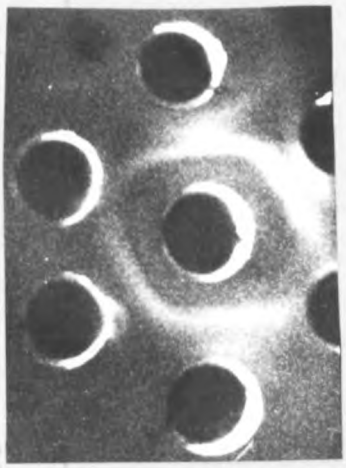
Before Staining.

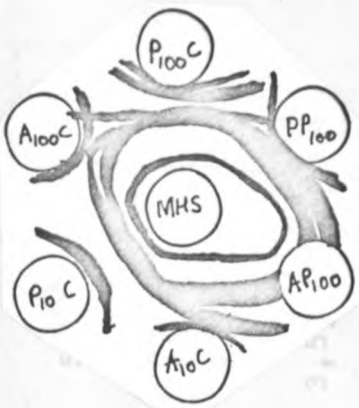




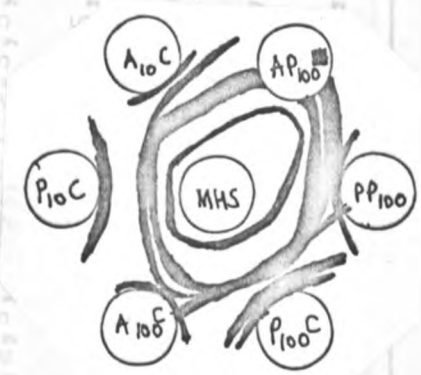
After Staining.

Figure 6: showing Reactions of MHS with various Antigenic Fractions Prior to and After the Staining of the Slides.





Before Staining.



After Staining.

Figure 6: showing Reactions of MHS with various Phenolised Antigenic Fractions Prior to After Staining of the Slides.

Table 5: Precipitin Lines seen in the AGIT with Acetonised Antigenic Fractions when Reacted to Various Dilutions of *B. melitensis* Hyperimmune Serum (MHS):

Antigenic Fractions	Dilutions of Hyperimmune Serum (MHS)					
	Neat	1/2	1/3	1/4	1/5	1/6
A_3	1,2,3,5,6	1,2,5,6	1,2,5,6	2,5,6	-	-
A_3C	1,2,3,5,6	1,2,3,5,6	1,5,6	5,6	-	-
AP_3	1,2,3,5,6	-	-	-	-	-
A_{10}	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	-	-	-
$A_{10}C$	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	5,6	-	-
AP_{10}	1,2,3,6	1,2,3,6	1,2,3	2,3	-	-
$A_{100}C$	1,2,3,5,6	1,2,5,6	5,6	5,6	-	-
AP_{100}	1,2,3,5,6	2,3,5,6	3,5,6	3,5,6	5,6	5,6

Table 6: Precipitin Lines seen in the AGIT with Phenolised Antigenic Fractions when Reacted to Various Dilutions of *B. melitensis* Hyperimmune Serum:

Antigenic Fractions	Dilutions of Hyperimmune Serum (MHS)					
	Neat	1/2	1/3	1/4	1/5	1/6
P_3	2,3,5,6	2,3,5,6	5,6	5,6	-	-
P_3C	1,2,3,5,6	2,3,5,6	2,3,5,6	5,6	5,6	5,6
PP_3	1,2,3,5,6	2,3,5,6	2,3,5,6	2,3,5,6	3,6	3,6
P_{10}	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	2,3,5,6	-	-
$P_{10}C$	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	5,6	5,6
PP_{10}	1,2,3,5,6	1,2,3,5,6	1,2,3,6	1,2,3	1,2,3	-
$P_{100}C$	1,2,3,4,5,6	1,2,3,4,5,6	2,3,4*,5,6	2,3,5,6	2,3,5,6	2,3,5,6
PP_{100}	1,2,5,6	1,2,5,6	5,6	5,6	5,6	5,6

*Faint line.

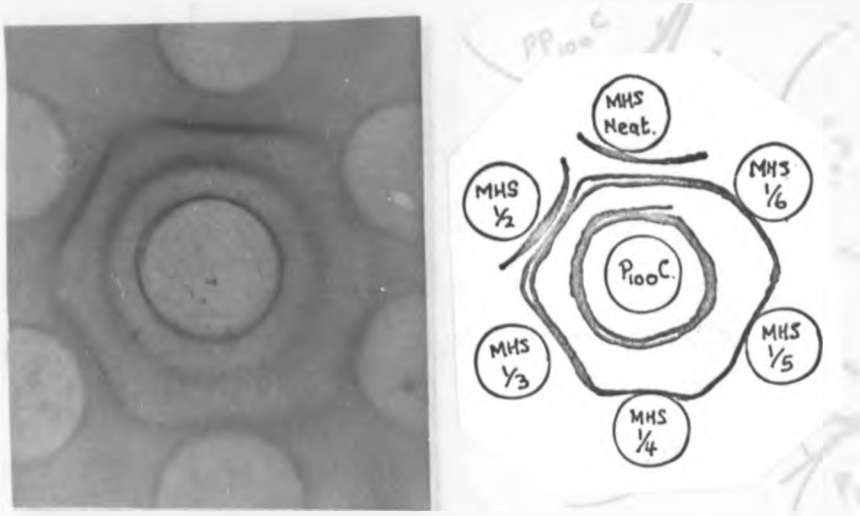


Figure 8: showing the Reaction of Antigenic Fraction $P_{100}C$ with varying Dilutions of MHS.

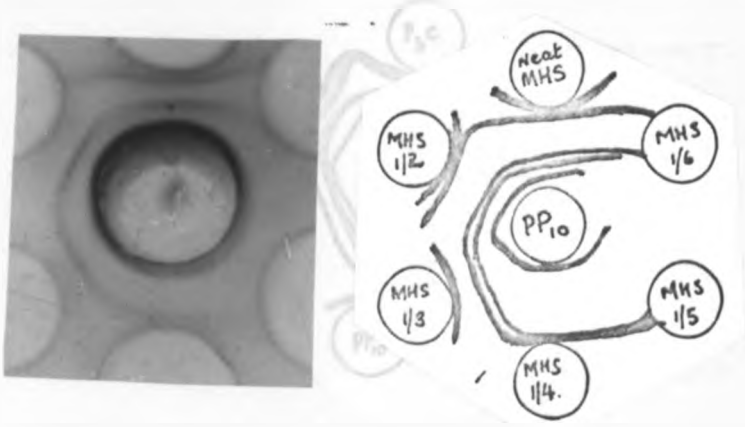


Figure 9: showing the Reaction of Antigenic Fraction PP_{10} with varying Dilutions of MHS.

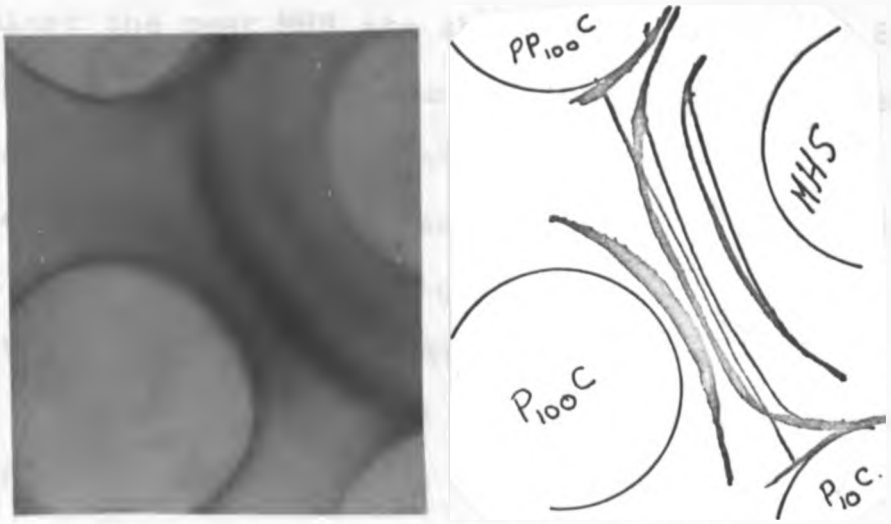


Figure 10: showing the Reaction betweem Fraction $P_{100}C$ and MHS. Precipitin Line 4 is seen clearly. Cross-reactions of Fractions $P_{10}C$, PP_{10} and $P_{100}C$ are also seen.

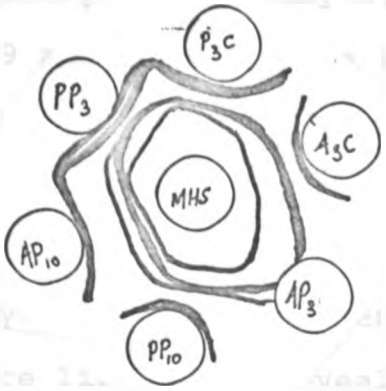


Figure 11: Diagrammatic representation of the Cross-reactions of Antigenic Fractions obtained by low centrifugation.

The AGIT results of various antigen dilutions against the neat MHS are shown in Tables 7 and 8. As before, the highest number of precipitin lines were obtained with the antigen fraction $P_{100}C$. Line number 4 was only seen with this antigen and not with other. Neat antigens tended to give the most lines against neat MHS (Figure 12).

In general, lines 5 and 6 appeared as a broad diffuse band close to the antigen well but were clearly disernible after staining. On dilution of the reagents it was found that some of the lines were separating out initially and then started to disappear on further dilutions.

The antigenic fractions were tested against the MHS in the AGIT, irregularly over a period of months. Tables 9 and 10 summarize the results of these tests. More lines and fewer negative reactions were observed with the concentrated fractions. Differential centrifugation did not help in the isolation of any antigenic component but was useful, in that more lines were revealed with the centrifuged fractions. Antigenic fractions extracted with phenol were more consistent in giving positive reactions and a higher number of lines than acetone fractions (Figures 13 to 15).

Similar findings were noted on the limited tests done with the B. melitensis monospecific

Table 7: Precipitin Lines seen in the AGIT with B. melitensis Hyperimmune Serum (MHS) when Reacted to Various Dilutions of Acetonised Antigenic Fractions:

Antigenic Fractions	Dilutions of Antigenic Fractions					
	Neat	1/2	1/3	1/4	1/5	1/6
A_3	1,2,3,5,6	1,2,5,6	1,2,5,6	1	1	1
A_3C	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	2,3,5,6	NT*	2,3,5,6
AP_3	2,3,5,6	2,3,5,6	2,3,5,6	2,3,5,6	NT	2,3,5,6
A_{10}	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6**	1,2,3,5,6**	1	1
$A_{10}C$	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6**	1,2,3	1,2,3
AP_{10}	1,2,3,6	1,2,3	1,2,3	1,2,3	1,2,3	-
$A_{100}C$	1,2,3,5,6	2,3,5,6	5,6	5,6	5,6	5,6
AP_{100}	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6**	1,2,3	-	-

*NT = Not tested. **Faint line.

Table 8: Precipitin Lines seen in the AGIT with *B. melitensis* Hyperimmune Serum (MHS) when Reacted to Various Dilutions of Phenolised Antigenic Fractions:

Antigenic Fractions	Dilutions of Antigenic Fractions					
	Neat	1/2	1/3	1/4	1/5	1/6
P_3	2,3,5,6	2,3,5,6	2,3,5,6	2,3,5	2,3	2,3
P_3C	1,2,3,5,6	1,2,5,6	1,2,5,6	1,2,5,6	NT*	1,2,5,6
PP_3	1,2,3,5,6	2,3,5,6	2,3,5,6	2,3,5,6	NT	2,3,5,6
P_{10}	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	1,2,5,6	1,2	1,2
$P_{10}C$	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	1,2,3	1,2,3**
PP_{10}	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	1,2,3,5,6	1,2,3*	-
$P_{100}C$	1,2,3,4,5,6	1,2,3,4,5,6	1,2,3,4,5,6	1,2,3,4**,5,6	1,2,3,5,6	1,2,3,5,6
PP_{100}	1,2,5,6	1,5,6	1,5,6	1,5,6	1,5,6	1,5,6

*NT = Not tested. **Faint line.

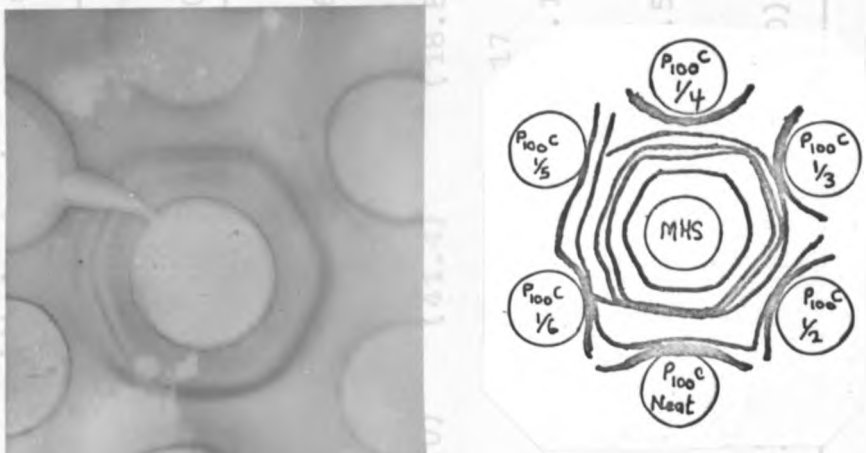


Figure 12: showing the Reaction of MHS with various Dilutions of Antigenic Fraction $P_{100}C$.

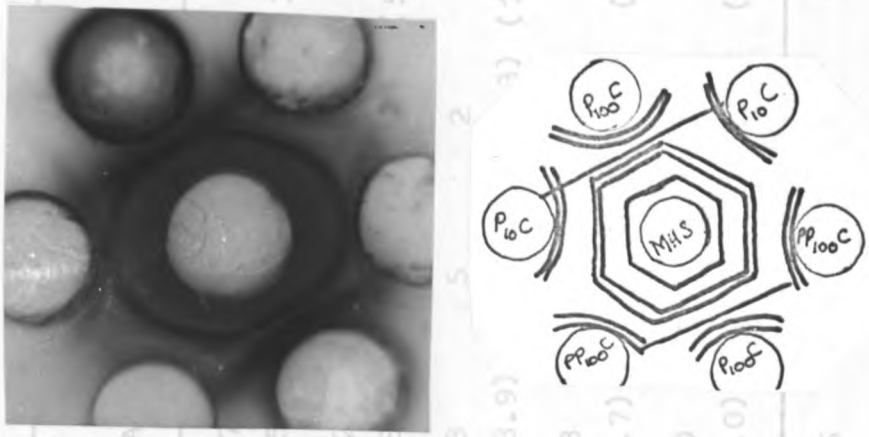


Figure 13: showing the Cross-reaction of various Phenol Antigenic Fractions when Reacted against MHS.

Table 9: Number of Times* Total Number of Precipitin Lines Obtained when B. melitensis Hyperimmune Serum (MHS) was Reacted Several Times with Acetonised Fractions:

Total No. of Lines	Antigenic Fractions							
	A_3	A_3C	AP_3	A_{10}	$A_{10}C$	AP_{10}	$A_{100}C$	AP_{100}
0	7 (15.5)	1 (5.5)	4 (26.7)	16 (32.0)	1 (1.2)	5 (13.9)	4 (5.7)	5 (15.6)
1	22 (48.9)	11 (61.2)	5 (33.3)	25 (50.0)	22 (25.6)	13 (36.0)	29 (41.4)	6 (18.8)
2	13 (28.9)	5 (27.8)	2 (13.3)	9 (18.0)	39 (45.3)	14 (38.9)	24 (34.3)	17 (53.1)
3	3 (6.7)	1 (5.5)	3 (20.0)	0 (0.0)	22 (25.6)	4 (11.1)	13 (18.6)	4 (12.5)
4	0 (0.0)	0 (0.0)	1 (6.7)	0 (0.0)	2 (2.3)	0 (0.0)	0 (0.0)..	0 (0.0)
Total No. Times Tested	45	18	15	50	86	36	70	32

*In parentheses, percentage of times tested.

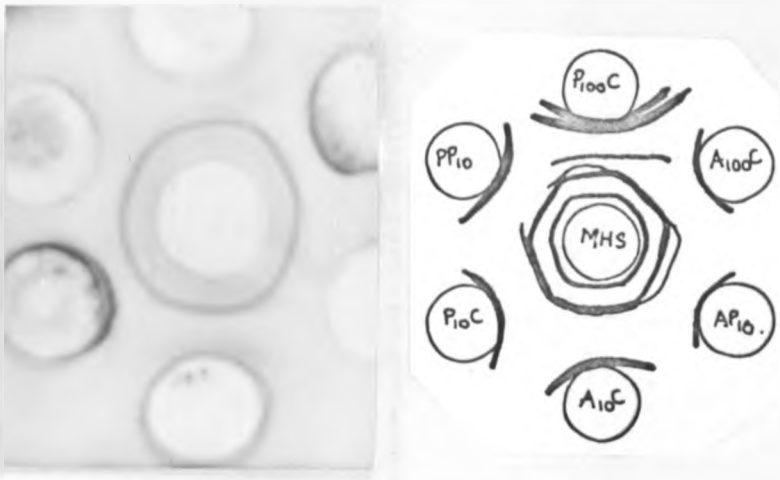


Figure 14a:

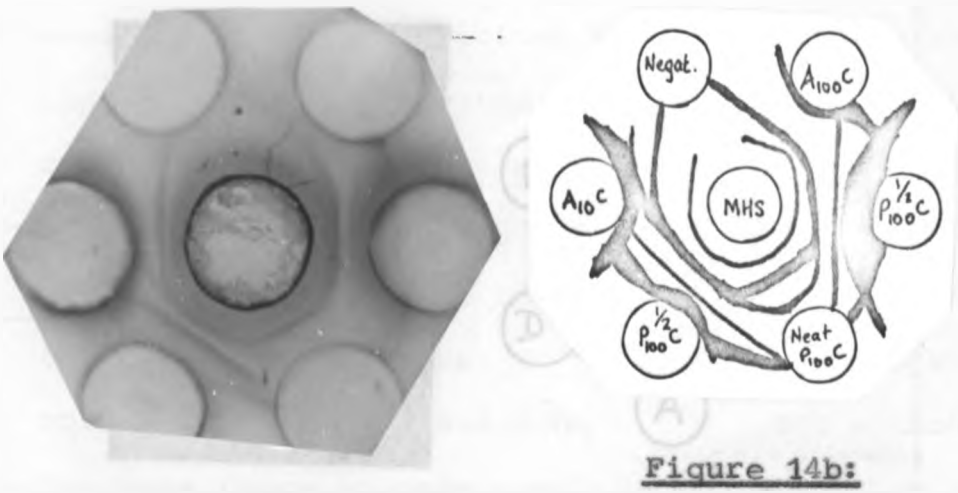


Figure 14b:

Figure 14: shows the Cross-reactions in the AGIT of various Phenol and Acetone Antigenic Fractions when Reacted against MHS.

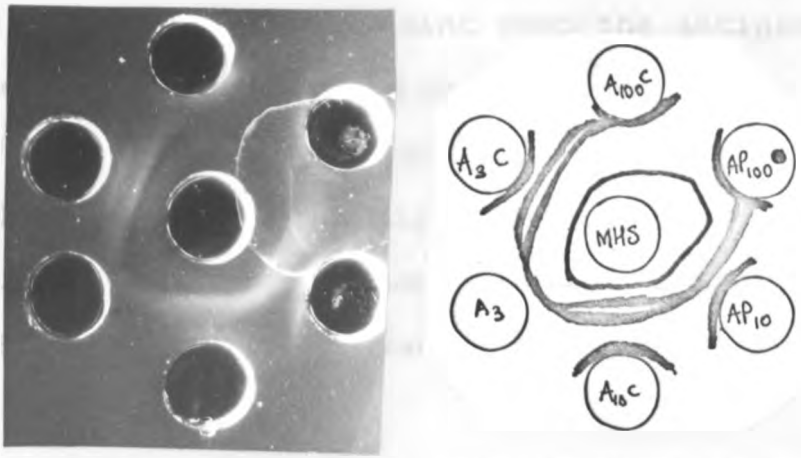


Figure 15: showing the Reaction of MHS with various Acetone Antigenic Fractions.

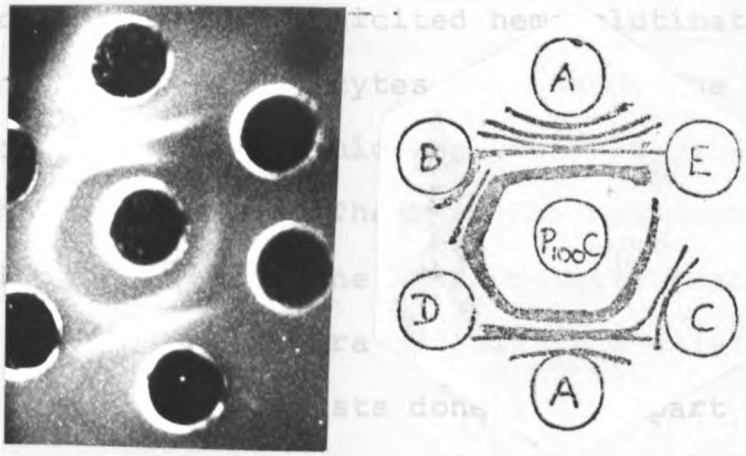


Figure 16: shows the Reaction in the AGIT of Antigenic Fraction $P_{100}C$ with various Hyperimmune Sera. (A = MHS).

serum (MMS). Fewer lines were seen with the MMS than with MHS(Table 11 and 12). Line number 5 was either missing or faint when the antigenic fractions were tested with MMS.

Hyperimmune sera prepared by different methods were tested against the antigens (Table 13). It was seen that serum A best, followed by B; sera C, D and E were not as good (Figure 16).

B). INDIRECT HEMAGGLUTINATING ACTIVITY OF THE ANTIGENS.

The antigenic fractions were checked for their hemagglutinating activity. All ten fractions failed to react when tested against the MHS in the IHAT, using fresh sheep red blood cells. However, the antigens elicited hemagglutination when tanned sheep erythrocytes were used. The optimum concentration of tannic was found to be 1:20,000 using PBS (pH 7.4). The same PBS was used for the antigen dilutions. The IHAT results were erratic; the titres of the sera and also the antigens would not be similar on tests done a day apart although the same reagents were used. Panagglutination also caused some problems. Lack of antigens and also lack of time prevented any further work on this test. Some of the results obtained are shown in Tables 14 and 15.

Table 11: Number of Times* Total Number of Precipitin Lines Obtained when B. melitensis Monospecific Serum (MMS) was Reacted Several Times with Acetonised Fractions+:

Total No. of Lines	Antigenic Fractions						
	A_3	A_3C	A_{10}	$A_{10}C$	AP_{10}	$A_{100}C$	AP_{100}
0	2 (33.3)	2 (50.0)	14 (77.8)	2 (7.4)	3 (30.0)	0 (0.0)	3 (42.8)
1	3 (50.0)	1 (25.0)	4 (22.2)	16 (59.3)	4 (40.0)	6 (66.7)	2 (28.6)
2	1 (16.7)	1 (25.0)	0 (0.0)	7 (29.9)	3 (30.0)	3 (33.3)	2 (28.6)
3	0 (0.0)	0 (0.0)	0 (0.0)	2 (7.4)	0 (0.0)	0 (0.0)	0 (0.0)
Total Times Tested.	6	4	18	27	10	9	7

*In parentheses, percentage of times tested.

+Fraction AP_3 was tested once only and gave 2 precipitin lines.

Table 12: Number of Times* Total Number of Precipitin Lines Obtained when B. melitensis Monospecific Serum (MMS) was Reacted Several Times with Phenolised Fractions+:

Total No. of Lines	Antigenic Fractions				
	P_3C	P_{10}	$P_{10}C$	$P_{100}C$	PP_{100}
0	2 (66.7)	11 (55.0)	3 (12.5)	0 (0.0)	2 (22.2)
1	0 (0.0)	7 (35.0)	14 (56.0)	3 (33.3)	1 (11.1)
2	1 (33.3)	2 (10.0)	6 (25.0)	4 (44.4)	1 (11.1)
3	0 (0.0)	0 (0.0)	1 (4.2)	1 (11.1)	5 (55.5)
4	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)
Total Times Tested	3	20	24	9	9

*In parentheses, percentage of times tested.

+Fractions P_3, PP_3 and PP_{10} were not tested.

Table 13: Comparision of Various Hyperimmune Sera in the AGIT when Tested Against Different Antigenic Fractions:

Hyperimmune Serum	Antigenic Fractions								
	A_3	AP_3	AP_{100}	$A_{10}C$	$A_{100}C$	PP_3	$P_{10}C$	PP_{100}	$P_{100}C$
A	1,2,3, 5,6	1,2,3, 5,6	1,2,3, 5,6	1,2,3, 5,6	1,2,3, 5,6	1,2,3, 5,6	1,2,3, 5,6	1,2,5, 6	1,2,3, 4,5,6
B	1,3	1,2,3, 5,6	1,2	1,2,3	1,2,3	1,2	1,2,3	2,3,5, 6	1,3,5, 6
C	2,3	1,2,3	-	1,2	2,5	NT*	1,2	NT	1,3,5, 6
D	2,3	3	-	2,3,5	NT	NT	5,6	1,2,5,6	1,5,6
E	NT	2,3	2	2,3,5,6	2,3	NT	2,3,5,6	NT	5,6
F**	-	-	-	-	-	-	-	-	-

*NT = Not tested. **Serum from a non-infected rabbit.

Table 14: Indirect Hemagglutination Test using Tanned Sheep Red Blood Cells.
Titration of Antigenic Fraction AP_{100}:

Antigen Dilutions	Serum Dilutions*									
	1: 10	20	40	80	160	320	640	1280	2560	5120
1:2	+	+	+	±	-	-	-	-	-	-
1:3	+	-	-	-	-	-	-	-	-	-
1:4	-	-	-	-	-	-	-	-	-	-
1:8	-	-	-	-	-	-	-	-	-	-
1:12	-	-	-	-	-	-	-	-	-	-
1:16	-	-	-	-	-	-	-	-	-	-
1:24	-	-	-	-	-	-	-	-	-	-

*Serum with SAT titre of 400 I.U. and CFT titre of 1:10.
Sensitized and unsensitized cell controls, and buffer control were negative.
- = negative; ± = suspicious; + = positive.

Table 15: Indirect Hemagglutination Test using Tanned Sheep Red Blood Cells.
Titration of Antigenic Fraction $P_{100}C$:

Antigen Dilutions	Serum Dilutions*									
	1: 10	20	40	80	160	320	640	1280	2560	5120
1:2	+	+	+	+	+	+	+	+	+	+
1:4	+	+	+	+	+	+	+	±	-	-
1:8	+	+	+	+	+	+	+	±	-	-
1:16	+	+	+	+	+	+	±	-	-	-
1:32	+	+	+	+	+	±	-	-	-	-
1:64	+	+	+	+	±	+	±	±	±	-
1:128	+	+	+	+	+	+	+	+	±	-

*Serum with SAT titre of 400 I.U. and CFT titre of 1:10.

Sensitized and unsensitized cell controls, and buffer control were negative.

- = negative; ± = suspicious; + = positive.

Hemagglutinating activity was best using the phenol antigens, especially $P_{100}C$ and PP_{100}. The acetone fractions did not show any significant hemagglutinating activity. The working dilution of antigen $P_{100}C$ was 1/32. The titre of the sera tested were higher in the IHAT than the SAT titres, for example, a serum with SAT titre of 400 I.U. had a IHAT titre of 1/1600. With the MMS, the titres were one or two wells higher than with MHS.

Gluteraldehyde treated, tanned sheep red blood cells were also employed. The optimal working dilution of the antigen $P_{100}C$ was found to be 1/50, and the titre of the MHS was 1/81920 (SAT - 12800 I.U. and CFT - 1/80). The results are shown in Table 16. A set of positive and negative sera from goats were tested (Table 17). It was seen that the IHAT titre was higher than the SAT titre in positive sera. Two animals which had an IHAT titre of 1/40 were either suspicious or negative in the other serological tests. Two animals with similar titres in the IHAT, that is 1/40, were negative in the other tests.

C). COMPLEMENT FIXING ACTIVITY OF THE ANTIGENS.

All the fractions were tested for the CF activity in a micro CFT. All ten fractions reacted

Table 16: Indirect Hemagglutination Test using Gluteraldehyde Treated Tanned Sheep Red Blood Cells. Titration of Antigenic Fraction $P_{100}C$:

Antigen Dilutions	Serum Dilutions*										
	1: 40	80	160	320	640	1280	2560	5120	10240	40960	81920
1:50	+	+	+	+	+	+	+	+	+	+	+
1:100	+	+	+	+	+	+	+	+	±	±	±
1:150	+	+	+	+	+	+	+	+	+	±	±
1:200	+	+	+	+	+	+	+	+	+	±	±
1:250	+	+	+	+	+	+	+	+	+	±	±

*Serum with SAT titre of 12800I.U. and CFT titre of 1:80.

Sensitized and unsensitized cell control and buffer control were negative.

- = negative; ± = suspicious; + = positive.

Table 17: Indirect Hemagglutination Test using Gluteraldehyde Treated Tanned Sheep Red Blood Cells Sensitized with Antigenic Fraction $P_{100}C$*. Results of Test Sera:

Sample No.	SAT** titre	CFT** titre	RBPT**	AGIT**	IHAT** titre
1	50	1:40	+	+	1:160
2	25	4:5	+	+	1:320
3	50	2:10	+	+	1:1280
4	100	1:20	+	+	1:640
5	0	0	-	-	1:40
6	0	0	-	-	1:40
7	25	3:5	+	+	1:40
8	0	4:2.5	-	+	1:40

**SAT titre in International Units (I.U.); CFT titres in degree of fixation at a particular serum dilution; IHAT titres in serum dilutions; RBPT and AGIT: - = negative and + = positive.

*Antigen $P_{100}C$ used at a titre of 1:50.

against the MHS in the CFT (Tables 18 and 19). Anticomplimentary activity was seen at low dilutions of the antigens. The acetone antigens tended to give a lower serum titre than the phenol antigens. The optimal dilution of the antigens was found to be between 1/8 to 1/16. At these dilutions, the antigens had no anticomplimentary activity.

The titre of MMS was higher than that of MHS when tested in the CFT using the fractions.

A few experimentally infected goat sera and negative sera were tested in the CFT using $P_{100}C$ as antigen. The results did not show any significant difference in the titres from those obtained by the whole cell antigen.

D). SEROLOGICAL RESULTS OF THE FIELD SERA.

Goat sera from two field outbreaks of brucellosis were tested in four serological tests (SAT, CFT, RBPT and AGIT). The results are given in Table 20. The categories depend on the results of the tests.

1). Farm 1.

The results of 136 goat sera from this farm are given in Table 20.

Fifty goats were negative and 17 goats positive to all the four tests (Categories 1 and 12 respectively).

Table 18: Complement Fixation Test. Titration of Antigenic Fractions 1) AP_{10} and 2) $A_{100}C$:

Antigen Dilutions	Serum Dilutions*									Complement Controls		
	1: 2.5	5	10	20	40	80	160	320	AC+	C	C/2	C/4
1) AP_{10}												
1:2	4	4	4	4	Tr	Tr	-	-	-	-	4	4
1:4	4	4	4	4	Tr	-	-	-	-	-	Tr	4
1:8	4	4	4	4	3	-	-	-	-	-	-	4
1:16	4	4	4	4	4	4	-	-	-	-	Tr	4
2) $A_{100}C$												
1:2	4	4	4	4	4	4	-	-	-	4	4	4
1:4	4	4	4	4	4	4	-	-	-	-	4	4
1:8	4	4	4	4	4	3	-	-	-	-	2	4
1:16	4	4	4	4	4	2	-	-	-	-	2	4
WHCA**	4	4	4	4	4	2	-	-	-	-	Tr	4
VBD***										-	Tr	4

*Degree of fixation: 4 = 100%; 3 = 75%; 2 = 25%; 1 = 25%; Tr = 5 to 10%.

+AC = Serum anticomplementary control.

**WHCA = Whole cell antigen.

***VBD = Complement control in Veronal Buffer Diluent.

Table 19: Complement Fixation Test. Titration of Antigenic Fractions 1) PP_{10} and 2) $P_{100}C$:

Antigen Dilutions	Serum Dilutions*									Complement Controls		
	1: 2.5	5	10	20	40	80	160	320	AC+	C'	C/2	C/4
1) PP_{10}												
1:2	4	4	4	4	4	4	4	4	-	Tr	4	4
1;4	4	4	4	4	4	4	4	Tr	-	-	1	4
1:8	4	4	4	4	4	4	4	-	-	-	Tr	4
1:16	4	4	4	4	4	4	4	4	-	-	Tr	4
2) $P_{100}C$												
1:2	4	4	4	4	4	4	3	3	-	4	4	4
1:4	4	4	4	4	4	4	2	-	-	2	4	4
1:8	4	4	4	4	4	4	1	-	-	-	Tr	4
1:16	4	4	4	4	4	4	2	-	-	-	Tr	4
WHCA**	4	4	4	4	4	3	-	-	-	-	Tr	4
VBD***										-	1	4

*Degree of fixation: 4 = 100%; 3 = 75%; 2 = 50%; 1 = 25%; Tr = 5 to 10%.

+AC = Serum anticomplementary control.

**WHCA = Whole cell antigen.

***VBD = Complement control in Veronal Buffer Diluent.

Fifty nine sera were negative in the AGIT (Categories 2 to 6), of which 24 were also negative in the RBPT (Categories 2 and 3). Sixteen of these were doubtful in the CFT, two positive in the CFT and five doubtful in the SAT.

Thirty five goats out of 59 negative in the AGIT were positive in the RBPT (Categories 4 to 6) but six of these animals were negative in both the SAT and CFT. Seventeen were doubtful either in the SAT or CFT or both the tests. Only one goat was positive in the SAT out of the 9 positive in the CFT; three other goats were positive in the SAT, of which 2 were doubtful in the CFT and the remaining one was negative in the CFT.

Ten animals were positive in the AGIT (Categories 7 to 11), of which 4 were negative (Categories 7 to 9) and 6 positive (Categories 10 to 11), in the RBPT. Two of the 4 goats negative in the RBPT were positive in the CFT; one was positive in both the SAT and CFT and one doubtful in both SAT and CFT. Two of the six animals positive in the RBPT were positive to the CFT only; 2 were doubtful in both the SAT and CFT; 1 doubtful in the SAT was positive in the CFT and finally 1 doubtful in the CFT was positive in the SAT.

Table 20: Analysis of Results of Caprine Sera from Field Outbreaks of Brucellosis.

Comparision of 1) AGIT negative and 2) AGIT positive sera in RBPT, SAT and CFT:

Category*	Results** of RBPT	SAT	CFT	1) AGIT negative sera Farm 1	Farm 2	Total	Category	2) AGIT positive sera Farm 1	Farm 2	Total	Grand Total
	-	-	-	50	42	92		0	1	1	93
1	-	-	o	16	6	22	7	0	0	0	22
	-	-	+	2	1	3		2	0	2	5
	-	o	-	1	2	3		0	0	0	3
2	-	o	o	5	0	5	8	1	0	1	6
	-	o	+	0	0	0		0	2	2	2
	-	+	-	0	0	0		0	0	0	0
3	-	+	o	0	0	0	9	0	0	0	0
	-	+	+	0	1	1		1	0	1	2
	+	-	-	6	0	6		0	0	0	6
4	+	-	o	11	0	11	10	0	0	0	11
	+	-	+	2	0	2		2	1	3	5
	+	o	-	4	0	4		0	0	0	4
5	+	o	o	2	0	2	11	2	0	2	4
	+	o	+	6	0	6		1	0	1	7
	+	+	-	1	1	2		0	0	0	2
6	+	+	o	2	0	2	12	1	0	1	3
	+	+	+	1	1	2		17	12	29	31
Total				109	54	263		27	16	43	306

*Category depends on the results. ** - = negative; o - suspicious; + = positive

2). Farm 2.

The results of the 70 goat sera are shown in Table 20.

Forty two animals were negative in the AGIT (Categories 2 to 6), of which 10 were negative in the RBPT (Categories 2 and 3); 8 of these 10 were doubtful in the CFT or the SAT. One was positive only in the CFT. One of the 2 goats positive in the RBPT (Categories 4 to 6) was positive in the SAT and the other was positive to both the tests (SAT and CFT).

Four sera were positive in the AGIT (Categories 7 to 11), of which 3 were negative (Categories 7 to 9) in the RBPT; two of these were positive in the CFT but doubtful in the SAT and the other was negative to both the SAT and CFT. One animal positive (categories 10 to 11) in the RBPT was also positive in the CFT but negative in the SAT.

E). SEROLOGICAL RESPONSE OF THE EXPERIMENTALLY INFECTED GOATS.

A total of 24 goats were divided into 3 groups as shown in Table 4 and two of these were infected with live B. melitensis; One by subcutaneous route and the other by conjunctival route. The third group was left as a control. All groups were observed for a period of 44 weeks.

Table 21 summarizes the clinical and bacteriological results of each goat.

The day of infection was considered day O. Each serum sample was assayed by four serological tests. The serological response of each goat is summarized in Figures 17 to 27.

1). Group One.

The results are shown in Figures 17 to 21. Antibody activity, as shown by all tests, was remarkably uniform in four of the five goats infected subcutaneously. Activity was initially detected by SAT in each of the four animals by the 4th day following infection. Peak titres were reached by day 15 and were maintained in most animals until about the 50th day. From this day, the titres started to fall gradually to low levels. By the 94th day, the titres were in the range of 25 to 100 I.U.

The RBPT first detected antibodies between days 4 and 10 in the four animals and remained positive throughout 304 days. Antibody reactive in the CFT rose rapidly from 8th and 10th day, reaching the peak by the 22nd day. The CFT also remained positive through 304 days. Precipitin activity, as shown by the AGIT, was detected about day 6 and remained positive throughout the experiment.

Table 21: Clinical Signs, the Milk Ring Test Results and Isolation of *B. melitensis* from Tissues of Experimental Goats:

Grp* No.	Goat No.	Kidded on Day‡	Aborted on Day‡	MRT+	Tissues**	Remarks
1	3120	107	-	+ve	-	-
	3132	-	33	+ve	V(31)F(33)	-
	3138	281	55	+ve	F(55)V(94)	-
	3140	-	-	-	V(8;31)	-
	3143	220	-	+ve	-	-
	3129	-	-	-	-	died(58)***
	3135	121	-	-ve	-	-
	3123	-	-	-	-	died(155)
2	3119	-	-	-	ILN(53)	died(53)
	3128	-	-	-	-	-
	3130	-	61;285	+ve	F(61;285) US(290)	died(290), metritis, mastitis.
	3134	161	-	-ve	-	-
	3139	221	-	±	-	-
	3126	-	-	-	-	-
	313	-	-	-	V(94)	-
	3122	-	-	-	-	-
3	3121	153	-	-ve	-	Twins
	3124	224	-	-ve	-	-
	3125	28;193	-	-ve	-	-
	3127	186	-	-ve	-	-
	3133	-	-	-	-	-
	3136	-	-	-	-	moved to Grp.1 (155)
	3141	-	-	-	-	-
	3142	-	-	-	-	-

*Grp = Group.

**Tissues: V = vaginal swab; F = fetal tissues: US = uterine smear; ILN = iliac lymph node. Only tissues from which *B. melitensis* recovered were recorded. In parentheses is the time (days) after infection, the organism was isolated.

***In parentheses, days after infection.

+MRT: -ve = negative; ± = suspicious; + = positive

‡Day after infection of goats in Groups 1 and 2.

KEY TO FIGURES 17 to 30:

SAT: Serum Agglutination Test. Titres in International Units (I.U.).

CFT: Complement Fixation Test. Titres in degree of fixation at a particular serum dilution.

AGIT: Agar Gel Immunodiffusion Test.

- = negative;

o = spur precipitin line;

o = 1 precipitin line;

oo = 2 precipitin lines;

ooo = 3 precipitin lines.

RBPT: Rose Bengal Plate Test.

- = negative; o = positive.

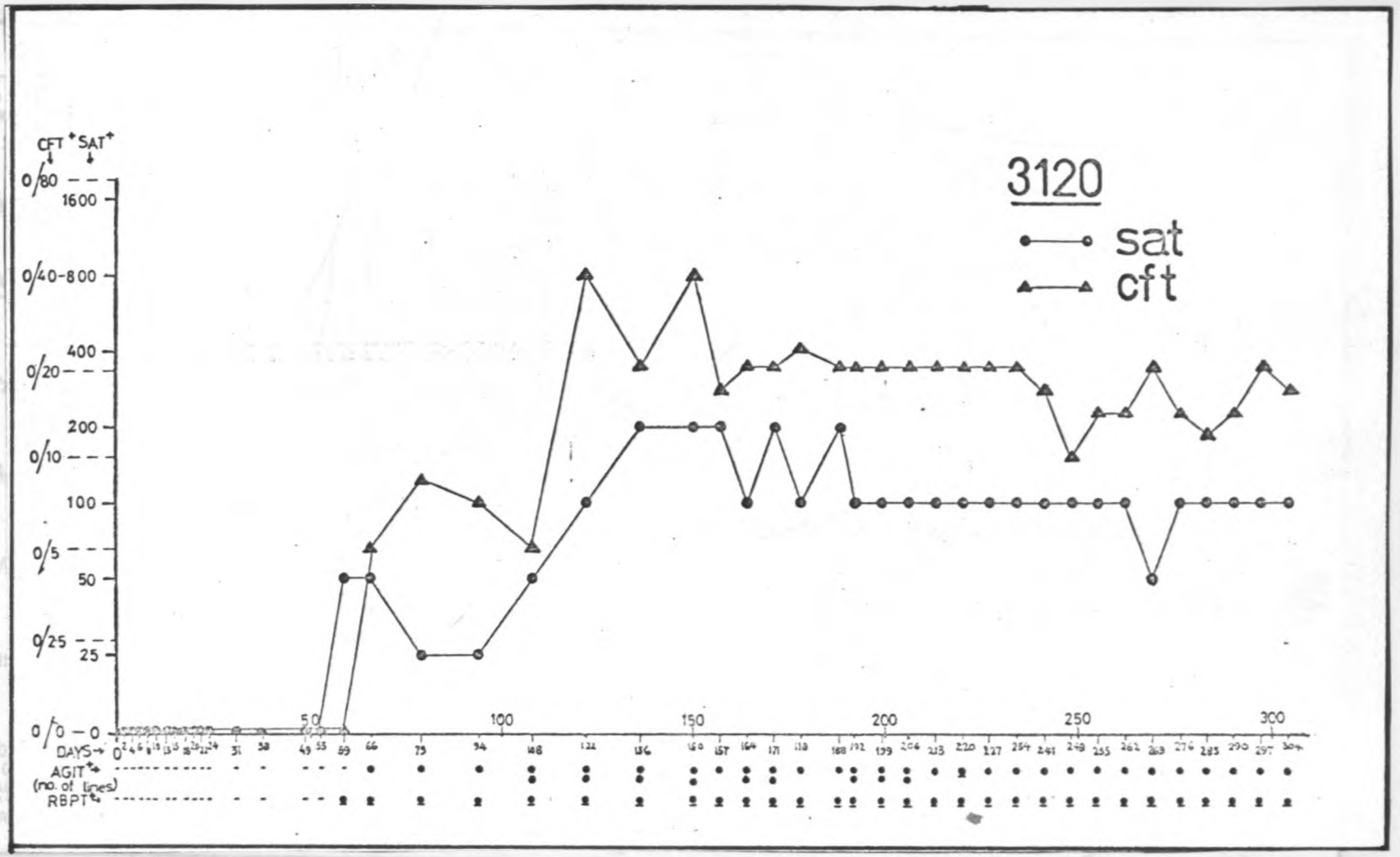


Figure 17: Serological Response of Group One Goats (Subcutaneous Infection). Goat No. 3120.

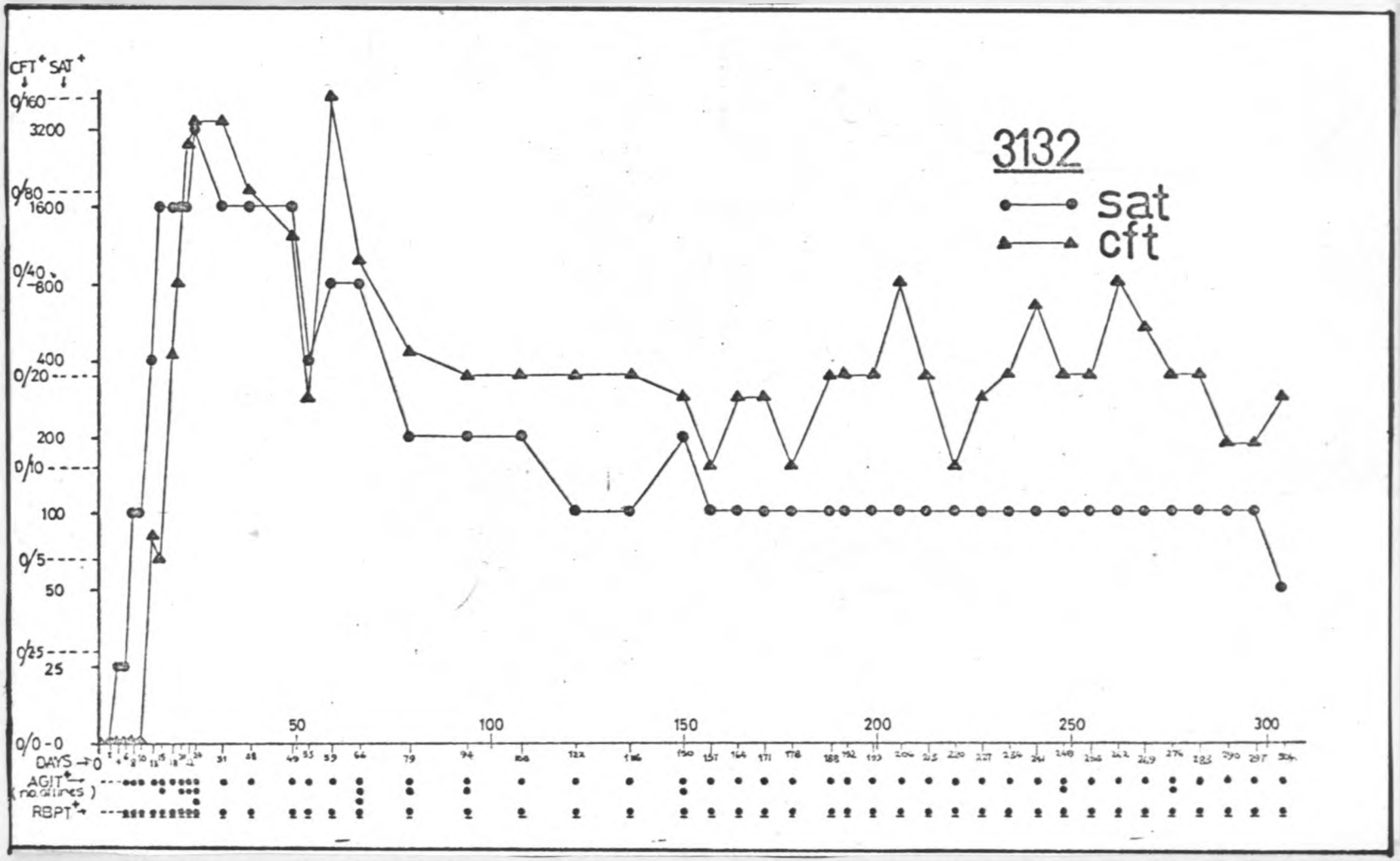


Figure 18: Serological Response of Group One Goats (Subcutaneous Infection). Goat No. 3132.

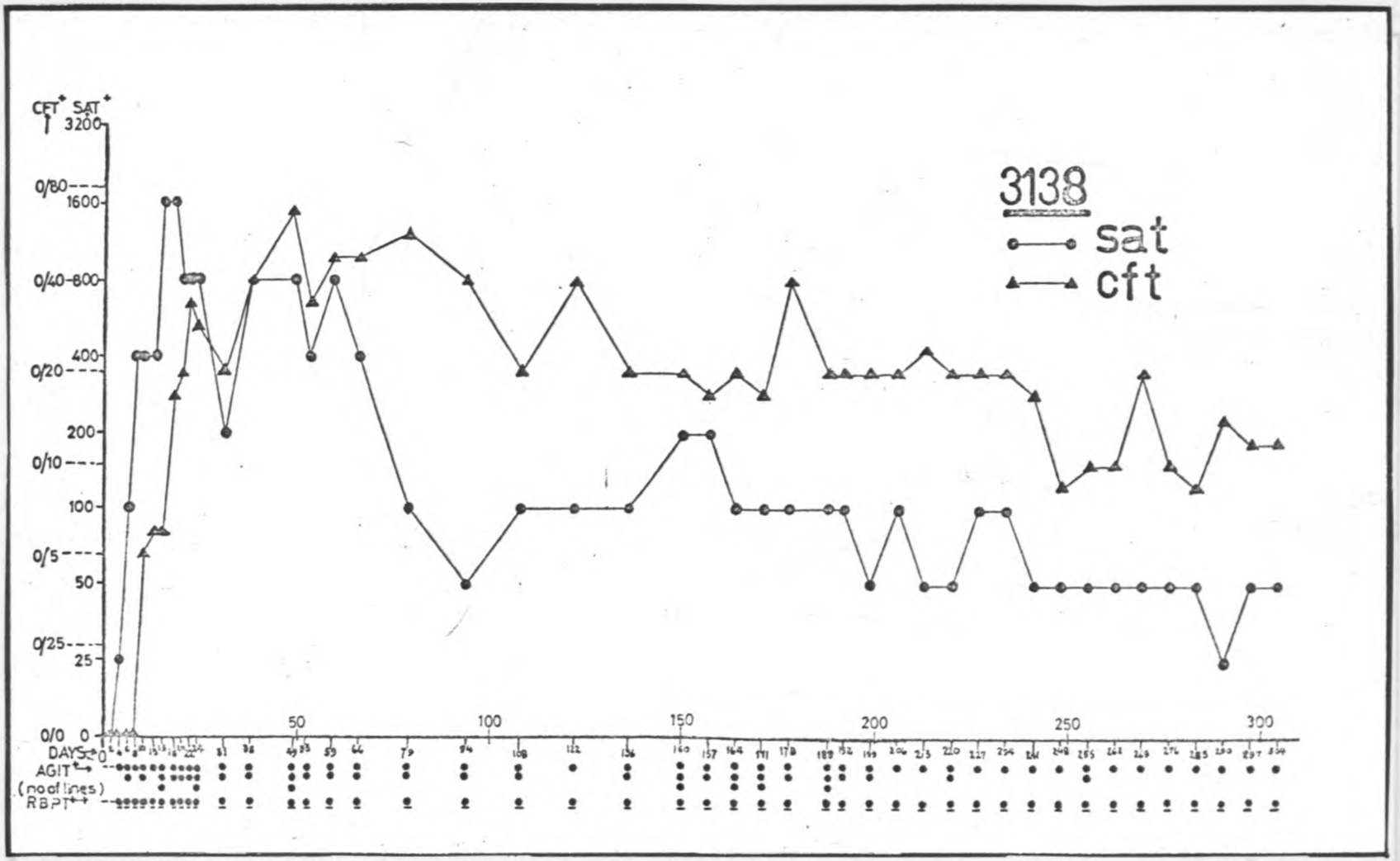


Figure 19: Serological Response of Group One Goats (Subcutaneous Infection) Goat No. 3138.

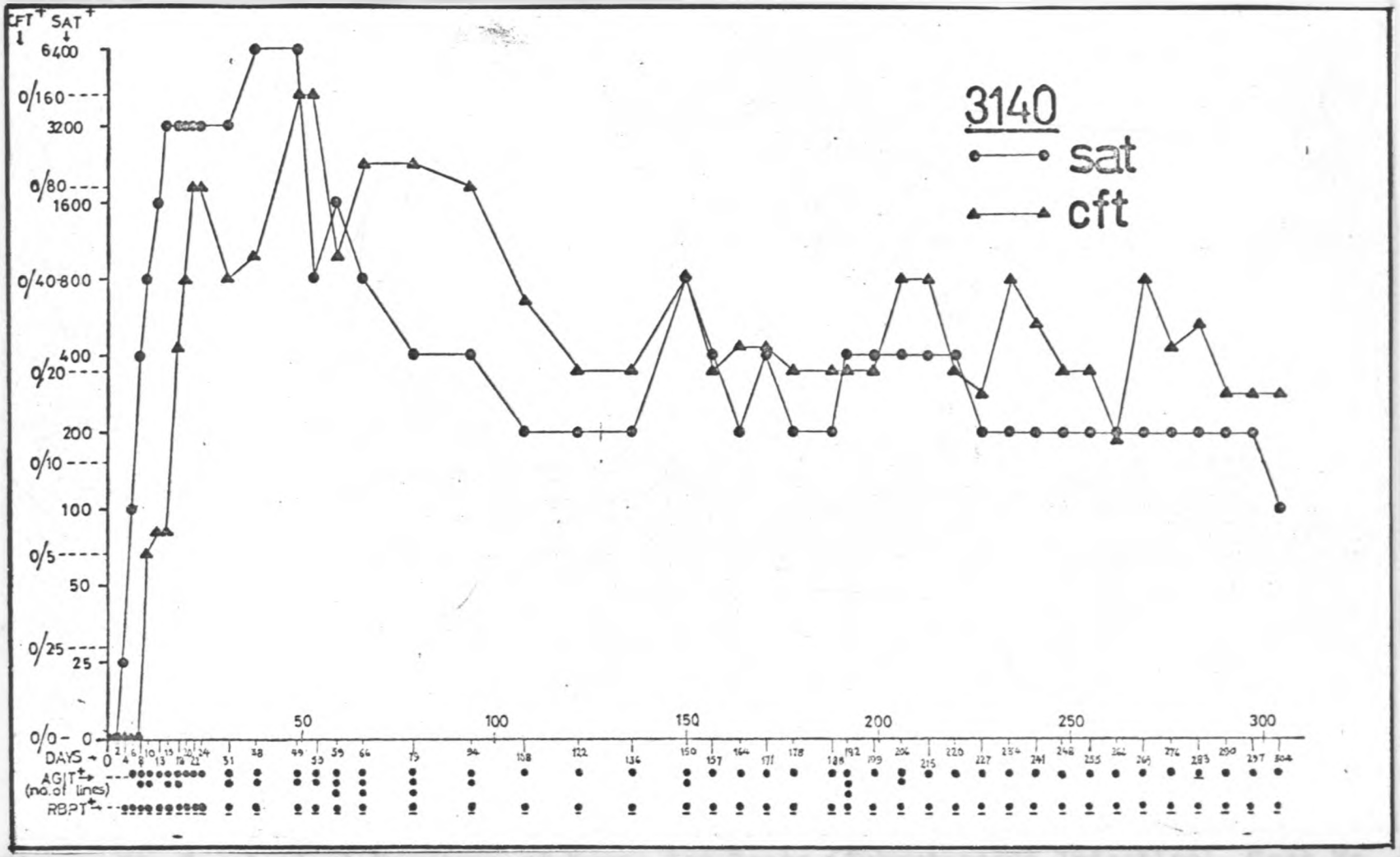


Figure 20: Serological Response of Group One Goats (Subcutaneous Infection). Goat No. 3140.

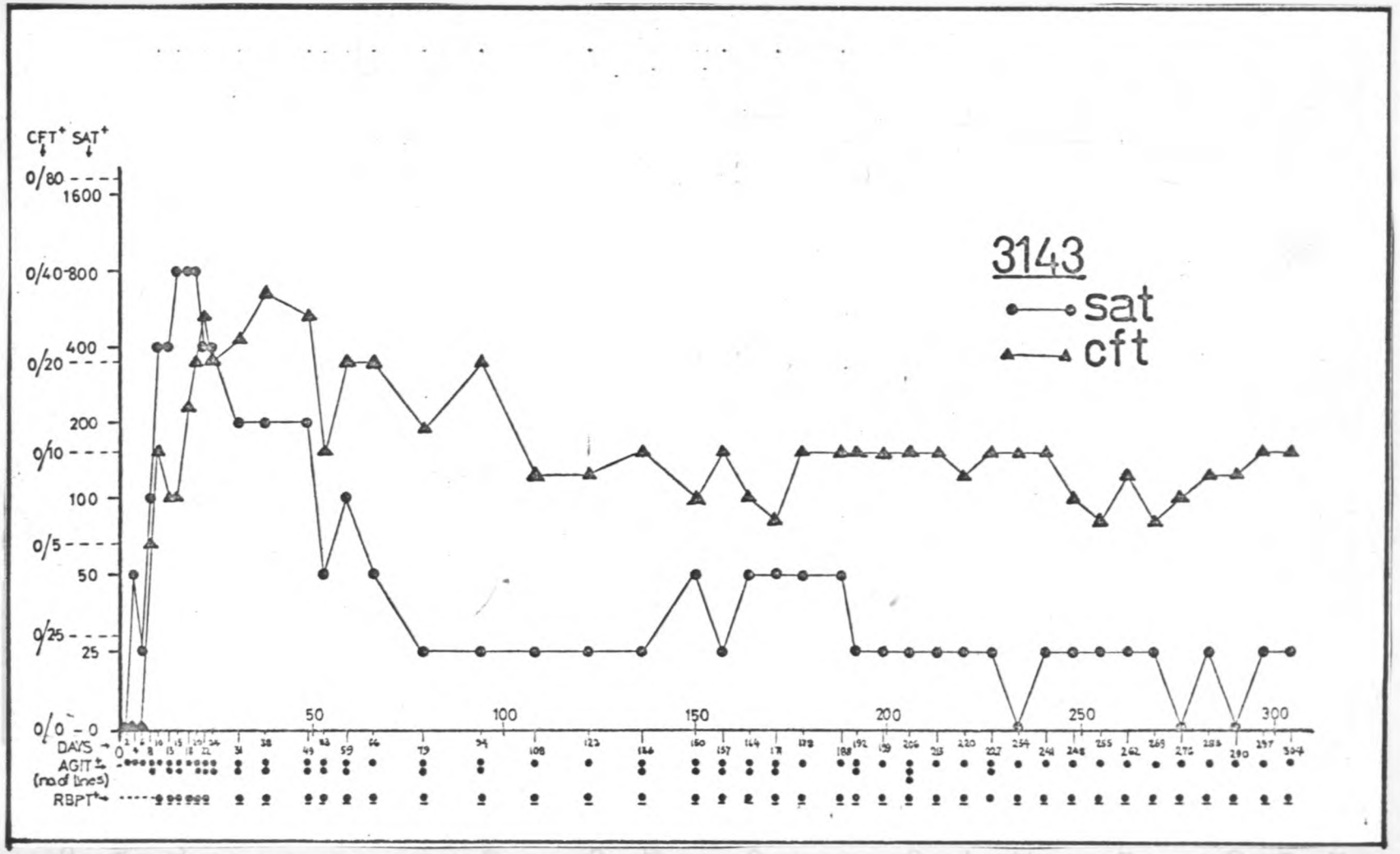


Figure 21: Serological Response of Group One Goats (Subcutaneous Infection). Goat No. 3143.

The fifth animal (No. 3120) in this group did not react until day 59 when both the SAT and RBPT became positive. By day 66, the AGIT and CFT were also positive.

Only two of the three incontact goats in this group (Group I) reacted serologically. In both, the SAT became positive on day 53 following infection of the other goats in the group. However, the SAT reactivity lasted only 14 to 30 days. Transient and suspicious reactions were also noted using the RBPT, CFT and AGIT. One of the pair (No. 3123) died on day 155 of aspiration pneumonia. The third incontact animal died on day 59, due to purulent pneumonia, without any detectable antibody activity. Another goat which was moved from Group 3 on 165th day to replace No. 3123 gave antibody reactivity in AGIT on day 164 and was irregularly positive until the 304th day. Both the SAT and CFT reacted on the 297th day. No antibody activity was detected in RBPT.

2). Group Two.

The results are shown in Figures 22 to 27.

Antibody activity was also fairly uniform in four goats of this group infected by conjunctival instillation of live B. melitensis. The agglutinins were detected first, between days

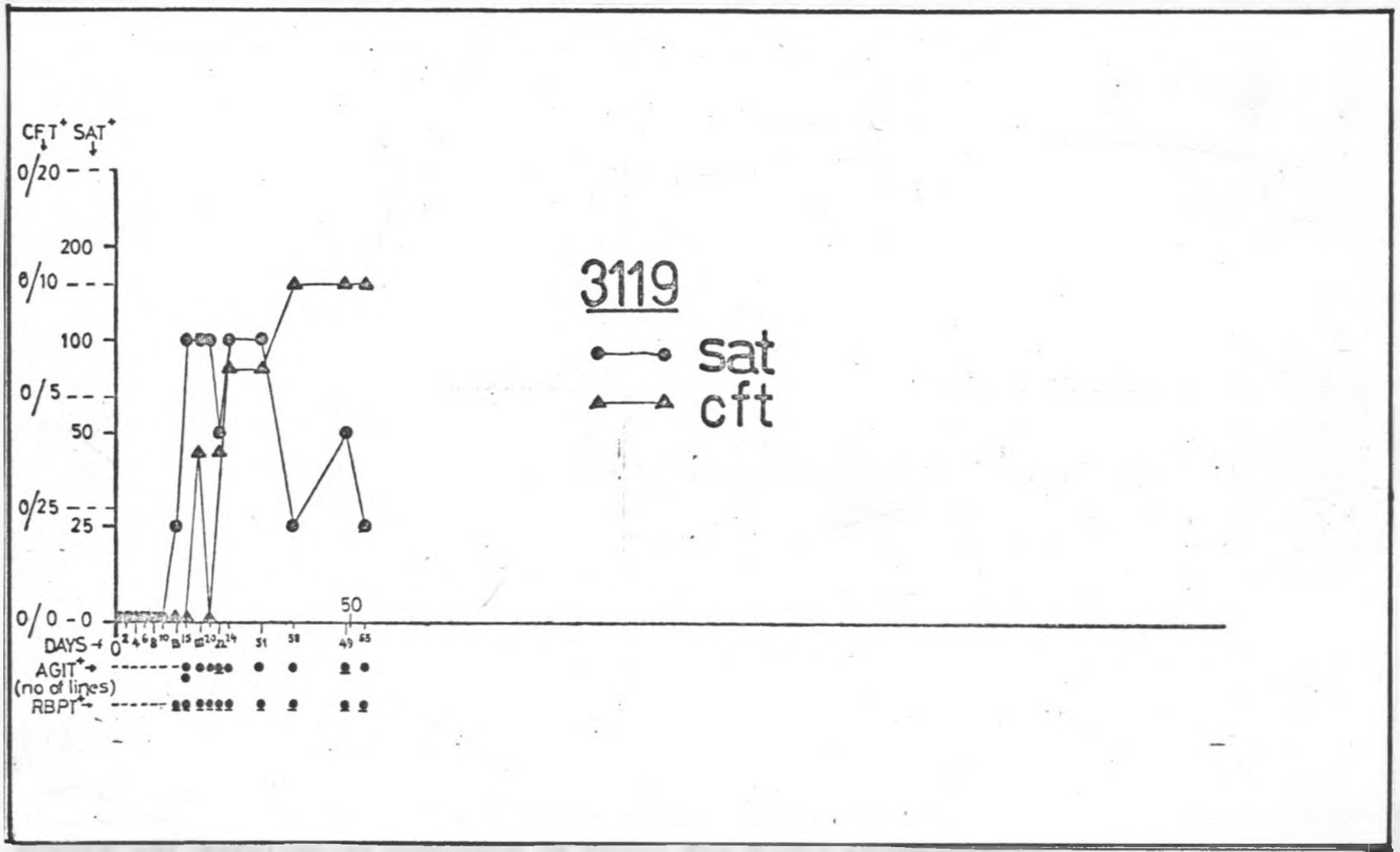


Figure 22: Serological Response of Group Two Goats (Conjunctival Infection). Goat No. 3119.

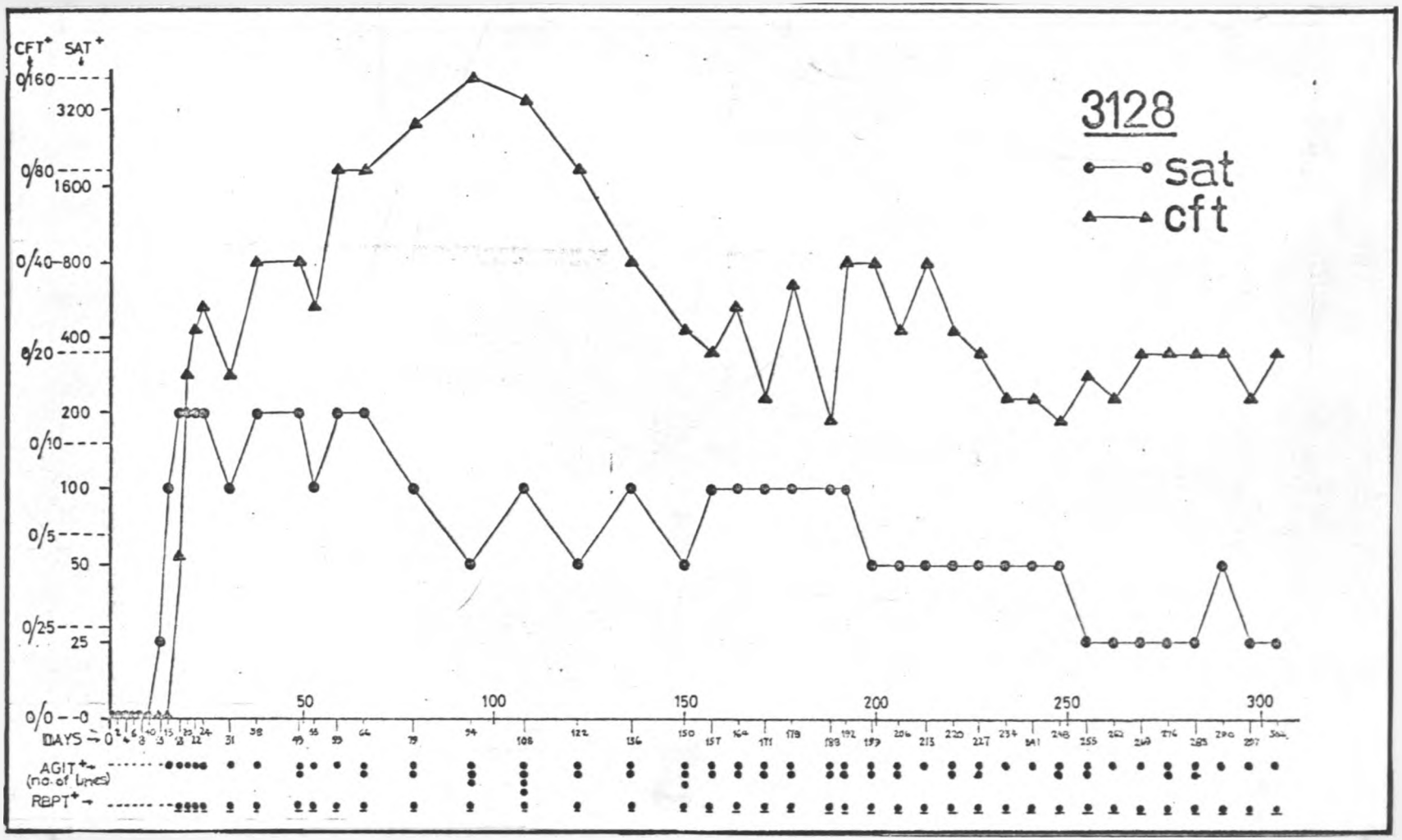


Figure 23: Serological Response of Group Two Goats (Conjunctival Infection). Goat No. 3128.

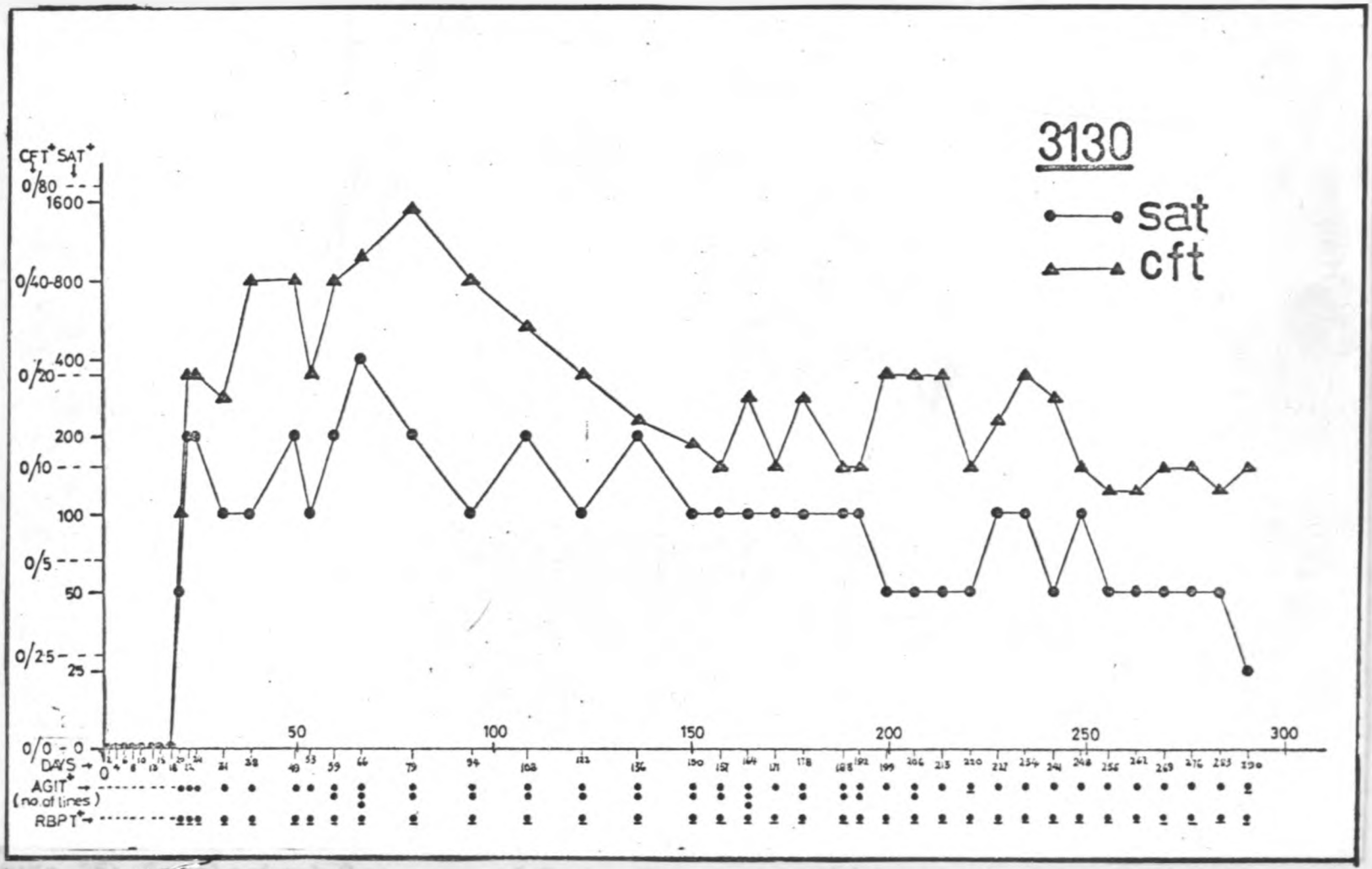


Figure 24: Serological Response of Group Two Goats (Conjunctival Infection). Goat No. 3130.

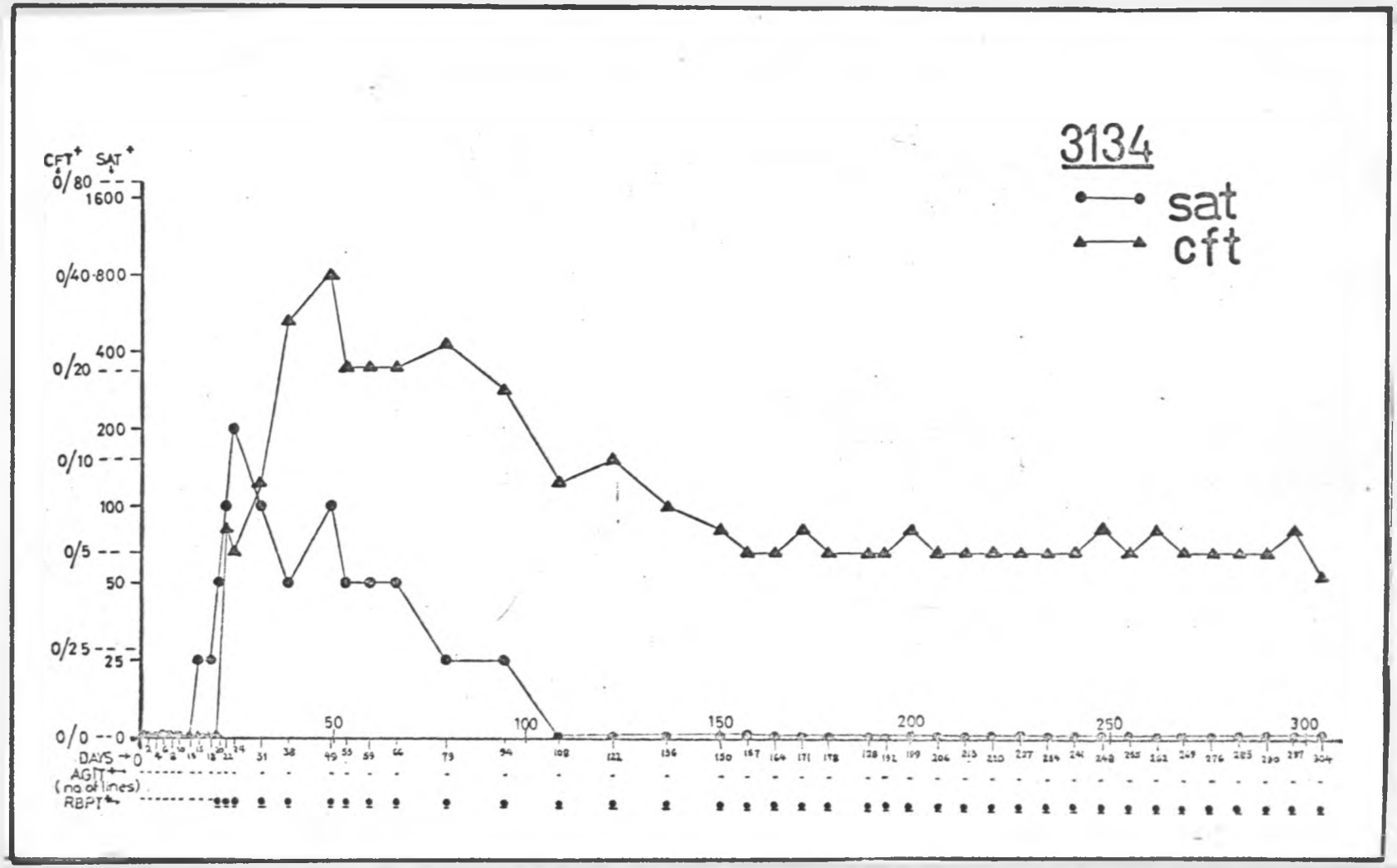


Figure 25: Serological Response of Group Two Goats (Conjunctival Infection) Goat No. 3134.

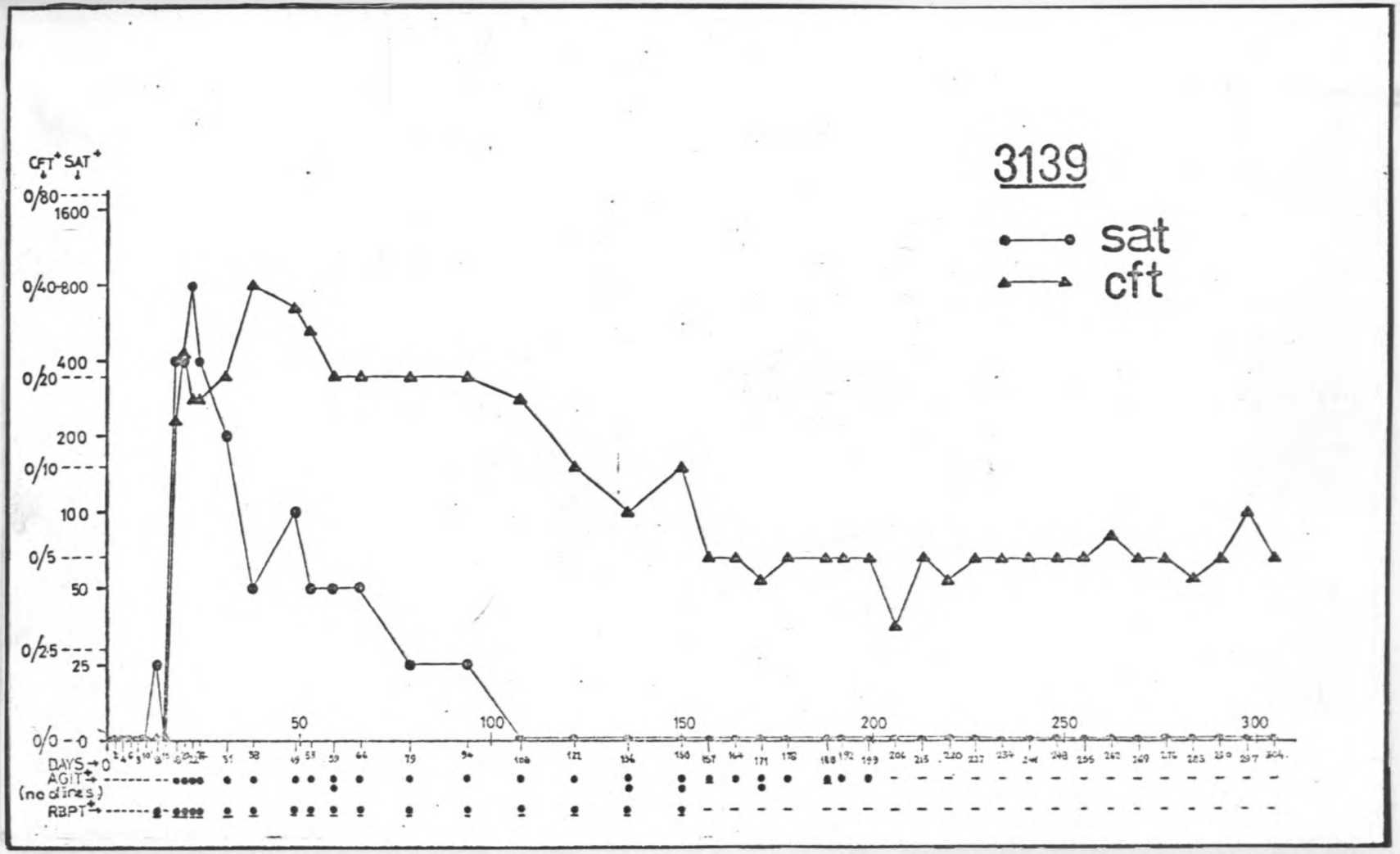


Figure 26: Serological Response of Group Two Goats. (Conjunctival Infection). Goat No. 3139.

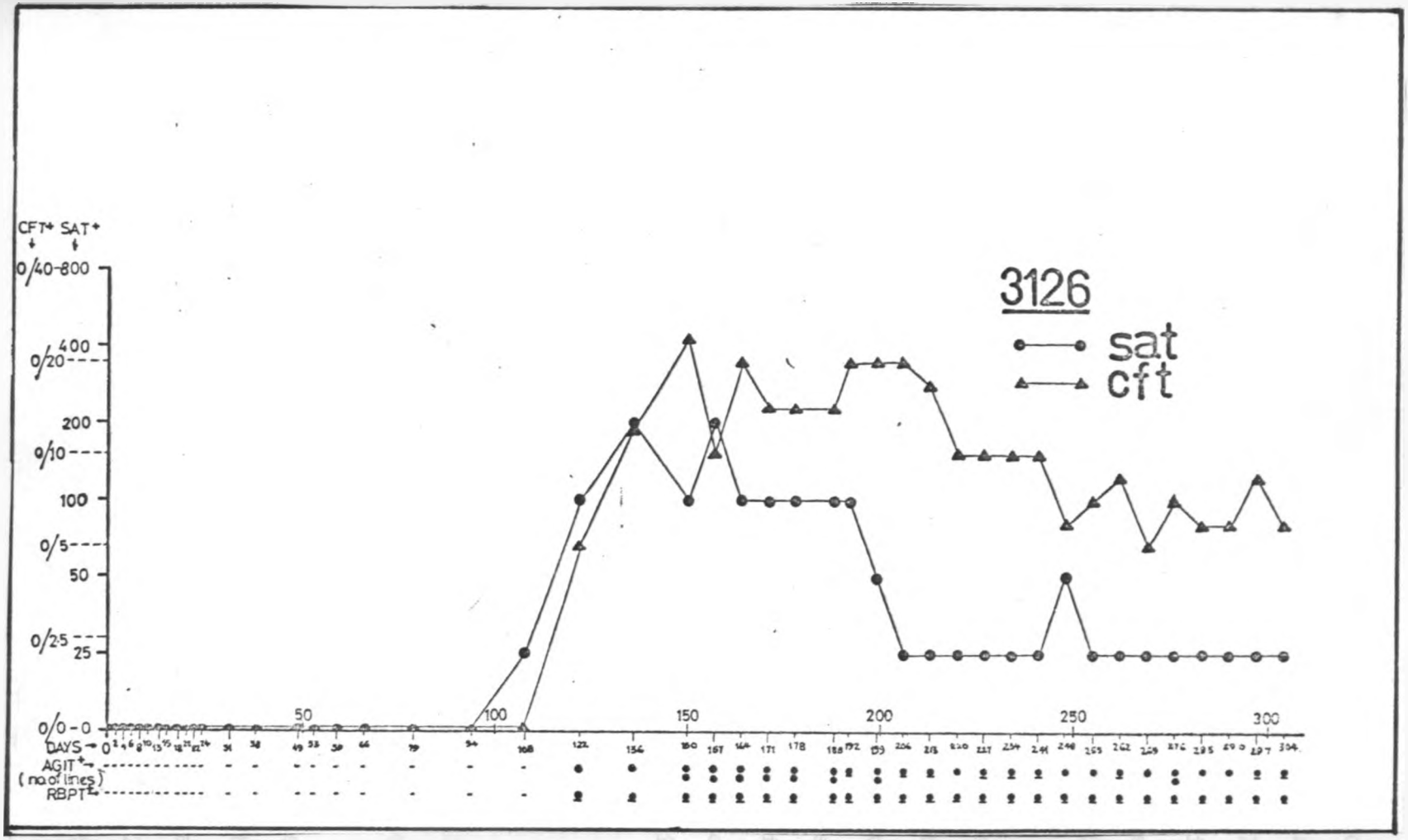


Figure 27: Serological Response of Goat No. 3126 (Incontact to Group Two Animals).

10 and 18 after infection. Peak titres, recorded by day 24, started to fall rapidly. The titres were down to 50 I.U. and 100 I.U. by day 38. Two animals (Nos. 3134 and 3139) became negative in the SAT on day 108 and remained negative until the end of experiment. In all four goats, the antibody activity to RBPT, CFT and AGIT was first detected between days 15 to 20. Serum from goat No. 3134 was negative in the AGIT throughout the experiment. A goat, No. 3139, became negative to the RBPT on day 157 and the AGIT on day 206 and remained negative thereafter. The CF activity of serum from these four goats fell to a low level by day 150. All animals remained CF positive until the 304th day.

One animal out of these four goats died on day 297 of septicemia resulting from endometritis following an abortion. Another animal No. 3119, that was also experimentally infected, died on the 53rd day of infection, of acute pneumonia. The animal reacted similarly to the others in the group in serological tests, that is, it was positive to all tests until its death.

One of the three incontact goats (No.3126) became positive in the SAT on day 108 and to the other three tests on day 122. The agglutinins reached a peak level on day 136 and fell rapidly

to a low titre of 25 I.U. by day 206. This low titre persisted until the end of the experiment. The RBPT, CFT and AGIT remained positive throughout the trial.

3). Group Three.

This control group consisted of eight goats. All the goats except No.3124 remained negative on all the four tests throughout the experiment. No.3124 gave a titre of 25 I.U. in the SAT, irregularly.

F). THE MEAN TITRES IN THE SAT AND CFT OF EXPERIMENTALLY INFECTED GOATS.

Comparing the mean titres of agglutinins in goats of both groups (Group 1 and Group 2), the titre rose to a higher level in Group 1 than in Group 2 (Figures 28 to 30). In addition, the SAT antibody of Group 1 was detected earlier (by day 4) when compared to Group 2 (by day 13). However, the kinetics of the response was similar for both the groups. The fall of titre was comparatively rapid in Group 2 animals.

In the CFT, antibody was detected sooner and peak titres were reached earlier in Group 1 goats than in Group 2. The fall of titre was also slower in Group 1. The titres in animals of both groups

maintained at a steady level in the later stages of the trial.

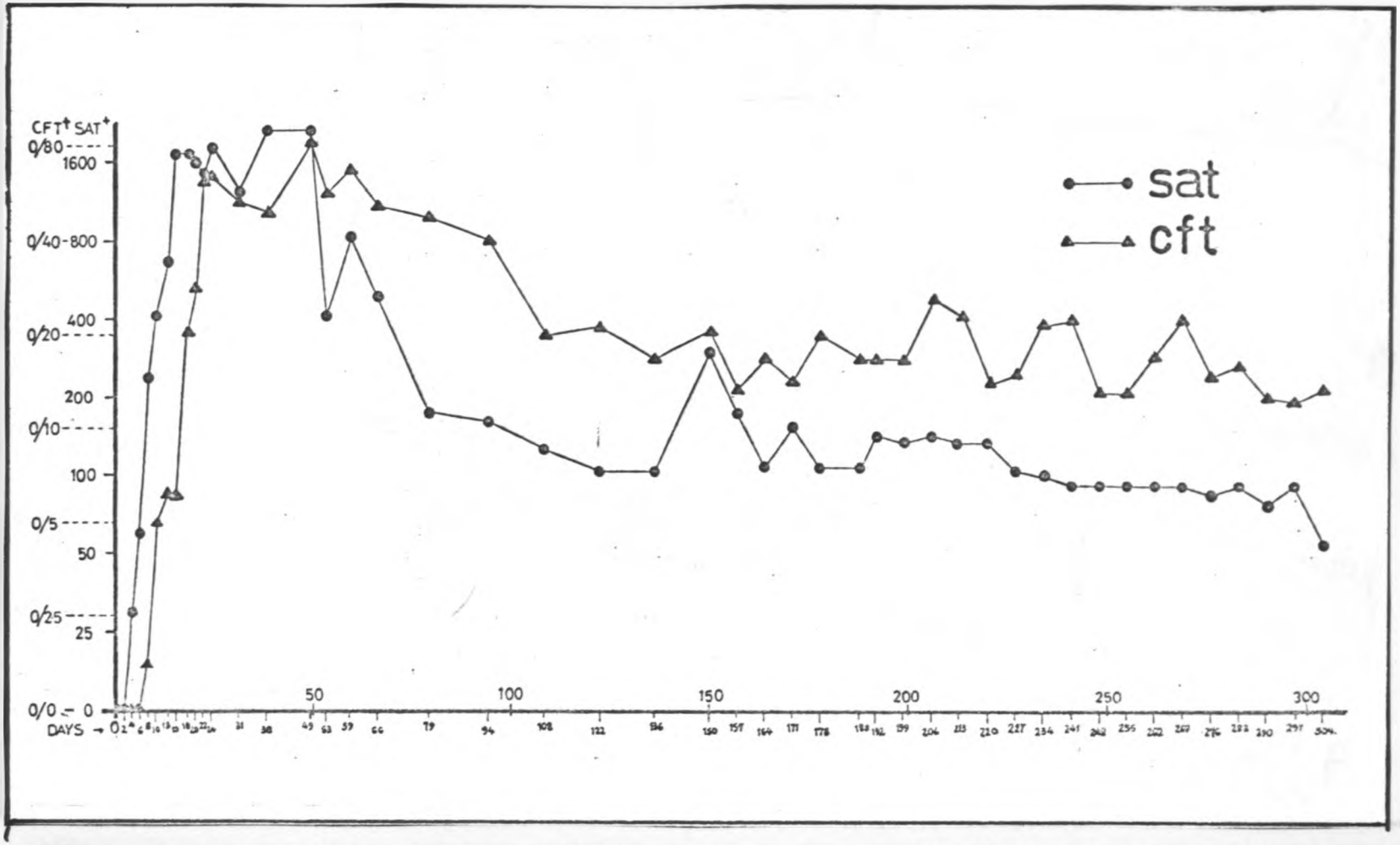


Figure 28: Mean Titres in SAT and CFT of Group One Goats (Subcutaneous Infection).

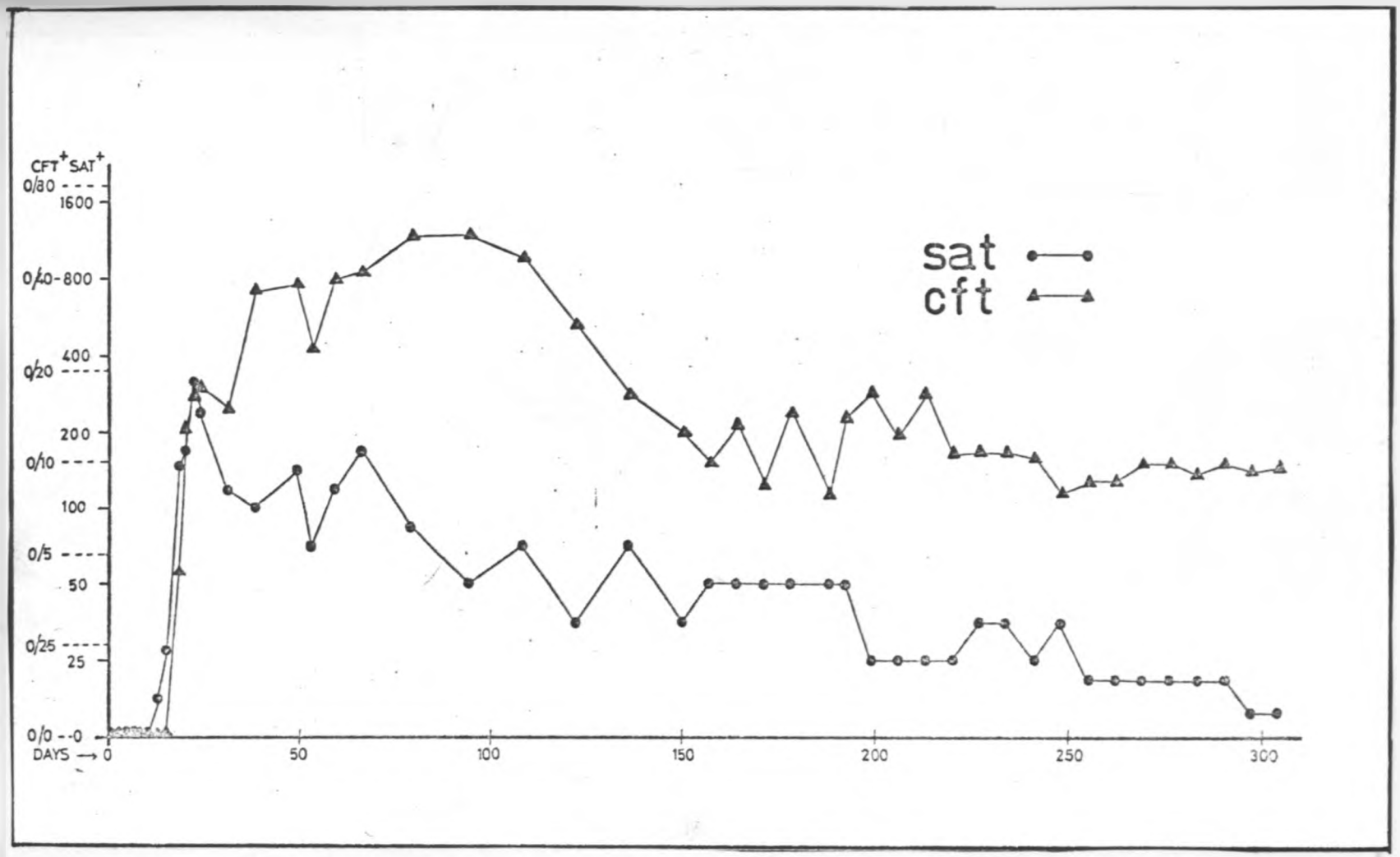


Figure 29: Mean Titres in SAT and CFT of Group Two Goats (Conjunctival Infection).

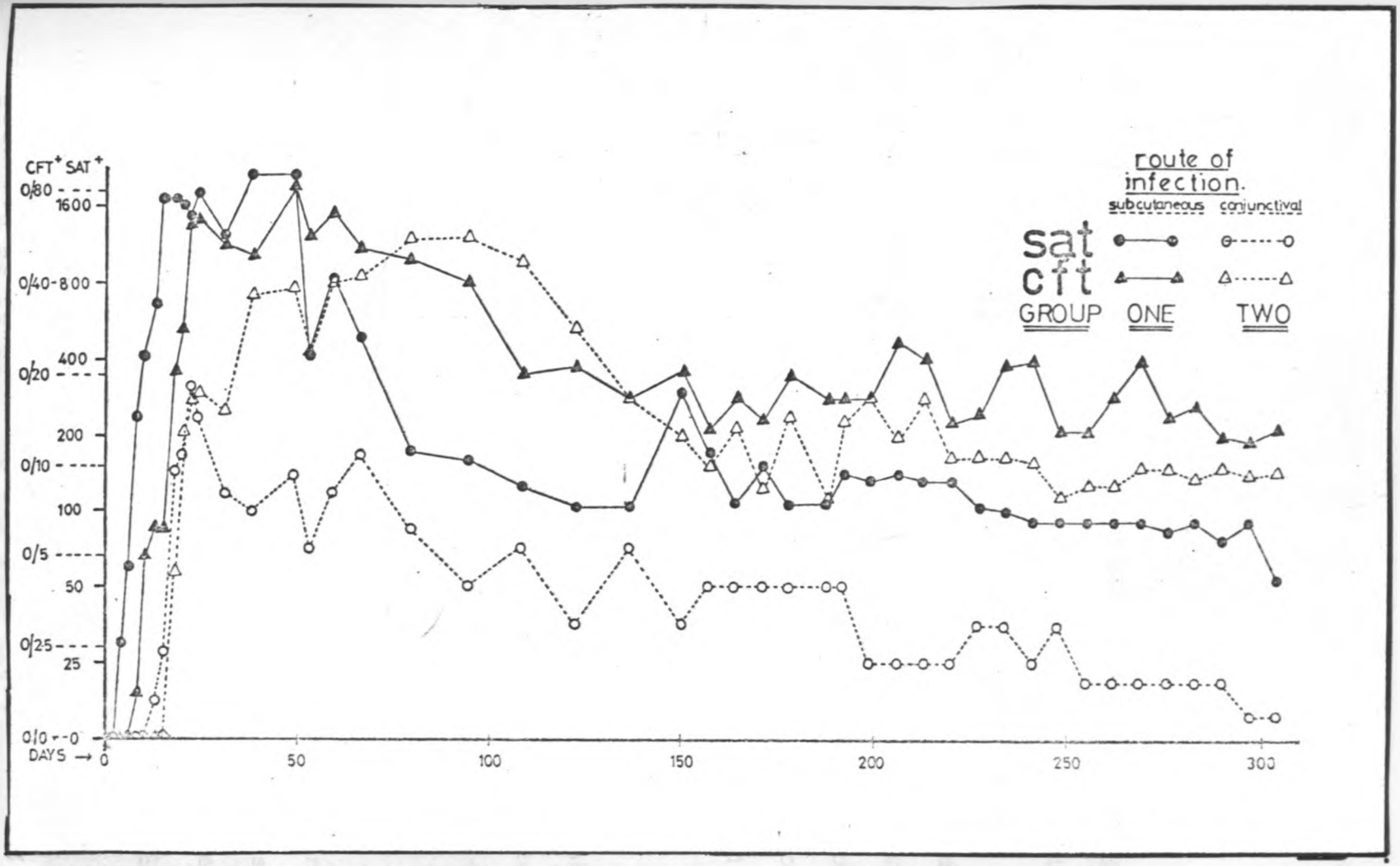


Figure 30: Comparision of Mean Titres of Group One and Two Animals.

SECTION V

DISCUSSION

A) ANTIGENS OF BRUCELLA MELITENSIS.

For several years, considerable attention has been devoted to the serological differentiation of the three main species of Brucella. It was only possible to distinguish B. melitensis form the other species by the use of agglutination tests. Recently, however, there has been an effort to obtain a complete scheme of antigenic structure of Brucella by agar gel immunodiffusion tests and immunoelectrophoresis on antigenic fractions extracted by different procedures.

In establishing the antigenic relationships and differences between the members of the genus Brucella. it has been reported that the surface antigens of smooth Brucella do not react with those of rough Brucella species, whereas the cytoplasmic antigens of smooth and rough variants of Brucella species possess common determinants (Diaz et al (31 and 32)). The antigenic differences between smooth B. abortus, B. suis and B. melitensis are quantitative rather than qualitative.

The number of antigenic components of smooth Brucella as revealed by the agar gel immunodiffusion techniques vary. The variation in the number of

precipitin lines has been due to the differences in the methods of antigen extraction and antibody production. According to Sanders (97), the avidity and specificity of antibodies to Brucella organism changes with hyperimmunisation.

Kaebrele (47) reported that the response of an animal to an inactivated microbial agent may be quite different from that to the infectious organism. Process of destruction and liberation of bacterial substances into host tissues occur besides the in vivo growth of intact Brucella organisms (Smith et al (101)). Several of these in vivo antigens have been found to be immunogenic. Glenchur et al (39) have shown that all constituents of the Brucella bacterial cell, with few exceptions, are capable of inducing antibodies for precipitation.

In this study, the maximum number of precipitin lines observed was six when the antigen fraction $P_{100}C$ was reacted with the standard hyperimmune serum (MHS). A broad diffuse band of precipitation seen around the antibody well was discounted as non-specific since it was also seen when negative sera were tested against the antigenic fractions. The other antigen fractions gave a varying number of precipitin lines (3 to 5) when tested against the MHS. Hyperimmune sera prepared by using live Brucella cells were better, that is,

gave more lines when compared to those prepared by using killed Brucella cells either with or without Freund's Adjuvant. Immunisation periods were very short for the live B. melitensis compared to the killed B. melitensis antigen with adjuvant. Immunisation periods for the latter were comparatively shorter than used by Olitzki and Sulitzeanu (72). This would suggest that live Brucella cells are capable of releasing antigenic components into the host circulation and initiating antibody formation which the killed cells are incapable of. If these components are present (or released by destruction) in the dead cells, than they are in such a low level that the antibodies formed are not demonstrated by the AGIT.

In the present study, precipitin lines 4, 5, and 6 demonstrated in the AGIT are of significance. Line number 4 was elicited only when higher concentrations of both the antigen $P_{100}C$ and the standard hyperimmune serum (MHS) were used for the reaction in the AGIT. No lines showing identity to this line 4 were seen with the other antigenic fractions when reacted against the MHS. This suggests that the antigenic component eliciting this line is either absent or if present, it is at low levels in the rest of the fractions. Since this line was not observed with the other hyperimmune

serum preparations, it can be assumed that the antibody specific for this component is either absent or it is at too low a level to be revealed in the AGIT.

A lipopolysacchride (LPS) antigen located in the cell wall of smooth Brucella species and consituting the inseparable components of the agglutinogens A and M has been reported to form a thick precipitin band close to the antigen well when antigen extracts were reacted against smooth Brucella antisera in immunodiffusion or immuno-electrophoresis (Diaz et al (31, 33 and 34), Kulshreshtha et al (48) and Corbel (23)). Diaz et al (34) have also described a polysacchride protein component (Component 1) which is not restricted to the surface of smooth Brucella and is not correlated with the smooth agglutinogen.

Two precipitin lines, 5 and 6, were observed to occur in a broad band close to the antigen well, in this study. The difference between these two was more noticeable after staining of the slides. These lines were revealed to be lipopolysacchride protein in nature. The extraction methods used here were not specifically for lipopolysacchrides as employed by the other workers. Line number 5 was not demonstrated or was very faint when the antigenic extracts were tested against the

monospecific serum for B. melitensis (MMS). This antigenic component was therfore considered to be a surface antigen common to both B. abortus and B. melitensis. It has been shown that intact bacterial cells react in vitro with antibodies to their surface antigens alone because these bacteria are impermeable to antibody molecules (Baughn and Freeman (12)). To support this finding, Waghela (unpublished data) has shown that a concentrated saline wash of B. melitensis cells treated with sodium hydroxide gave only two lines when tested against the standard hyperimmune serum (MHS). These two lines showed identity to precipitin lines 5 and 6 (Figure 31). One of the lines corresponding to line 5 did not appear with the monospecific serum for B. melitensis (MMS). This findings suggest that line number 6 corresponds to the LPS antigen of Diaz et al (34) which may carry the specificity of the Brucella group, namely, the antigens Am in B. abortus and Ma in B. melitensis. Line 5 is probably similar to Component 1 obseved by Diaz et al (34).

The rest of the precipitin lines are assumed to be due to the reaction of antigen components which are subsurface or cytoplasmic in origin, since the antibodies against them are not absorbed by whole cells of Brucella. These antigens are

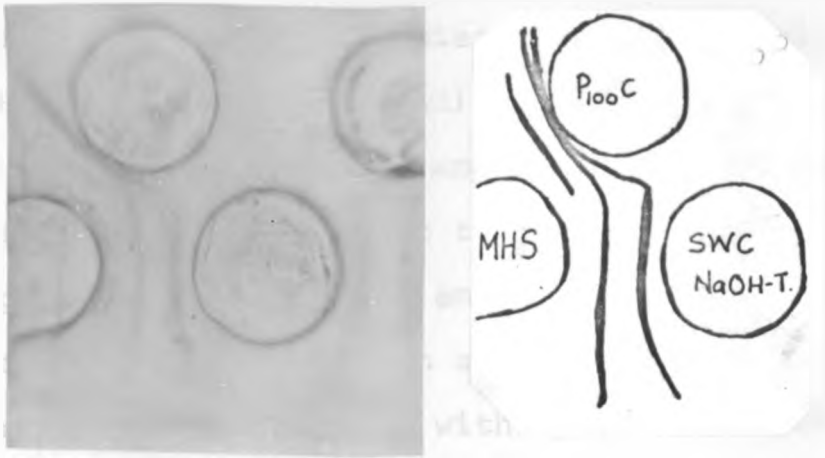


Figure 31a: showing the Cross-reactions of Precipitin Lines 5 and 6 of Fraction $P_{100}C$ and Precipitin Lines of Saline Wash Concentrate of B. melitensis Treated with NaOH.

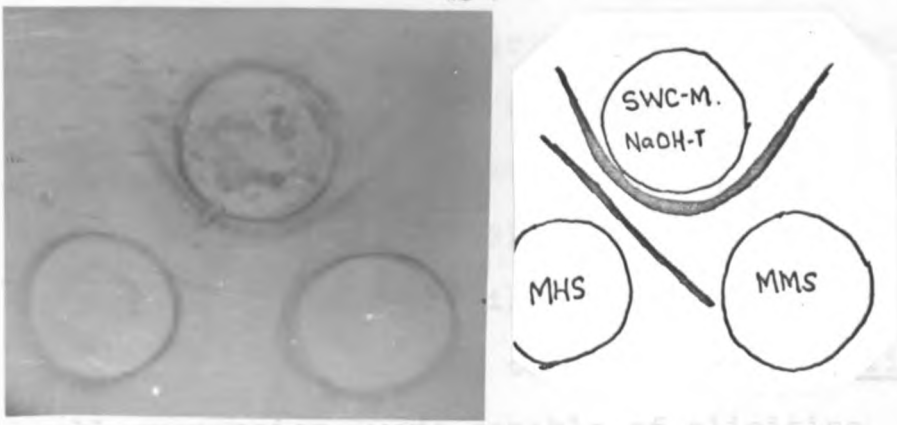


Figure 31b: showing the absorption of Precipitin Line in B. melitensis Monospecific Serum, corresponding to Line 5.

thought to be group specific since they also occur in rough Brucella species (Diaz et al (33 and 34), Hinsdill and Berman (41) and Freeman et al (36)).

The convenience and efficiency of any serological diagnostic technique depends primarily on the test employed and secondarily on the stability of an antigen to give the same reaction on each test with a standard serum. In this study, three different designs of the AGIT, minimicromethod, micromethod and macromethod, were compared. The micromethod was found to be better than the other designs, giving results in a shorter time with the use of less reagents, and was easier to read. Best reactions were obtained using 1 percent Noble Agar at a pH between 7.2 and 7.4. Preservation of agar either with merthiolate or sodium azide did not influence the results of reaction and avoided contamination.

Glenchur et al (39) reported that most of the fractions obtained by differential centrifugation and DEAE-chromatography of sonicated B. melitensis cell suspension, were capable of eliciting secondary immunological reactions (agglutination, precipitation, blocking antibody test and delayed hypersensitivity). Kulshreshtha et al (49) reported that B. melitensis Strain 16M appeared to liberate its precipitinogens better if phenol was used for

extraction. In this study, antigenic fractions extracted with phenol were more consistent than acetone fractions in giving similar results. It was also noted that the phenol fractions elicited more precipitin lines with a greater frequency than the acetone fractions. The other factors affecting the efficacy of an antigen were concentration and differential centrifugation. Both of these procedures not only increased the rate of positive reactions but also enhanced the frequency of observations of more lines. Antigen $P_{100}C$ was found to give the most number of lines with the highest rate of positive reactions. The antigenicity of phenol antigenic fractions, especially of $P_{100}C$, was higher than that of acetone fractions. The differential centrifugation did not help in the fractionation of the antiigens since most of the precipitin lines were revealed by all the fractions. The only difference noted was the appearance of precipitin line 4 when antigen $P_{100}C$ was reacted against the hyperimmune serum (MHS). The significance of this line has been discussed above. However, it could be that this antigenic component was extracted by phenol and concentrated by centrifugation at 100,000 g. Although, quantitative and qualitative analyses were not performed on each fraction, it can be assumed that every antigenic fraction was

contaminated with every other fraction.

The serological activity of Brucella antigenic fractions has been reported. Diaz et al (33) showed that the LPS component of the Brucella cell wall was specific for agglutination and hemagglutination with fresh sheep erythrocytes and diffused poorly through agar gel. This antigenic component has been found to play an important role in the SAT, Coomb's reaction, RBPT (Diaz and Levieux (39)) and CFT (Diaz and Jones (30)). The subsurface or cytoplasmic antigens diffused through agar gel freely and sensitized only tanned sheep red blood cells for hemagglutination (Diaz et al (33)). These authors also found that sonically treated suspensions only sensitized tanned cells. In general, polysacchride antigens are absorbed by untreated red blood cells. Protein antigens on the other hand, attach only to pretreated erythrocytes (Burnett (18)). The ether-water extract of B. melitensis, containing the lipopolysacchride (LPS), did not sensitize red blood cells for hemagglutination unless it was pretreated with sodium hydroxide (Diaz et al (33)). A similar finding has been observed by Kulshreshtha and Ramanchandran (50) in that some antigenic extracts of B. abortus only worked in hemagglutination after hydrolysis with sodium hydroxide. Neter (67) has

reported that certain lipids in antigens inhibit sensitisation of red blood cells and that the activation from a non-modifying to a modifying antigen can be accomplished by treatment with sodium hydroxide.

In this study, the various antigenic fractions did not sensitise fresh sheep red cells. None of the antigen fractions were treated with sodium hydroxide. Most of the fractions extracted with phenol sensitised tanned sheep red blood cells readily whereas only two of the acetone fractions sensitised the tanned cells, and at low titres. It was also noted that the gluteraldehyde treated tanned cells adsorbed the fraction $P_{100}C$ even better than tanned cells, since the titre of the antigen obtained with tanned cells was lower than that obtained with gluteraldehyde treated, tanned red blood cells.

The IHA reaction has been found to be very sensitive in diagnosis of brucellosis (Renoux *et al* (91), Corbel and Day (24), and Versilova (109)). Corbel and Day (24) have reported that the only potential advantage of the IHA reaction, its high specificity, was offset by the difficulty of interpreting reactions produced at low dilutions of sera and also by the preparation and standardisation of freshly sensitised erythrocytes

for each batch of the tests. The IHA reaction has been found to be valuable in the diagnosis of Brucella infections in man and animals; and may be worthy of inclusion in the battery of serological tests (Anon (10)).

In this study, lack of antigen $P_{100}C$ plus lack of time prevented the follow up of the IHA reaction. However, on limited tests done on caprine sera, the IHA was observed to be very sensitive. Interpretation of reactions at low serum dilutions was difficult since some of the negative and positive sera both gave similar titres.

Brucella antigenic extracts have been reported to demonstrate complement fixing activity (Jones et al (46) and Renoux and Alton (88)) but no special advantage was found over the whole cell antigens. One disadvantage reported has been the anticomplementary activity of the extracts (Myers et al (64)). A similar problem was noted in this study. In addition, comparatively low antigen titres meant large amounts of reagents were used. Slightly higher titres were recorded inthe CFT employing fractions compared to the whole cell antigen. This difference can be explained by the fact that the antigenic fractions were homologous to the antisera (B. melitensis) whereas the whole cell antigen was made up of B. abortus.

Nevertheless, the whole cell antigen is more efficient and a simpler antigen for use in the CFT.

B). SEROLOGICAL DIAGNOSIS OF CAPRINE BRUCELLOSIS.

Diagnostic tests, especially serological, are an invaluable asset for the detection, definition and removal of the infection foci during control, eradication and surveillance procedures for brucellosis. The tests employed should be simple, efficient and sensitive so that the rate of false positive and false negative results remains minimal.

Evidence as to the efficiency of the various diagnostic tests for the detection of B. melitensis infection in sheep and goats is limited (Anon (10)). It has been stressed (Anon (10), Renoux (86), Morgan(60) and Unel et al (107)) that no serological test for brucellosis is exclusively superior to any of the others and that they should be used in conjunction. The known limitations of the SAT have discussed by Unel et al (107) and Renoux (84). The CFT has been found to be highly specific and sensitive test for Brucella antibodies. It has shown higher correlations between infection and positive reactions than the SAT. The RBPT has been reported to be oversensitive with cattle sera

(Anon (9), Davies (28) and Morgan et al (61)). Jones et al (45) have found that the Card test was not sensitive enough to detect infected goats. Philpott and Auko (79) observed that the RBPT failed to detect a number of goats reacting at a titre of 1:5 in the CFT, but negative in the SAT. The card test has been found to be positive only when the agglutination titre was high (Varela-Diaz et al (108)). Corbel (22) has shown that in cattle sera the reaction to the CFT and RBPT is mediated by the same immunoglobulin, IgG. He suggested that a complete correlation between the results of the two tests should not be expected since the RBPT may detect antibody of another class. Varela-Diaz et al (108) have reported that CF and Mercaptoethanol reactions were developed exclusively by the IgG containing serum fractions in B. melitensis Rev 1 vaccinated and non-vaccinated (but challanged later) goats. The card test activity was found in either or both IgM and IgG fractions. Bruce and Jones (16) found 90 percent agreement between the results of the SAT and AGIT on bovine sera. The disagreement usually occured in sera with low agglutination titres. The AGIT was found to correlate well with the CFT and was more practical for the diagnosis of B. ovis infection in sheep (Myers and Sinuik (65), Myers et al (64) and

Myers (63)). However, the AGIT did not appear to be as sensitive as the established methods for the diagnosis of B. melitensis and B.abortus infections.

In the present study, by combining the results of all four serological tests (SAT, CFT RBPT and AGIT) on caprine sera from two field outbreaks of brucellosis, it was found that 114 positive or doubtful reactors were detected out of a total of 206 goats. Eighty four of these 114 goats were positive in one or more tests, and 29 out of the 84 were positive in all four tests. Individually, the SAT detected 38 positive goats, the CFT detected 52, the RBPT detected 73 and the AGIT detected 43 goats. The RBPT tended to be oversensitive, especially in sera from Farm 1 where 41 sera were positive in the RBPT but negative in one or more of the other tests. The RBPT also failed to detect 27 animals suspicious in the CFT, most of which were negative in the SAT. The AGIT failed to detect several reactors, for example, 56 of the CFT reactors. Twenty nine sera were positive in all the other tests out of the 43 sera positive in the AGIT. Only one sera of the remaining 14 sera was negative in the CFT whereas 6 were negative in the SAT and 7 in the RBPT.

No meaningful correlation between the tests can be obtained because of the high number of equivocal reactions in the CFT and SAT. If only positive reactions are considered, the best correlation was between the CFT and RBPT, followed by CFT and AGIT and finally RBPT and AGIT. This suggests that of the four tests, the SAT would prove to be least useful. The RBPT and AGIT appear to be useful in confirmation of the suspicious reactors in the CFT. However, the use of all four tests would help in the detection of a maximum number of infected animals in caprine and ovine brucellosis due to B. melitensis.

Glenchur et al (39) reported a sequence in the appearance of various antibody reactions in the serum of a Brucella infected host. Agglutinins appeared soon after infection, precipitins followed and with continued infection, the blocking phenomenon appeared. Bruce and Jones (16) found that the complement fixing and activity appeared at about the same time but not simultaneously. Agglutinins consistently preceded complement fixing and precipitating antibodies. In this study, antibodies were usually detected first by the SAT, followed by the AGIT, RBPT and finally CFT. In a few animals the appearance of the reaction overlapped for two or more tests.

In a Brucella infected animal, the agglutinins decline in the chronic stages of the disease (Anderson et al (6)). The precipitins persist for very long periods, possibly for the duration of infection (Corbel (23)). Complement fixing antidodies are also known to persist during very long periods of infection (Morgan (59)) and sometimes in the chronic stages of the infection CFT is the only test positive. In this study, in general, the SAT was also the first test to become negative or doubtful. The CFT remained the only test capable of detecting antibodies in all the infected animals throughout the trial period.

Polding (81) reported that the serological response in pregnant goats with contact infection was erratic, with several minor peaks before the titres reached higher levels whereas in non-pregnant goats the reaction was low and usually disappeared within a short time. In this study, apart from one animal, the serological response of incontact goats was irregular. The exceptional case reacted to all the four tests and remained so until the end of the experiment.

It has been reported that an animal which is definitely infected and excreting Brucella may be negative in the standard serological tests,

especially, the agglutination reaction (Renoux (86)). In this study, one in-contact goat shedded Brucella but never reacted in any of the serological tests. Another goat infected conjunctivally never reacted in the AGIT although it was positive in the other tests.

Factors contributing to the response of an individual animal to Brucella infection would be the nature and persistence of the antigen and for the animal's immunological responsiveness (Morgan and McDiarmid (62)). Stress factors are also known to play a role in the antibody response (Cullen and Corbel (27)). The magnitude of response to the SAT, and CFT of Group One animals was considerably higher than that of Group Two, although the kinetics of the response was similar. There was a significant difference (P for the SAT = 1 percent to 5 percent from 4th to 20th day and P for the CFT = 0.1 percent to 5 percent from 10th to 18th day) in the meantitres between the two groups only in the early part of the infection. The lack of significant differences in the later stages is probably because of the small number of animals involved in the trial. It could also be that once an animal's defence is overcome during Brucella infection, the immune response is similar and follows the same course in a group of animals

under the similar environmental and experimental conditions. The results of this study suggest that the route of infection and dosage of the infective organism played a major role in the magnitude of the serological response. The variations of the individual serological response were probably due to the stress factors and individual immunological responsiveness and resistance to the infection.

Abortion is the principal manifestation of B. melitensis infection in goats and sheep (Alton (4)). Mastitis is known to occur in a percentage of the infected animals. In a chronic state, the organisms tend to localise in the udder and associated lymph nodes, where in the absence of abortions, the disease has little effect on the milk yield. Infertility may occur in goat herds following a brucellosis outbreak (Anon (10)). Waghela (Unpublished data) has observed that infertility was a problem in a herd of 2000 goats following an abortion outbreak due to B. melitensis infection involving nearly 70 to 80 percent of the female goats. The infertility was related to endometritis following abortion. Most of these animals, kidded normally, except for a few abortions, following a sexual rest of 3 to 4 months. There was a suggestion of infertility in the infected goats of the present study. A goat died of a septicemic synderome,

a sequele to the necrotising endometritis following an abortion. Abortion was the prominent sign in the infected goats. Mastitis was observed in one goat.

Brucella isolation was possible in the early part of the infection. Later on the goats were negative. Unel et al (106) reported failure to recover B. melitensis from either vaginal swabs or milk samples from most of the aborting ewes. Due to an opsonising effect of antibody to smooth Brucella the dissemination of large numbers of organisms in a host's body resulting from bacteremic showers is minimised by the restriction of localisation of Brucella to regional lymph nodes (Sulitzeanu (105)). This probably explains the failure to recover Brucella during chronic stages of the disease.

SECTION VI

CONCLUSIONS

The variability in the number of *Brucella* precipitinogens obtained has been related to the procedures employed for the preparation of antigens and production of hyperimmune sera and the method of demonstration of the components. In this study, six precipitinogens revealed by the agar gel immunodiffusion technique, in fractions of sonicated *Brucella melitensis* Strain 16M were classified as numbers 1 to 6. It was difficult to classify these antigenic components in relation to any other study because of the variation in designation used by the other workers. It is suggested that a standardized system of nomenclature of *Brucella* antigenic components should be adopted. This system would entail the use of a standard hyperimmune serum and a standard procedure for testing of the antigenic fractions prepared by different techniques. At present, the only antigens which have been correlated by several authors are the surface lipopolysacchride antigens which have been demonstrated by either immunodiffusion or immunoelectrophoresis.

The antigenic fractions used in this study were comparatively crude and contamination of

each fraction with another was evident. Purification of these crude fractions by further differential centrifugation, chemical and enzymatic treatment and gel or ion-exchange chromatography should reveal more information as to the antigenic structure of Brucella. This would involve the preparation of immune sera against each fraction for a proper characterzation. In this study, the AGIT probably did not reveal all the antigenic components in each fraction. Therefore, it is suggested that a more sensitive method, preferably immunoelectrophoresis should be employed.

The phenol antigenic fractions were found to be more efficacious. Thus, before preparing any antigenic fractions, Brucella cell suspensions should be treated with phenol, instead of using acetone dried cells.

The specificity of Brucella species lies in the lipopolysacchride protein complex of the surface antigens. This antigen enables the identification of smooth B. melitensis from the other smooth species of Brucella; and also differentiates smooth from rough Brucella. An isolated LPS-protein complex may help in the differentiation of antibodies to rough Brucella from those to smooth Brucella in the AGIT. The same antigen may help to differentiate antibodies due to infection

from those from B. abortus Strain 45/20 vaccination in cattle. On further purification of the LPS-protein complex, the specific antigens M and A may be released and then this antigens may be used for diagnosis and immunoprotections and should, therefore, continue to be used. The phenolised fractions were found to give good hemagglutinating reaction with tanned sheep red blood cells but the reaction was even better with gluteraldehyde fixed, tanned cells. However, more research is necessary before this system is adopted.

In the study of serological tests, the micromethod of AGIT was found to be simple and more practical than the minimicromethod and macromethod of the same test. The test was useful in the confirmation of SAT and CFT suspicious reactor animals although it failed detect a few positive reactors. The results of the AGIT would suggest $P_{100}C$ to be a good antigen but to make the test more sensitive, further fractionation of this antigen may be needed.

The RBPT has been described as a simple test which gives good results with cattle sera. In this study, the test failed to detect a number of CFT suspicious reactors and was found to be oversensitive. However, the test helped in confirmation of

suspicious reactors of CFT. The RBPT alone may not be the best test for the detection of B. melitensis infections in goats and sheep.

The SAT has been widely used as a standard diagnostic test for brucellosis. Several authors have discussed limitations of the test for the diagnosis of brucellosis, especially that of caprine and ovine brucellosis. In this study, it was observed that the SAT is of limited value when used in conjunction with the other tests; but as a single test it helps in the detection of brucellosis, especially when reagents for the other tests are not available.

The CFT is the most specific test for the diagnosis of brucellosis but is cumbersome and laborious. In a small laboratory or where trained staff are not available the test would be a problem. However, the microCFT offsets some of these disadvantages. Once established, the test can provide good results with a high effeciency ratio if performed by a semi-skilled person. In this study, the microCFT was found to give the maximum number of reactors and with good correlation with the other tests. It was the only test remaining reactive in all animals in the later stages of infection. A modification of this test for field use in Kenya would be very useful.

From the results of this study, it is suggested that a combination of at least two tests, especially CFT - RBPT and CFT - AGIT, would be able to detect most of the reactors. Where no CFT facilities are available, the use of RBPT - AGIT combination will detect most reactors.

This study suggests that the initiation and magnitude of serological response in an infected animal would depend on the dose and route of infection. Once the infection overcomes the body defence of an individual animal, the response is similar in all the animals under the same set of management and environmental conditions.

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APPENDIX

SEROLOGICAL TEST* RESULTS OF SERUM SAMPLES FROM NATURALLY AND EXPERIMENTALLY INFECTED GOATS.

A) NATURALLY INFECTED GOAT SERA.

1) Farm 1:

No.	SAT	CFT	RBPT	AGIT	No.	SAT	CFT	RBPT	AGIT
1	0	0	N	N	11	100	3:20	P	1
2	50	2:20	P	S	12	0	0	N	N
3	0	0	N	N	13	0	2:10	N	S
4	0	0	N	N	14	400	4:320	P	2
5	0	0	N	N	15	0	4:5	N	1
6	0	0	N	N	16	0	0	N	N
7	0	0	N	N	17	0	0	N	N
8	100	1:20	P	S	18	0	0	N	N
9	0	1:5	P	S	19	0	0	N	N
10	200	4:20	P	2	20	25	0	P	N

*SAT titres in International Units (I.U.).

CFT titres in degree of fixation at a particular serum dilution.

RBPT: N = negative; P = positive.

AGIT: N = negative; S = spur line; Nos. 1 to 4 = Number of precipitin lines formed with each serum.

21	25	0	P	N	48	0	0	N	N
22	0	4:20	N	N	49	0	0	N	N
23	0	0	N	N	50	0	0	N	N
24	400	2:20	P	N	51	0	4:2.5	N	N
25	0	0	P	N	52	0	3:2.5	N	N
26	0	3:2.5	P	N	53	400	3:10	P	2
27	100	4:2.5	P	1	54	0	0	N	N
28	50	3:2.5	P	N	55	0	0	N	N
29	800	4:40	P	1	56	0	1:2.5	P	N
30	0	0	N	N	57	0	0	N	N
31	25	4:5	P	N	58	0	4:2.5	P	N
32	0	0	N	N	59	0	4:40	P	2
33	0	0	N	N	60	25	3:10	P	S
34	0	0	N	N	61	0	0	N	N
35	0	0	N	N	62	25	2:2.5	N	1
36	0	0	N	N	63	25	0	N	N
37	0	1:2.5	P	N	64	400	4:80	P	1
38	200	4:20	P	1	65	400	4:80	P	2
39	0	3:2.5	P	N	66	0	2:2.5	N	N
40	25	2:5	P	N	67	0	4:2.5	P	N
41	0	2:10	P	N	68	0	4:2.5	P	N
42	0	4:5	P	N	69	0	0	N	N
43	0	0	N	N	70	0	0	N	N
44	0	0	N	N	71	25	0	P	N
45	400	4:40	P	1	72	0	0	N	N
46	25	0	P	N	73	25	1:5	N	N
47	0	0	P	N	74	0	4:5	N	N

75	0	0	N	N	102	0	4:2.5	N	N
76	0	0	N	N	103	0	4:2.5	N	N
77	0	0	N	N	104	0	4:2.5	N	N
78	0	0	N	N	105	100	4:40	P	1
79	25	4:2.5	N	N	106	0	0	N	N
80	0	0	N	N	107	0	0	N	N
81	0	0	N	N	108	0	4:2.5	N	N
82	25	4:2.5	P	1	109	25	4:2.5	N	N
83	0	0	N	N	110	0	2:2.5	N	N
84	25	4:5	P	N	111	0	4:2.5	N	N
85	0	0	N	N	112	0	0	N	N
86	25	3:5	P	N	113	0	0	N	N
87	200	4:10	P	1	114	0	4:2.5	P	N
88	0	0	N	N	115	0	0	P	N
89	0	0	N	N	116	0	4:2.5	N	N
90	50	4:10	P	1	117	25	2:5	P	N
91	0	1:5	P	N	118	25	4:2.5	P	N
92	0	1:5	P	N	119	25	4:2.5	P	N
93	0	1:2.5	N	N	120	0	4:2.5	N	N
94	25	4:5	P	N	121	0	0	N	N
95	800	4:40	P	2	122	0	0	P	N
96	0	4:2.5	N	N	123	0	4:2.5	P	N
97	400	4:20	P	1	124	200	4:10	P	1
98	0	0	N	N	125	25	4:2.5	P	S
99	0	4:2.5	N	N	126	0	4:2.5	P	N
100	0	0	N	N	127	0	2:2.5	N	N
101	25	4:2.5	N	N	128	25	1:5	N	N

129	0	1:5	N	N	133	50	1:5	P	N
130	0	4:2.5	P	N	134	0	0	N	N
131	200	3:40	N	1	135	0	2:2.5	N	N
132	50	0	P	N	136	0	0	N	N

2) Farm 2:

No.	SAT	CFT	RBPT	AGIT	No.	SAT	CFT	RBPT	AGIT
1	200	4:80+	P	2	25	0	0	N	N
2	0	2:2.5	N	N	26	200	0	P	N
3	0	0	N	N	27	0	2:10	N	N
4	50	2:10	N	N	28	0	0	N	N
5	0	2:2.5	N	N	29	200	4:20	P	2
6	400	4:80+	P	2	30	0	0	N	N
7	0	0	N	N	31	0	0	N	N
8	50	3:20	P	S	32	0	0	N	N
9	0	0	N	N	33	0	0	N	N
10	0	0	N	N	34	0	0	N	N
11	0	0	N	N	35	0	0	N	N
12	0	0	N	N	36	0	0	N	N
13	100	4:20	P	N	37	0	0	N	N
14	0	0	N	N	38	200	4:80+	P	1
15	0	0	N	N	39	25	4:10	N	1
16	0	4:5	P	1	40	0	0	N	N
17	0	0	N	N	41	0	0	N	N
18	0	0	N	N	42	0	0	N	N
19	400+	4:80+	P	2	43	100	4:80+	P	3
20	0	0	N	N	44	0	0	N	N
21	100	1:80	P	2	45	0	0	N	N
22	25	0	N	N	46	0	0	N	N
23	0	0	N	N	47	0	0	N	N
25	0	0	N	N	48	0	0	N	N

49	100	4:80+	P	1	60	0	0	N	N
50	100	4:80+	P	2	61	50	4:10	P	1
51	0	0	N	N	62	200	4:80	P	1
52	0	0	N	N	63	0	0	N	N
53	0	0	N	1	64	0	3:2.5	N	N
54	25	4:20	N	1	65	0	3:2.5	N	N
55	0	0	N	N	66	0	4:2.5	N	N
56	25	0	N	N	67	0	4:2.5	N	N
57	0	0	N	N	68	0	0	N	N
58	0	0	N	N	69	0	0	N	N
59	0	0	N	N	70	0	0	N	N

B) SERUM SAMPLES FROM EXPERIMENTALLY INFECTED GOATS.

1) Complement Fixation Test:

Group No.	Goat No.	Route of Infection*	Time in Days**						
			0	2	4	6	8	10	13
1	3120	A	-	-	-	-	-	-	-
	3132	A	-	-	-	-	-	-	1:5
	3138	A	-	-	-	-	-	4:2.5	1:5
	3140	A	-	-	-	-	-	4:2.5	1:5
	3143	A	-	-	-	-	4:2.5	4:5	2:5
	3129	C	-	-	-	-	-	-	-
	3135	C	-	-	-	-	-	-	-
	3123	C	-	-	-	-	-	-	-
	3136***								
2	3119	B	-	-	-	-	-	-	-
	3128	B	-	-	-	-	-	-	-
	3130	B	-	-	-	-	-	-	-
	3134	B	-	-	-	-	-	-	-
	3139	B	-	-	--	-	-	-	-
	3122	C	-	-	-	-	-	-	-
	3126	C	-	-	-	-	-	-	-
	313	C	-	-	-	-	-	-	-
3	3121	D	-	-	-	-	--	-	-
	3124	D	-	-	-	-	-	-	-
	3125	D	-	-	-	-	--	-	-
	3127	D	-	-	-	-	-	-	-
	3133	D	-	-	-	-	-	-	-
	3141	D	-	-	-	-	-	-	-
	3142	D	-	-	-	-	-	-	-
	3136***	D	-	-	-	-	-	-	-

*A = subcutaneous; B = conjunctival; C = Incontact; D = non-infected. **Days after infection of goats in Groups 1 and 2. ***Moved to Group 1 on day 155.

15	18	20	22	24	31	38	49	53
-	-	-	-	-	-	-	-	-
4:2.5	1:20	4:20	2:80	3:80	3:80	4:40	2:40	3:20
1:5	3:10	4:10	3:20	2:20	4:10	4:20	3:40	3:20
1:5	1:20	4:20	4:40	4:40	4:20	1:40	4:80	4:80
2:5	2:10	4:10	2:20	4:10	1:20	3:20	2:20	4:5
NT[+]	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	2:2.5	-	2:2.5	1:5	1:5	4:5	4:5	4:5
-	3:2.5	3:10	1:20	2:20	3:10	4:20	4:20	2:20
-	-	2:5	4:10	4:10	3:10	4:20	4:20	4:10
-	-	-	1:5	4:2.5	3:5	2:20	4:20	4:10
-	2:10	1:20	3:10	3:10	4:10	4:20	3:20	2:20
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	--	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-

[+]NT = not tested.

59	66	79	94	108	122	136	150	157
-	4:2.5	3:5	3:5	4:2.5	4:20	4:10	4:20	3:10
4:80	1:40	1:20	1:20	4:10	4:10	4:10	3:10	4:5
1:40	1:40	2:40	4:20	4:10	4:20	4:10	4:10	3:10
1:40	1:80	1:80	4:40	3:20	4:10	4:10	4:20	4:10
4:10	4:10	1:10	4:10	3:5	3:5	4:5	2:5	4:5
D+								
-	4:2.5	4:2.5	4:2.5	3:2.5	-	-	1:2.5	-
-	-	4:2.5	4:5	4:2.5	-	4:2.5	3:2.5	D
								-
D								
4:40	4:40	2:80	4:80	3:80	4:40	4:20	1:20	4:10
4:20	1:40	3:40	4:20	2:20	4:10	2:10	1:10	4:5
4:10	4:10	1:20	3:10	3:5	4:5	2:5	1:5	4:2.5
4:10	4:10	4:10	4:10	3:10	4:5	2:5	4:5	4:2.5
-	-	-	-	-	-	-	-	-
-	-	-	-	-	4:2.5	1:10	1:20	4:5
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	

+D = died.

164	171	178	188	192	199	206	213	220
4:10	4:10	1:20	4:10	4:10	4:10	4:10	4:10	4:10
3:10	3:10	4:5	4:10	4:10	4:10	4:20	4:10	4:5
4:10	3:10	4:20	4:10	4:10	4:10	4:10	1:20	4:10
1:20	1:20	4:10	4:10	4:10	4:10	4:20	4:20	4:10
2:5	1:5	4:5	4:5	4:5	4:5	4:5	4:5	3:5
–	3:5	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
2:20	2:10	3:20	1:10	4:20	4:20	1:20	4:20	1:20
4:5	3:5	4:5	4:5	4:10	4:10	4:10	4:10	4:5
4:2.5	1:5	4:2.5	4:2.5	4:2.5	1:5	4:2.5	4:2.5	4.2.5
4:2.5	3:2.5	4:2.5	4:2.5	4:2.5	4:2.5	1:2.5	4:2.5	3:2.5
–	–	–	–	–	–	–	–	–
4:10	2:10	2:10	2:10	4:10	4:10	4:10	3:10	4:5
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–

227	234	241	248	255	262	269	276	283
4:10	4:10	3:10	4:5	2:10	2:10	4:10	2:10	1:10
3:10	4:10	3:20	4:10	4:10	4:20	2:20	4:10	4:10
4:10	4:10	3:10	3:5	4:5	4:5	4:10	4:5	3:5
3:10	4:20	2:20	4:10	4:10	1:10	4:20	1:20	2:20
4:5	4:5	4:5	2:5	1:5	3:5	1:5	2:5	3:5
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
4:10	2:10	2:10	1:10	3:10	2:10	4:10	4:10	4:10
2:10	4:10	3:10	4:5	3:5	3:5	4:5	4:5	3:5
4:2.5	4:2.5	4:2.5	1:5	4:2.5	1:5	4:2.5	4:2.5	4:2.5
4:2.5	4:2.5	4:2.5	4:2.5	4:2.5	1:5	4:2.5	4:2.5	3:2.5
-	-	-	-	-	-	-	-	-
4:5	4:5	4:5	1:5	2:5	3:5	4:2.5	2:5	1:5
-	D							
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-

290	297	304
2:10	4:10	3:10
1:10	1:10	3:10
3:5	4:5	4:5
3:10	3:10	3:10
3:5	4:5	4:5
-	-	-
-	-	-
4:10	2:10	4:10
4:5	D	
4:2.5	1:5	3:2.5
4:2.5	2:5	4:2.5
-	-	-
1:5	3:5	1:5
-	-	-
-	-	-
-	-	-
-	-	-
-	-	-
-	-	-
-	-	-

2) Serum Agglutination Test:

Group No.	Goat No.	Route of Infection*	Time in Days**						
			0	2	4	6	8	10	13
	3120	A	-	-	-	-	-	-	-
	3132	A	-	-	25	25	100	100	400
	3138	A	-	-	25	100	400	400	400
	3140	A	-	-	25	100	400	800	1600
	3143	A	-	-	50	25	100	400	400
1	3129	C	-	-	-	-	-	-	-
	3135	C	-	-	-	-	-	-	-
	3123	C	-	-	-	-	-	-	-
	3136***								
	3119	B	-	-	-	-	-	-	25
	3128	B	-	-	-	-	-	-	25
	3130	B	-	-	-	-	-	-	-
	3134	B	-	-	-	-	-	-	-
2	3139	B	-	-	-	-	-	-	-
	3122	C	-	-	-	-	-	-	-
	3126	C	-	-	-	-	-	-	-
	313	C	-	-	-	-	-	-	-
	3121	D	-	-	-	-	-	-	-
	3124	D	-	-	-	-	-	25	-
	3125	D	-	-	-	-	-	-	-
	3127	D	-	-	-	-	-	-	-
3	3133	D	-	-	-	-	-	-	-
	3141	D	-	-	-	-	-	-	-
	3142	D	-	-	-	-	-	-	-
	3136***	D	-	-	-	-	-	-	-

*A = subcutaneous; B = conjunctival; C = Incontact; D = Non-infected. *Days after infection of goats in Groups 1 and 2. **Moved to group 1 on day 155.

2) Serum Agglutination Test:

Group No.	Goat No.	Route of Infection*	Time in Days**						
			0	2	4	6	8	10	13
	3120	A	-	-	-	-	-	-	-
	3132	A	-	-	25	25	100	100	400
	3138	A	-	-	25	100	400	400	400
	3140	A	-	-	25	100	400	800	1600
	3143	A	-	-	50	25	100	400	400
1	3129	C	-	-	-	-	-	-	-
	3135	C	-	-	-	-	-	-	-
	3123	C	-	-	-	-	-	-	-
	3136***								
	3119	B	-	-	-	-	-	-	25
	3128	B	-	-	-	-	-	-	25
	3130	B	-	-	-	-	-	-	-
	3134	B	-	-	-	-	-	-	-
2	3139	B	-	-	-	-	-	-	-
	3122	C	-	-	-	-	-	-	-
	3126	C	-	-	-	-	-	-	-
	313	C	-	-	-	-	-	-	-
	3121	D	-	-	-	-	-	-	-
	3124	D	-	-	-	-	-	25	-
	3125	D	-	-	-	-	-	-	-
	3127	D	-	-	-	-	-	-	-
3	3133	D	-	-	-	-	-	-	-
	3141	D	-	-	-	-	-	-	-
	3142	D	-	-	-	-	-	-	-
	3136***	D	-	-	-	-	-	-	-

*A =subcutaneous; B = conjunctival; C = Incontact; D = Non-infected. **Days after infection of goats in Groups 1 and 2. ***Moved to group 1 on day 155.

15	18	20	22	24	31	38	49	53	59
-	-	-	-	-	-	-	-	-	50
1600	1600	1600	1600	3200	1600	1600	1600	400	800
1600	1600	800	800	800	200	800	800	400	800
3200	3200	3200	3200	3200	3200	6400	6400	800	1600
800	800	800	400	400	200	200	200	50	100
NT[+]	-	-	-	-	-	-	-	-	D[‡]
-	-	-	-	-	-	-	-	25	50
-	-	-	-	-	-	-	-	25	25
100	100	100	50	100	100	25	50	25	D
100	200	200	200	200	100	200	200	100	200
-	-	50	200	200	100	100	200	100	200
25	25	50	100	200	100	50	100	50	50
-	400	400	800	400	200	50	100	50	50
-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	25	-	-	-	25
-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	--
-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	--	-	-
-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	--

[+]NT = not tested. [‡]D = died.

66	79	94	108	122	136	150	157	164	171
50	25	25	50	100	200	200	200	100	200
800	200	200	200	100	100	200	100	100	100
400	100	50	100	100	100	200	200	100	100
800	400	400	200	200	200	800	400	200	400
50	25	25	25	25	25	50	25	50	50
25	–	–	–	–	–	–	–	–	–
25	100	25	–	–	25	–	D		
							–	–	–
200	100	50	100	50	100	50	100	100	100
400	200	100	200	100	200	100	100	100	100
50	25	25	–	–	–	–	–	–	–
50	25	25	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	25	100	200	100	200	100	100
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	25	–	–	–	25	25	25	25	25
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–			

178	188	192	199	206	213	220	227	234	241
100	200	100	100	100	100	100	100	100	100
100	100	100	100	100	100	100	100	100	100
100	100	100	50	100	50	50	100	100	50
200	200	400	400	400	400	400	200	200	200
50	50	25	25	25	25	25	25	–	25
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
100	100	100	50	50	50	50	50	50	50
100	100	100	50	50	50	50	100	100	50
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
100	100	100	50	25	25	25	25	25	25
–	–	–	–	–	–	–	–	D	
–	–	–	–	–	–	–	–	–	–
25	–	25	25	25	25	25	25	25	25
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–

248	255	262	269	276	283	290	297	304
100	100	100	50	100	100	100	100	100
100	100	100	100	100	100	100	100	50
50	50	50	50	50	50	25	50	50
200	200	200	200	200	200	200	200	100
25	25	25	25	–	25	–	25	25
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	25	25
25	25	25	25	25	25	50	25	25
100	50	50	50	50	50	25	D	
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
50	25	25	25	25	25	25	25	25
–	–	–	–	–	–	–	–	–
25	–	–	–	25	25	25	25	25
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–

3) Rose Bengal Plate Test:

Group No.	Goat No.	Route of Infection*	Time in Days**										
			0	2	4	6	8	10	13	15	18	20	22
	3120	A	-	-	-	-	-	-	-	-	-	-	-
	3132	A	-	-	-	P	P	P	P	P	P	P	P
	3138	A	-	-	P	P	P	P	P	P	P	P	P
	3140	A	-	-	P	P	P	P	P	P	P	P	P
	3143	A	-	-	-	-	-	P	P	P	P	P	P
1	3129	C	-	-	-	-	-	-	-	NT*	-	-	-
	3135	C	-	-	-	-	-	-	-	-	-	-	-
	3123	C	-	-	-	-	-	-	-	-	-	-	-
	3136***												
	3119	B	-	-	-	-	-	-	P	P	P	P	P
	3128	B	-	-	-	-	-	-	-	-	P	P	P
	3130	B	-	-	-	-	-	-	-	-	-	P	P
	3134	B	-	-	-	-	-	-	-	-	-	P	P
2	3139	B	-	-	-	-	-	-	P	-	P	P	P
	3122	C	-	-	-	-	-	-	-	-	-	-	-
	3126	C	-	-	-	-	-	-	-	-	-	-	-
	313	C	-	-	-	-	-	-	-	-	-	-	-
	3121	D	-	-	-	-	-	-	-	-	-	-	-
	3124	D	-	-	-	-	-	-	-	-	-	-	-
	3125	D	-	-	-	-	-	-	-	-	-	-	-
	3127	D	-	-	-	-	-	-	-	-	-	-	-
3	3133	D	-	-	-	-	-	-	-	-	-	-	-
	3141	D	-	-	-	-	-	-	-	-	-	-	-
	3142	D	-	-	-	-	-	-	-	-	-	-	-
	3136***	D	-	-	-	-	-	-	-	-	-	-	-

*A = subcutaneous ; B = conjunctival; C = incontact; D = non-infected. ** Days after infection of goats in groups 1 and 2. *** Moved to group 1 on day 155.

24	31	38	49	53	59	66	79	94	108	122	136	150	157	164	171
-	-	-	-	-	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
-	-	-	-	-	D+										
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	P	-	P	P	-	P	-	-	D		
													-	-	-
P	P	P	P	P	D										
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	P	P	P	P	P
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-			

+NT = not tested; D+ = died.

178	188	192	199	206	213	220	227	234	241	248	255	262	269
P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P
–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P
P	P	P	P	P	P	P	P	P	P	P	P	P	P
–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
p	p	p	p	p	p	p	p	p	p	p	p	p	p
–	–	–	–	–	–	–	–	D					
–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–

276	283	290	297	304
P	P	P	P	P
P	P	P	P	P
P	P	P	P	P
P	P	P	P	P
P	P	P	P	P
–	–	–	–	–
–	–	–	–	–
P	P	P	P	P
P	P	P	D	
P	P	P	P	P
–	–	–	–	–
–	–	–	–	–
P	P	P	P	P
–	–	–	–	–
–	–	–	–	–
–	–	–	–	–
–	–	–	–	–
–	–	–	–	–
–	–	–	–	–
–	–	–	–	–

4) Acar Gel Immunodiffusion Test:

Group No.	Goat No.	Route of Infection*	Time in Days**										
			0	2	4	6	8	10	13	15	18	20	22
	3120	A	–	–	–	–	–	–	–	–	–	–	–
	3132	A	–	–	–	1	1	1	1	2	1	2	2
	3138	A	–	–	1	2	1	2	1	3	2	2	2
	3140	A	–	–	–	1	2	2	1	2	2	1	1
	3143	A	–	–	1	1	2	1	2	2	1	2	2
1	3129	C	–	–	–	–	–	–	–	NT+	–	–	–
	3135	C	–	–	–	–	–	–	–	–	–	–	–
	3123	C	–	–	–	–	–	–	–	–	–	–	–
	3136***												
	3119	B	–	–	–	–	–	–	2	1	1	S	1
	3128	B	–	–	–	–	–	–	1	1	1	1	1
	3130	B	–	–	–	–	–	–	–	–	1	1	1
	3134	B	–	–	–	–	–	–	–	–	–	–	–
2	3139	B	–	–	–	–	–	–	–	1	1	1	1
	3122	C	–	–	–	–	–	–	–	–	–	–	–
	3126	C	–	–	–	–	–	–	–	–	–	–	–
	313	C	–	–	–	–	–	–	–	–	–	–	–
	3121	D	–	–	–	–	–	–	–	–	–	–	–
	3124	D	–	–	–	–	–	–	–	–	–	–	–
	3125	D	–	–	–	–	–	–	–	–	–	–	–
	3127	D	–	–	–	–	–	–	–	–	–	–	–
3	3133	D	–	–	–	–	–	–	–	–	–	–	–
	3141	D	–	–	–	–	–	–	–	–	–	–	–
	3142	D	–	–	–	–	–	–	–	–	–	–	–
	3136***	D	–	–	–	–	–	–	–	–	–	–	–

*A = subcutaneous; B = conjunctival; C = incontact; D = non-infected. **Days after infection of goats in Groups 1 and 2. ***Moved to group 1 on day 155. +NT = not tested.

24	31	38	49	53	59	66	79	94	108	122	136	150	157	164	171
–	–	–	–	–	–	1	1	1	2	2	2	2	1	2	2
3	1	1	1	1	1	3	2	2	1	1	1	2	1	1	1
3	2	2	3	2	2	2	2	2	2	1	2	3	2	3	3
1	2	2	2	2	3	3	3	2	1	1	1	2	1	1	1
2	2	2	2	2	2	1	2	2	1	1	2	2	2	2	2
–	–	–	–	–	D+										
–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–
–	–	–	–	–	–	–	1	1	–	–	–	1	D		
													–	–	–
1	1	1	S	1	D										
1	1	1	2	1	1	2	2	3	4	2	2	3	2	2	2
1	1	1	1	1	2	3	2	2	2	2	2	2	2	3	1
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1	1	1	1	1	2	1	1	1	1	1	2	2	S	1	2
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	1	1	2	2	2	2
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–			

178	188	192	199	206	213	220	227	234	241	248	255	262
1	1	2	2	2	1	S	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	2	1	1
2	3	2	2	1	1	2	1	1	1	1	2	1
1	1	3	1	2	1	1	1	1	1	1	1	1
1	1	2	1	3	1	1	2	1	1	1	1	1
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
2	2	2	2	2	1	2	2	1	1	2	2	1
2	2	2	1	2	1	S	1	1	1	1	1	1
–	–	–	–	–	–	–	–	–	–	–	–	–
1	S	1	1	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
2	2	S	2	S	S	1	S	S	S	1	1	S
–	–	–	–	–	–	–	–	D				
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–	–	–

269	276	283	290	297	304
1	1	1	1	1	1
1	2	1	1	1	1
1	1	1	1	1	1
1	1	S	1	1	1
1	1	1	1	1	1
–	–	–	–	–	–
–	–	S	1	1	–
1	2	2	1	1	1
1	1	1	S	D	
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	–	–
1	2	1	1	S	S
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	–	–
–	–	–	–	–	–